Volume 2, Issue 3

RARE DISEASE AND ORPHAN DRUGS JOURNAL

RNA antisense and silencing strategies using synthetic drugs for rare muscular and neuromuscular diseases

Daniel Scherman

Open Access

ISSN 2771-2893 (Online)

www.rdodjournal.com

EDITORIAL BOARD

GENERAL INFORMATION

About the Journal

Rare Disease and Orphan Drugs Journal (*RDODJ*), is an international peer-reviewed, open access, online journal.The *Rare Disease and Orphan Drugs Journal* (*RDODJ*) is an international, peer-reviewed, open access journal for the publication of innovative research works on various aspects of this rapidly growing multidisciplinary and interdisciplinary field.
RDODJ will report on scientific advances in the genetics of rare diseases, the molecular basis of the pathologies, and translational research on diagnosis, prevention and treatment.
In addition, *RDODJ* aims to provide a forum for scientific studies and discussion covering the important regulatory, socio-economic and human science issues related to rare diseases and orphan drugs.
The ultimate objective of *RDODJ* is to promote the dissemination of research results and scientific discussion among the research community, practitioners, and patient-advocacy organizations.

Information for Authors

Manuscripts should be prepared in accordance with Author Instructions.
Please check www.rdodjjournal.com/pages/view/author_instructions for details.
All manuscripts should be submitted online at https://oaemesas.com/login?JournalId=rdodj.

Copyright

Permissions

For information on how to request permissions to reproduce articles/information from this journal, please visit www.rdodjjournal.com.

Disclaimer

The information and opinions presented in the journal reflect the views of the authors and not of the journal or its Editorial Board or the Publisher. Publication does not constitute endorsement by the journal. Neither the *RDODJ* nor its publishers nor anyone else involved in creating, producing or delivering the *RDODJ* or the materials contained therein, assumes any liability or responsibility for the accuracy, completeness, or usefulness of any information provided in the *RDODJ*, nor shall they be liable for any direct, indirect, incidental, special, consequential or punitive damages arising out of the use of the *RDODJ*. The *RDODJ*, nor its publishers, nor any other party involved in the preparation of material contained in the *RDODJ* represents or warrants that the information contained herein is in every respect accurate or complete, and they are not responsible for any errors or omissions or for the results obtained from the use of such material. Readers are encouraged to confirm the information contained herein with other sources.

Publisher

OAE Publishing Inc.
245 E Main Street st112, Alhambra, CA 91801, USA
Website: www.oaepublish.com

Contacts

E-mail: editorialoffice@rdodjjournal.com
Website: www.rdodjjournal.com

Rare Disease and Orphan Drugs Journal

CONTENTS

Volume 2 / Issue 3 / Sep 2023 /

Case Report

11 **Biallelic cubilin pathogenic variants as a cause of «benign » proteinuria: implications for clinical management**
Valentine Gillion, Karin Dahan, Nathalie Godefroid

Review

12 **RNA antisense and silencing strategies using synthetic drugs for rare muscular and neuromuscular diseases**
Daniel Scherman

Original Article

13 **Coenzyme Q10, Vitamin E and Polyvitamin B: an exploratory double-blind randomized cross-over study in Phelan-McDermid Syndrome**
Antonio M. Persico , Arianna Ricciardello,Francesca Cucinotta, Laura Turriziani, Giorgia Calabrese, Pasquale Tomaiuolo, Tiziana Di Bella, Fabiana Bellomo, Maria Boncoddo, Giada Turturo, Silvestro Mirabelli, Lisa Asta, Federico Banchelli, Riccardo Cuoghi Costantini, Roberto D'Amico

Review

14 **Cathepsin C: structure, function, and pharmacological targeting**
Milena Stojkovska Docevska, Marko Novinec

Correction

15 **Correction: A report and review of the recurrent c.811C > T variant and mutation spectrum of Kindler syndrome in East Asians: a diagnostic odyssey of 2 weeks versus 49 years**
Valentine Gillion, Karin Dahan, Nathalie Godefroid

Systematic Review

16 **A systematic review of real-world applications of genome sequencing for newborn screening**
Giuditta Magnifico, Irene Artuso, Stefano Benvenuti

Opinion

17 **Could federated data analysis be the catalyst accelerating the introduction of newborn genome screening for the detection of genetic disease?**
Petros Tsipouras, Maria Chatzou Dunford, Hadley Sheppard, Hannah Gaimster, Theoklis Zaoutis

Case Report

18 **Factors influencing pulmonary arterial pressure in three related patients with Cantú syndrome: glyburide may provide precision care**
Ronald W. Day, Benjamin F. Call

Gillion *et al. Rare Dis Orphan Drugs J* 2023;2:11
DOI: 10.20517/rdodj.2022.23

Rare Disease and Orphan Drugs Journal

Case Report

Open Access

Biallelic cubilin pathogenic variants as a cause of « benign » proteinuria: implications for clinical management

Valentine Gillion[1,2], Karin Dahan[1,3], Nathalie Godefroid[2,4]

[1]Division of Nephrology, Cliniques universitaires Saint-Luc, Avenue Hippocrate 10, Brussels B-1200, Belgium.
[2]Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Brussels B-1200, Belgium.
[3]Centre de Génétique Humaine, Institut de Pathologie et de Génétique, Gosselies, Belgium.
[4]Division of Pediatric Nephrology, Cliniques universitaires Saint-Luc, Brussels B-1200, Belgium.

Correspondence to: Dr Valentine Gillion, Division of Nephrology, Cliniques universitaires Saint-Luc, Avenue Hippocrate 10, Brussels B-1200, Belgium. E-mail: valentine.gillion@uclouvain.be

How to cite this article: Gillion V, Dahan K, Godefroid N. Biallelic cubilin pathogenic variants as a cause of « benign » proteinuria: implications for clinical management. *Rare Dis Orphan Drugs J* 2023;2:11. https://dx.doi.org/10.20517/rdodj.2022.23

Received: 15 Nov 2022 **First Decision:** 23 Feb 2023 **Revised:** 24 Apr 2023 **Accepted:** 8 May 2023 **Published:** 15 May 2023

Academic Editors: Daniel Scherman, Jacques S Beckmann **Copy Editor:** Yanbing Bai **Production Editor:** Yanbing Bai

Abstract

The recent description of a cohort with both adults and children harboring biallelic pathogenic variants of *CUBN* changed the paradigm of the management of isolated proteinuria. Indeed, the detection of proteinuria in a patient, regardless of age, often leads to an exhaustive check-up including kidney biopsy but also the prescription of renin-angiotensin system (RAS) blockers to slow the progression of kidney disease. Patients with *CUBN* variants have nondetrimental proteinuria and are non-responsive to RAS blockers. We herein describe 2 siblings treated for isolated proteinuria for several years, eventually diagnosed with *CUBN* biallelic pathogenic variants (c.703 C > T and c.10363-3A > G). We review the physio-pathological mechanisms of this newly discovered disease and discuss implications for clinical management.

Keywords: Proteinuria, genetic kidney disease, cubilin, isolated albuminuria

INTRODUCTION

Discovering isolated proteinuria in a patient should lead to an appropriate work-up, including biological

analysis, kidney ultrasound, and sometimes kidney biopsy. Proteinuria may be glomerular (mainly albuminuria with or without microscopic hematuria), tubular (low molecular weight proteins such as α1-microglobulin, retinol-binding protein, or β2-microglobulin), or both. Regardless of the cause of proteinuria, blockers of the renin-angiotensin (RAS) system, such as angiotensin receptor blockers or angiotensin-converting enzyme (ACE) inhibitors, are usually prescribed to decrease proteinuria and therefore slow down kidney damage. Indeed, proteinuria is a well-established risk factor for progressive kidney disease[1,2]. Increased glomerular capillary pressure may induce excessive filtration of plasma proteins and podocyte dysfunction. The increased reabsorption of plasma proteins by proximal tubular cells may be toxic and lead to their apoptosis and to interstitial fibrosis[1,3]. This paradigm recently changed with the discovery of pathogenic variants in the cubilin (*CUBN*) gene[4] that may cause nondetrimental proteinuria. We report the case of 2 siblings with such variants and discuss their clinical management.

CLINICAL CASE

An 8-year-old girl of Turkish origin with no relevant medical history presented to the Pediatric Nephrology clinic for nocturnal enuresis. An initial check-up revealed isolated mild proteinuria (protein/creatinine ratio 1.2 g/g) made up of albuminuria. The estimated glomerular filtration rate (eGFR) was normal as was the ultrasound of the kidneys. A kidney biopsy was performed but only contained a single glomerulus with a normal aspect by light microscopy. Immunofluorescence microscopy was negative. Enalapril was started at 2.5 mg/day. She remained on this medication for 15 years and proteinuria remained stable at 0.7 g/g to 1.2 g/g despite an increase in enalapril dosage up to 15 mg/day. Enalapril was poorly tolerated, with frequent episodes of orthostatic hypotension. eGFR remained completely normal.

Her younger brother was also diagnosed with moderate isolated proteinuria (0.9 g/g) during a check-up for hyperactive bladder. His kidney biopsy showed eight normal glomeruli by light microscopy. Immunofluorescence microscopy was negative. Enalapril was started and proteinuria remained stable at 0.6-0.9 g/g during 8 years with preserved eGFR. Both parents have normal urinalysis without proteinuria. The first genetic testing was performed in 2018 in both siblings with a panel including 20 genes related to proteinuria. Results were not contributive as a single variant of unknown origin (VUS) of the *ACTN4* gene was found in the sister but not in her brother. Updated genetic testing performed in 2022 (NGS panel enriched in 40genes important for proteinuric renal diseases)[5] revealed in both siblings the presence of a pathogenic nonsense variant (ACMG/AMP class 5) together with a potential splice disrupting variant (ACMG/AMP class 3) in the *CUBN* (NM_001081.4):c.703 C > T (p.Arg235ter) and c.10363-3A > G (p.?). Cubulin (CUBN) is dominated by 27 contiguous CUB domains, and variants occurring within the C-terminal (after the CUB8 domain) are usually associated with isolated proteinuria. Here, p.R235* led to truncation before CUB8, but none of the patients presented with low levels of vitamin B12 and megaloblastic anemia Enalapril was therefore discontinued in both patients.

DISCUSSION

The proximal tubule reabsorbs large amounts of low-molecular-weight proteins but also albumin and electrolytes from the glomerular filtrate. Megalin (LRP2), cubilin (CUBN), and amnionless protein (AMN) are located in the apical part of proximal tubular cells and are responsible for receptor-mediated endocytosis of proteins filtered through the glomerular barrier[3]. Cubilin has been shown to have an essential role in albumin reabsorption and is encoded by the *CUBN* gene [Figure 1][6]. Biallelic pathogenic variants in the *CUBN* gene cause Imerslund-Gräsbeck syndrome (OMIM 261100), also called selective vitamin B12 (cobalamin) malabsorption with proteinuria[6]. In this syndrome resulting in megaloblastic anemia responsive to parenteral vitamin B12 therapy, half of the patients present with mild proteinuria and normal eGFR. The mechanism of megaloblastic anemia is a defect in the receptor of the vitamin B12-intrinsic factor

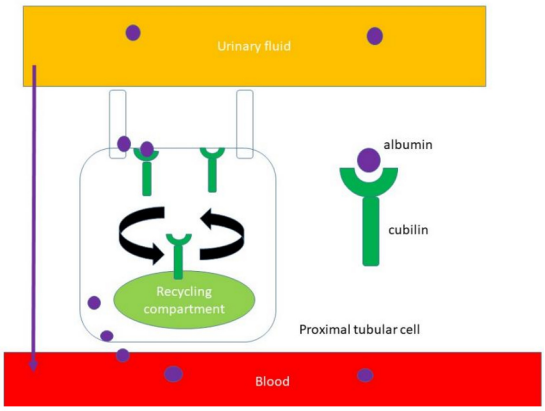


Figure 1. Albumin reabsorption in proximal tubular cells. Cubilin is located in the apical part of proximal tubular cells and is responsible for receptor-mediated endocytosis of albumin filtered through the glomerular barrier. After the decoupling of cubulin-albumin ligation, albumin is then released from the basolateral cell surface into the circulation.

complex in the ileal enterocyte. CUBN and AMN proteins represent the two subunits of this receptor. In patients with Imerslund-Gräsbeck syndrome, proteinuria persists over decades[7,8]. Most *CUBN* pathogenic variants are located in the N-terminal half of the cubilin gene [Figure 2].

Recently, bilallelic pathogenic variants in the C-terminal domain of *CUBN* were described as leading to isolated chronic proteinuria[4]. Indeed, Bedin et al. identified 39 patients with biallelic *CUBN* variants among 2216 individuals with suspected genetic kidney disease including proteinuric patients. Proteinuria ranged from 0,5-3 g/day with an average age at discovery of 10.9 years. When measured, albuminuria represented more than half of proteinuria and β2-microglobulin urine level was low or absent. Kidney biopsies were available in 19 patients and did not show any specific lesion in 11 patients. Four kidney biopsies had electronic microscopy (EM) evaluation, two were normal and two revealed glomerular synechiae. The use of ACE inhibitors did not lower proteinuria which remained stable over years. eGFR was normal in all patients, even those older than 50 years. Bedin et al. also identified a phenotype-genotype correlation. Indeed, variants located after the CUB8 domain (included in the vitamin B12/intrinsic factor binding region) lead to isolated proteinuria, whereas variants located before the CUB8 domain lead to Imerslund-Gräsbeck syndrome, a finding suggesting that there are separate binding sites in cubilin for vitamin B12–intrinsic factor (VitB12-IF) and albumin but the precise location of the binding sites for albumin remains unclear [Figure 2]. The latter should thus bind to more carboxy-terminal CUB domains. However, the isolated proteinuria caused by the p.R235* variant located before CUB8 and leading to premature truncation of cubilin illustrates how it remains complex to determine with certainty the phenotype. In addition, four specific C-terminal variants previously showed strong associations with albuminuria in GWAS[9-13]. These *CUBN* variants were associated with higher eGFR in Bedin *et al.* study[4].

In another recent cohort, Domingo-Gallego *et al.*[14] identified 15 patients with mild proteinuria (0.5-1.8 g/day) having homozygous or compound heterozygous pathogenic variants in the C-terminal CUBN protein. In most cases, proteinuria was detected incidentally, as in our patients. They confirmed the glomerular nature of proteinuria, normal kidney histology, lack of response to RAS blockade, and preserved eGFR in adulthood. Six children from Turkey were also identified with biallelic *CUBN* pathogenic variants located at the C-terminal domain of the protein[15]. One child had a second kidney biopsy 3 years after the first normal kidney biopsy. This second biopsy revealed one periglomerular fibrosis among 27 glomeruli. Yang *et al.*[16] also reported glomerulosclerosis and effacement of foot processes in podocytes on electronic microscopy

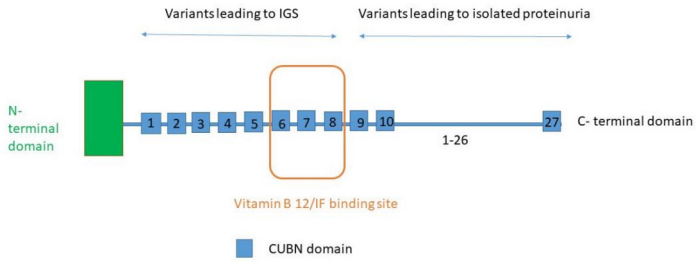


Figure 2. Cubilin protein structure. Cubilin (CUB) is a 460 kDa glycoprotein without transmembrane domain. It acts as a receptor for intrinsic factor-vitamin B12 complexes. There are 27 CUB domains. Intrinsic factor and vitamin B12 binding region is located in domains 5 to 8. The precise location of the binding sites for albumin remains unclear, but it is supposed to be located in the CUB domains near the C-terminal area. IGS : Imerslund-Grasbëck syndrome; IF : intrinsic factor.

(EM) in three children with *CUBN* pathogenic variants. The authors suggested a role of podocyte dysfunction together with a defect in the re-uptake of albumin in the proximal tubule of patients with CUBN mutations. These data need to be confirmed. Indeed, these structural changes in podocytes were absent in two children on EM[17]. These authors showed that *CUBN* variants induce changes in the scaffolding capabilities of cubilin protein *in vitro*. These changes reduce the interactions between CUB and AMN, leading to an aberrant localization of AMN in the cytoplasm of proximal tubular cells instead of the cell membrane. This may interrupt the receptor-mediated endocytosis that re-uptakes filtered albumin.

In conclusion, in the absence of functional cubilin in proximal tubular cells, albumin reabsorption is incomplete and this leads to mild albuminuria. This mechanism acts downstream of the glomerular barrier and does not affect the intraglomerular pressure and is thus not expected to damage podocytes. This explains why anti-proteinuric agents such as ACE inhibitors do not succeed in lowering this particular proteinuria. Detection of *CUBN* pathogenic variants is crucial in clinical nephrology because it prevents unnecessary kidney biopsies but also the use of RAS blockers and their potential side effects such as symptomatic hypotension or rarely angioneurotic edema. The benign course of this disease needs to be confirmed by a longer follow-up.

DECLARATIONS

Authors' contributions

Contributed to the concept, design, draft, and revision of this manuscript: Gillion V, Dahan K, Godefroid N

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Roscioni SS, Lambers Heerspink HJ, de Zeeuw D. Microalbuminuria: target for renoprotective therapy PRO. *Kidney Int* 2014;86:40-9. PubMed
2. Hemmelgarn BR, Manns BJ, Lloyd A, et al; Alberta Kidney Disease Network. Relation between kidney function, proteinuria, and adverse outcomes. *JAMA* 2010;303:423-9. DOI
3. Quinlan C. CUBN variants uncouple proteinuria from kidney function. *Nat Rev Nephrol* 2020;16:135-6. DOI PubMed
4. Bedin M, Boyer O, Servais A, et al. Human C-terminal CUBN variants associate with chronic proteinuria and normal renal function. *J Clin Invest* 2020;130:335-44. DOI PubMed PMC
5. Knoers N, Antignac C, Bergmann C, et al. Genetic testing in the diagnosis of chronic kidney disease: recommendations for clinical practice. *Nephrol Dial Transplant* 2022;37:239-54. DOI PubMed PMC
6. Amsellem S, Gburek J, Hamard G, et al. Cubilin is essential for albumin reabsorption in the renal proximal tubule. J Am Soc Nephrol; 2010. 1859-67 DOI PubMed PMC
7. Gräsbeck R. Imerslund-Gräsbeck syndrome (selective vitamin B(12) malabsorption with proteinuria). *Orphanet J Rare Dis* 2006;1:17. DOI
8. Aminoff M, Carter JE, Chadwick RB, et al. Mutations in CUBN, encoding the intrinsic factor-vitamin B12 receptor, cubilin, cause hereditary megaloblastic anaemia 1. *Nat Genet* 1999;21:309-13. DOI
9. Ahluwalia TS, Schulz CA, Waage J, et al. A novel rare CUBN variant and three additional genes identified in Europeans with and without diabetes: results from an exome-wide association study of albuminuria. *Diabetologia* 2019;62:292-305. DOI PubMed PMC
10. Böger CA, Chen MH, Tin A, et al; CKDGen Consortium. CUBN is a gene locus for albuminuria. *J Am Soc Nephrol* 2011;22:555-70. DOI
11. Haas ME, Aragam KG, Emdin CA, et al; International Consortium for Blood Pressure. Genetic association of albuminuria with cardiometabolic disease and blood pressure. *Am J Hum Genet* 2018;103:461-73. DOI PubMed PMC
12. Zanetti D, Rao A, Gustafsson S, Assimes TL, Montgomery SB, Ingelsson E. Identification of 22 novel loci associated with urinary biomarkers of albumin, sodium, and potassium excretion. *Kidney Int* 2019;95:1197-208. PubMed PMC
13. Teumer A, Tin A, Sorice R, et al; DCCT/EDIC. Genome-wide association studies identify genetic loci associated with albuminuria in diabetes. *Diabetes* 2016;65:803-17. DOI
14. Domingo-Gallego A, Pybus M, Madariaga L, et al. Clinical and genetic characterization of a cohort of proteinuric patients with biallelic CUBN variants. *Nephrol Dial Transplant* 2022;37:1906-15. DOI
15. Cicek N, Alpay H, Guven S, et al. Clinical and genetic characterization of children with cubilin variants. *Pediatr Nephrol* 2023;38:1381-5. DOI
16. Yang J, Xu Y, Deng L, et al. CUBN gene mutations may cause focal segmental glomerulosclerosis (FSGS) in children. *BMC Nephrol* 2022;23:15. DOI PubMed PMC
17. Gan C, Zhou X, Chen D, et al. Novel pathogenic variants in CUBN uncouple proteinuria from renal function. *J Transl Med* 2022;20:480. DOI PubMed PMC

Scherman. *Rare Dis Orphan Drugs J* 2023;2:12
DOI: 10.20517/rdodj.2023.01

Rare Disease and Orphan Drugs Journal

Review

Open Access

RNA antisense and silencing strategies using synthetic drugs for rare muscular and neuromuscular diseases

Daniel Scherman[1,2]

[1]Foundation for Rare Diseases, 96 rue Didot, Paris 75014, France.
[2]Université Paris Cité, Faculté de sciences pharmaceutiques et biologiques, Unité de Technologies Chimiques et Biologiques pour la Santé (UTCBS), CNRS UMR8258, Paris 75014, France.

Correspondence to: Prof. Daniel Scherman, Faculté de sciences pharmaceutiques et biologiques, Unité de Technologies Chimiques et Biologiques pour la Santé (UTCBS), CNRS UMR8258, Inserm U1267, 4, avenue de l'observatoire, Paris 75014, France. E-mail: daniel.scherman@parisdescartes.fr

How to cite this article: Scherman D. RNA antisense and silencing strategies using synthetic drugs for rare muscular and neuromuscular diseases. *Rare Dis Orphan Drugs J* 2023;2:12. https://dx.doi.org/10.20517/rdodj.2023.01

Received: 12 Jan 2023 **Revised:** 16 May 2023 **Accepted:** 25 May 2023 **Published:** 5 Jun 2023

Academic Editor: Gillian Butler-Browne **Copy Editor:** Yanbing Bai **Production Editor:** Yanbing Bai

Abstract

Rare diseases occur in their large majority from a genetic cause, which makes them good candidates for genetic RNA drugs. The basic concepts, principles, mechanisms of action and chemical optimizations of synthetic antisense oligonucleotides (ASO) and small interfering RNA (siRNA) are illustrated. These drugs act either by leading to RNA degradation, or as steric blockers of RNA translation, microRNA antagonists, splicing modulators or inducers of exon skipping. Chemical modifications and delivery techniques differ and are adapted to their distinct functions. The successes, potential, and challenges of synthetic RNA drugs are illustrated for several muscular and neuromuscular diseases: Duchenne muscular dystrophy, spinal muscular atrophy, transthyretin amyloidosis, Type 1 myotonic dystrophy, centronuclear myopathy, oculopharyngeal muscular dystrophy.

Keywords: Antisense oligonucleotide, neuromuscular disorders, rare disease, RNA drug, RNA interference, small interfering siRNA

INTRODUCTION

In the last century, Paul Ehrlich and Emil Fischer introduced the concepts of chemotherapy, magic bullet, and lock-key, in which a drug is defined as a small molecule that specifically binds to a biological target through a three-dimension spatial recognition pattern[1,2]. With the advancement of genetics and the sequencing of the human genome, the genetic defects being the cause of rare genetic diseases are being increasingly elucidated. This has led to the emergence of a new class of "genetic" drugs. In addition to binding to their target through a 3-dimensional hydrogen bonding recognition pattern, these drugs recognize a 1-dimensional linear genetic sequence. In such cases, drug design is based on genetic information, which opens the perspective of the fast and rational development of drugs against a considerable number of diseases characterized by a specific genetic defect.

Theoretically, any gene or mRNA can be targeted by a proper nucleotide sequence selected to be unique on a given genome or transcriptome. Thus, such a sequence recognition mechanism opens tremendous perspectives in medicinal chemistry. Instead of a painstaking customized search for specific spatial recognition of a targeted biological ligand by a chemical compound or a monoclonal antibody, genetic drugs are based on a robust universal platform that can be used for a very large number of applications. Protein targets that were until now considered "non-druggable" can be challenged on their genetic expression. In addition, the use of a common validated technology for all antisense oligonucleotides (ASO) or all small interfering RNA (siRNA) allows for shortening pharmaceutical development steps and reducing costs, which is of paramount importance for rare diseases. In the case of ultrarare diseases, personalized therapy can now be envisioned with these revolutionary platforms for an n-of-1 patient[3].

The basic concepts, principles, mechanisms of action, and chemical optimizations of ASOs and siRNAs are illustrated in the present review. These RNA-targeted drugs can cause RNA degradation or act as steric blockers. In the latter case, they can inhibit RNA translation, antagonize a miRNA, modulate splicing, or induce exon skipping. Their specific divergent functions correspond to adapted chemical and delivery optimizations. Thus, RNA drugs are able either to restore a therapeutic mRNA or, in most cases, suppress mRNA or block mRNA translation to correct "gain-of-function" dominant genetic disorders. But another important application is that of splice modulator, which can overcome a nonsense mutation and lead to the expression of a functional, although shortened, therapeutic protein.

Out of the scope of the present review are the cell and gene therapy approaches compensating for a genetic deficiency by replacing the wild-type correct gene. We will not detail either the more recent revolution of genome editing, in which a genetic defect is corrected "*in situ*" by tools such as clustered regularly interspaced short palindromic repeats (CRISPR) associated with the Cas9 (CRISPR-Cas9)[4]. Finally, the present review does not intend at an extensive description of the clinical trials realized so far, as this aspect has been extensively reviewed elsewhere[5-8].

REVIEW

Steric blocker ASOs

The first RNA drug was introduced in 1978 against Rous sarcoma viral replication[9,10]. It used a 13-mer synthetic antisense oligonucleotide (ASO). It was an oligodeoxynucleotide complementary to 13 nucleotides of the 3'- and 5'-reiterated terminal sequences of Rous sarcoma virus 35S RNA and annealing to it through Watson-Crick recognition. This creates an intracellular DNA/RNA heteroduplex which may either function as a translation steric blocker or lead to mRNA degradation. The ASOs acting as steric blockers of translation target the mRNA translation initiation site (start codon). This inhibits the binding of ribosomal subunits to the mRNA through steric hindrance, thus blocking protein synthesis [Figure 1A]. While this

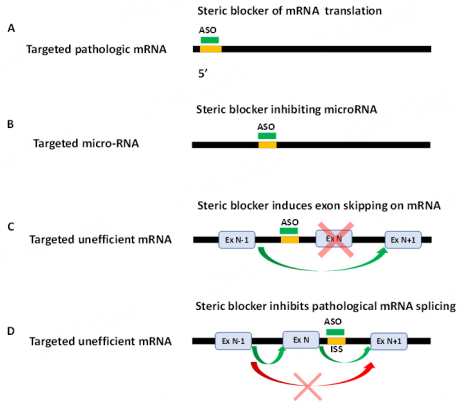


Figure 1. Non-RNase H-dependent ASO steric blocking effects. In yellow is indicated the complementary targeted sequence on the mRNA. ISS: Intronic splice silencer. A: mRNA Translation inhibition; B: Antagomir; C: Inducing skipping of a pathologic exon carrying stop or out-of-frame mutations; D: Splice modulator inhibiting a non-desired naturally occurring exon skipping.

antisense blocking effect on mRNA translation might be responsible for part of the observed decreased protein expression, it was later found that the loss of protein expression was also due to mRNA degradation induced by RNase H nuclease. This RNase specifically recognizes heteroduplexes and cleaves the associated mRNA. This nucleolytic mechanism of action drives most of the actual ASOs' clinical applications.

Antisense ASOs have the potential to induce numerous other steric blocking effects, which has led to major therapeutic successes. These are schematized in Figure 1.

MicroRNA (miRNAs) are a group of one to two thousand small non-coding RNA molecules containing 21 to 23 nucleotides. They play important biological functions as post-transcriptional regulators of gene expression. The miRNAs anneal to complementary sequences on mRNA molecules, leading to gene silencing by several mechanisms: mRNA cleavage mediated by the RNA-induced silencing complex RISC (see below the detailed section on RNA interference), mRNA destabilization by poly(A) tail shortening, or blocking of mRNA translation. As shown in Figure 1B, ASO steric blocking can antagonize these cellular "bandmasters", and thus display an antagomir action, which presents interesting therapeutic applications in neuronal and neuromuscular diseases[11].

An antisense blocker ASO can be used by sterically hindering a splice acceptor or splice enhancer site, thus promoting exon-skipping [Figure 1C]. This mechanism of action is of great interest for treating genetic diseases caused by stop or out-of-frame missense variants, and where the skipping of one or o several exons leads to a still functional or partially functional truncated form of the protein product. This strategy has led to clinically approved ASO RNA drugs for Duchenne Dystrophy. Exon skipping can also be of interest in cases where a pathologic alternative splicing of variant genes occurs, which leads to the inclusion of an additional exon, causing the expression of a non-functional protein. Finally, blocker ASOs can also treat cases where an intronic pathogenic variant results in aberrant inclusion of an intron segment into the mRNA transcripts, thus abolishing protein function.

Figure 1D displays the use of an ASO to inhibit a naturally occurring undesired splicing event leading to exon N skipping and translation of a non-functional truncated protein. By blocking this undesired exon skipping, therapeutic restoration of a complete functional protein is obtained. As will be described in Spinal muscular atrophy, this occurs in the case of spinal muscular atrophy (SMA), where a non-functional

truncated form of the SMN2 mRNA is produced. An ASO which sterically blocks the intronic splice silencer present on the SMN2 gene favors pre-mRNA maturation towards the complete form of the SMN2 mRNA, thus leading to the expression of a functional SMN protein. This approach has represented a historically major success as the first therapy for SMA [5,7,8, and more references in *Spinal muscular atrophy*].

ASO-induced mRNA degradation

RNA primers are required to initiate the synthesis of both the leading strand and Okazaki fragments on the lagging DNA strand during genome replication. Moreover, DNA replicases occasionally incorporate ribonucleotides into DNA. Finally, R-loops generated as a by-product of transcription when nascent mRNA molecules hybridize with the template DNA represent another example of naturally occurring RNA/DNA duplexes. Cells are ubiquitously equipped with ribonucleases H type 1 (called here RNase H), whose function is to remove RNA moieties from DNA, because such RNA/DNA duplexes might cause chromosomal instability and cell lethality during replication[12,13]. The RNases H nuclease belongs to the nucleotidyl transferase superfamily which relies on divalent cations to catalyze nucleophilic substitution reactions. These enzymatic reactions specifically hydrolyze either a single ribonucleotide or stretches of RNA in a diverse range of nucleic acids, such as RNA/DNA hybrids, R-loops, and double-stranded DNA with an embedded single ribonucleotide, *etc.*[14]. Other enzymes such as transposase, retroviral integrase, Holliday junction resolvase, and RISC nuclease Argonaute involved in the mechanism of RNA silencing belong to the same nucleotidyl transferase superfamily as RNase H.

As already mentioned, antisense oligodeoxynucleotides annealing to complementary mRNA sequence create an intracellular DNA/RNA heteroduplex to which RNase H binds, leading to cleavage and subsequent degradation of the targeted mRNA [Figure 2A]. After mRNA cleavage at the complementary site, the mRNA strand is released from the ASO, which thus becomes available for further association with another target mRNA.

RNase H-dependent ASOs are of interest for treating diseases caused by dominant-negative genetic variants. An important feature is that RNase H requires a non-modified deoxyribose moiety on the center of the complementary ASO sequence (the "seed" sequence) to maintain its catalytic efficiency. Thus, an efficient ASO must be either a pure deoxy oligomer bearing only unmodified ribose sugars, or a gapmer containing a stretch of about 10 natural ribose sugars with a variable number of modified sugars on the 3' and 5' ends.

mRNA degradation by RNA interference RNAi

The second strategy to suppress a targeted mRNA in a sequence-specific manner makes use of the RNA interference process (RNAi)[15-18]. As indicated above, interference is the natural microRNA-mediated process (mi-RNA) that is central to the post-transcriptional silencing regulation of many basic cellular and developmental programs. This process is schematized in Figure 3.

The basic steps of miRNA maturation involve the transcription by RNA polymerase II of primary miRNAs (pri-miRNAs). The miRNAs derive from regions of RNA transcripts that fold back on themselves to form short hairpins whose main characteristic is the presence of one or several mismatches in the self-complementary sequence. The primary transcript known as pri-miRNA is processed in the cell nucleus into ~70 nucleotides pre-miRNA by the microprocessor complex Drosha/DGCR8. The microprocessor complex subunit DGCR8 (DiGeorge syndrome critical region 8) contains an RNA-binding domain that stabilizes the primary miRNA for processing by the second microprocessor subunit Drosha, an RNase III enzyme. The pri-miRNA is cleaved by the Drosha/DGCR8 complex to a characteristic stem-loop structure known as a pre-miRNA. Pre-miRNAs have a hairpin structure with stems containing interspersed mismatches and are

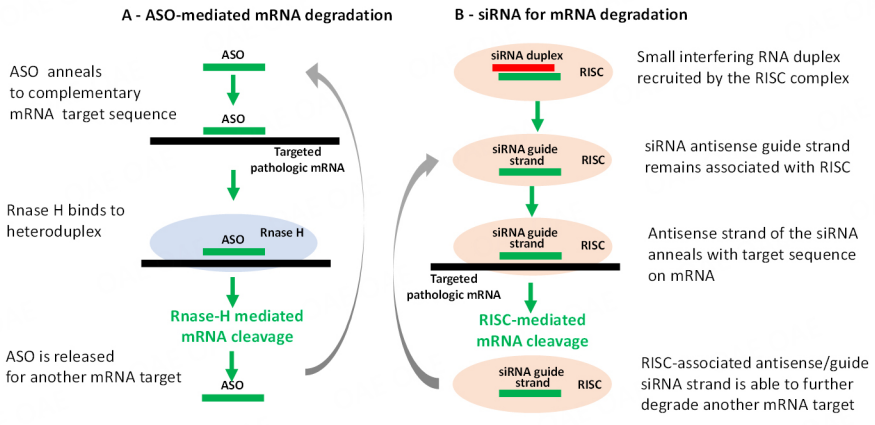


Figure 2. Comparison between the mechanism of action of genetically targeted mRNA degradation. A (left): ASO-mediated mRNA degradation through RNase H1 nucleolytic activity. ASO (green rod) binds to complementary sequences on mRNA. This heteroduplex induces RNase H binding and leads to mRNA cleavage. After mRNA cleavage, the mRNA fragments, ASO and RNase H are released from each other. Grey arrow: free ASO is recycled for binding to another mRNA on the target sequence, which induces a new cycle of RNase H binding and mRNA cleavage; B (right): siRNA-mediated mRNA degradation. Double-stranded homoduplex siRNA is recruited by the RISC complex. The passenger "sense" strand (red rod) is released from the complex. The guide strand (green rod) remains positioned in the RISC complex and stabilizes it on the targeted complementary mRNA sequence by Watson-Crick hybridization. This induces mRNA cleavage. The cleaved mRNA is dissociated, and the RISC/guide siRNA complex can associate and cleave another mRNA (grey arrow).

exported across the nuclear envelope to the cytosol by binding to exportin 5.

The next step occurs through a cytoplasmic process where the riboendonuclease Dicer mediates the cleavage of the pre-miRNA at the base of the stem (removing the terminal loop) to form the double-stranded mature miRNA duplex[19,20]. Then, the RNA Induced Silencing Complex (RISC) eliminates the miRNA sense passenger strand and keeps binding to the antisense strand. This antisense strand is also called the "guide" strand because it allows the specific binding of the RISC complex to the complementary Watson-Crick sequence on the targeted mRNA. The RISC complex is composed of the protein TRBP and the argonaute 2 nuclease (Ago).

Natural miRNA target sites tend to cluster in the mRNA 3' untranslated region, although other miRNA sites are being found in different mRNA regions. The binding of miRNA/RISC leads to the repression of mRNA translation and, in a certain proportion, to mRNA cleavage by Ago. The absence of mismatch induces complete mRNA cleavage, while the presence of mismatch favors translation repression.

For therapeutic use of the endogenous miRNA process, two strategies have been employed. In the first one, an expression cassette containing a short gene encoding a self-complementary mRNA is delivered to the cells. The resulting mRNA transcript forms a short hairpin (shRNA) [Figure 3 right side], which is processed by the Drocha/DGCR8 complex. The shRNAs have a completely base-paired 19-29 nt stem, ensuring the Dicer cleavage step to produce the final siRNA. The resulting siRNA duplex has symmetric 2 nucleotide 3' overhangs with a 19-21 base-paired region. It associates with the RISC ribonucleoprotein complex leading RNAi[21]. In most published cases, shRNA transgenes are delivered using either an AAV or a lentiviral gene delivery vector. The shRNA technique is widely used at the laboratory stage because it is available through standard expertise and ensures continuous intracellular siRNA production and efficient silencing.

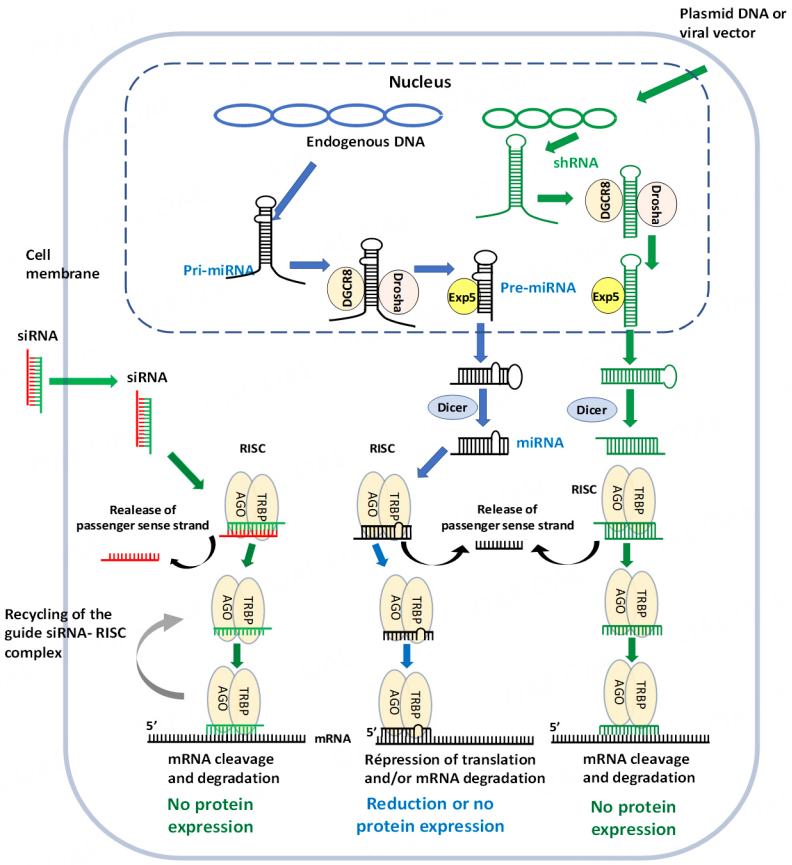


Figure 3. Mechanism of miRNA, siRNA, and shRNA action. The Center (blue arrows) represents the canonical miRNA maturation and mechanism of action pathway. Transcription leads to a pri-miRNA, which folds into a small hairpin RNA with one or several mismatches. The pri-miRNA is processed by Drosha and DGCR8 to a pre-miRNA. After association to exportin 5, the pre-miRNA is transported through the nuclear envelope by a process dependent upon the small GTPase ran-GTP. The pre-miRNA is finally processed by the Dicer endoribonuclease (a class III RNase), which deletes the hairpin loop. This generates an RNA duplex made of a passenger and a guide strand. After miRNA homoduplex association with the RISC complex composed of protein TRBP and the argonaute 2 nuclease, the passenger strand is eliminated. The RISC/guide strand complex binds to the target mRNA, in the 5' non-translated region, inducing a steric blocking of ribosome entry and translation. In some instances, miRNA binding can also lead to mRNA cleavage and degradation, but the mismatch generally make this less likely. Left side: siRNA mechanism. (green arrows), a double-strand siRNA with no mismatch is administered to the cells. All action takes place in the cytosol. After siRNA binding to the RISC complex composed of protein TRBP and the argonaute 2 nuclease, the passenger strand is eliminated. The RISC/guide strand complex binds to the target mRNA. The target sequence is generally chosen within the translated region. Because of the complete siRNA matching with the mRNA target sequence, argonaute 2 is now able to cleave the target mRNA with great efficiency. After mRNA cleavage and elimination, the RISC/guide siRNA complex can bind and cleave another mRNA (grey arrow). Right side: (green arrows): shRNA mechanism. A gene expression cassette coding a small hairpin RNA is administered either as a plasmid or by a viral gene delivery vector. The shRNA transcript is then processed as for the miRNA. However, no mismatch is introduced, which leads to fully efficient mRNA degradation and silencing.

The second RNAi therapeutic strategy uses exogenous siRNAs, which are made of synthetic 20-25 base pairs. As shown in Figure 2B and Figure 3 left side, their mechanism of action also involves RISC. The siRNAs are administered as two-strand perfect matching Watson-Crick homoduplexes. One "antisense" strand, which is also called the "guide" strand, is complementary to a sequence on the targeted mRNA. In addition, both 3' ends possess two supplementary over-hanging non-hybridized nucleotides.

Such an siRNA homoduplex is recognized by the RISC nucleoprotein complex which dissociates the sense siRNA strand. As with miRNAs, the RISC complex is then guided by the antisense strand to bind to the complementary sequence on the targeted mRNA, leading to mRNA nucleolytic cleavage and degradation [

Figure 2B and Figure 3, left side]. Since the guide sense is designed for a perfect match with its complementary sequence on the targeted mRNA, the Ago-mediated mRNA cleavage is complete.

Once this has occurred, the RISC /guide siRNA complex can be used again to target and cleave another pathologic mRNA. This recycling process associated with optimized chemistry enabling high siRNA metabolic stability ensures a long duration of action[22]. Indeed, *in vivo* effects have been reported for the most recently developed siRNA drugs with a spectacular duration of action superior to 6 months after a single administration[23].

Comparison between ASO and RNAi properties

The differences between natural phosphodiester ASOs and siRNAs are summarized in Table 1.

The ASO and siRNA characteristics and specificities listed in Table I are responsible for marked differences in physicochemical and pharmacokinetic properties which dictate different required chemical optimization, depending on the nature of the drug and the targeted tissue. A single-stranded ASO is more flexible and accessible to an endonuclease and thus necessitates complete protection of each phosphodiester linkage against nucleases. By contrast, a double-helix siRNA shows more resistance in its internal phosphodiester linkages. A single-stranded ASO also displays hydrophobic moieties from its nucleic bases, which is not the case for double-helical siRNA. Hence, ASOs will bind to plasma proteins such as albumin, which leads to longer circulation time and enhanced biodistribution to tissues. On the contrary, natural siRNAs are rapidly eliminated by kidney filtration and need a delivery vector such as a lipid nanoparticle (LNP) formulation or a functional targeting moiety towards an extracellular receptor.

Other differences originate from the fact that only binding to the target mRNA is required to achieve a blocker ASO function, as for mRNA degradation, the catalytic activity of RNase H (for ASOs) and of Argonaute 2 (for siRNAs) must be maintained. Finally, it is generally considered that ASOs can block or cleave both pre-mRNA and mRNA either in the nucleus or cytosol, while siRNA ensures the degradation of the mature mRNA in the cytosol only. This represents an important point for diseases caused by nuclear aggregation of variant mRNA, such as Myotonic Dystrophy Type 1 (MD1) (see section *Type 1 myotonic dystrophy: different ASO modes of action*). This is, however, a disputed point, because several siRNAs and shRNAs have been shown to lead to nuclear foci degradation in MD1 cellular and animal models[24-26] thus suggesting that the RISC-induced cleavage and subsequent degradation could also occur in the nucleus.

Chemical optimization of steric blocker ASOs

After their initial discovery, the first enthusiastic attempts towards the clinical use of both ASOs and siRNAs were very disappointing, leading to a drastic decrease in the investment into these genetic pharmacology drugs. These initial approaches used natural oligonucleotides, which have poor pharmacokinetics because they undergo fast degradation by endo- and exonucleases. They are degraded in less than 20 minutes after intravenous administration. In addition, these first-generation ASOs and siRNAs were rapidly eliminated by kidney filtration. This decreases tissue biodistribution and might induce undesirable glomerular toxicity resulting from temporary elevated local concentrations of these biologically active agents. Moreover, natural oligonucleotides are small hydrophilic polyanionic compounds due to the phosphodiester linkages, which hinder cell penetration through the lipophilic plasma membrane. Finally, innate immune response to double-stranded RNA, especially through the TLR3 receptor[27-30], was found to represent a strong bottleneck for the development of siRNA drugs. All these considerations led to the conclusion that chemical modifications were required for the ultimate success of an ASO or siRNA drug.

Table 1. Comparison between natural phosphodiester ASOs and Double-stranded siRNA

ASO	siRNA
Single-stranded	Double-stranded
14-20 bases - linear deoxynucleotide	21-23 base pairs RNA
Flexible with ~ 1 nm width	Rigid duplex ~ 2 nm diameter
Single-stranded nature requires full backbone modification with phosphorothioate (PS) linkages to protect from nucleases	Double-stranded nature ensures relative protection against nucleases
Design must retain ribose sugars moieties in the seed center sequence to allow RNase H activity	Ribose sugars can be modified to a certain limit with respect to Argonaute efficiency
Sugar modifications are tolerated only on wings of a gapmer	Sugar modifications tolerated
Hydrophobic surfaces accessible for protein interactions allow binding to plasma proteins such as albumin and increase blood circulation and biodistribution to tissues.	Little exposed hydrophobic surface since aromatic bases are paired and buried in duplex. Hydrophilic surface causes rapid kidney clearance.

ASO: antisense oligonucleotides; siRNA: small interfering RNA.

Many comprehensive excellent reviews have been dedicated to the intensive chemistry efforts performed on ASO or siRNA derivatives, which have concerned their different components: backbone, sugar, and base)[31-33]. The state-of-the-art will be briefly described here. The most successful chemical modifications introduced so far on ASO and siRNA nucleotides are displayed in Figure 4. Because of their different properties and mode of action, different chemical modifications have been selected after more than 20 years of intensive research for each class of RNA drug.

The single-stranded nature of ASOs requires full phosphodiester backbone modification because of their high exposure to exo- and endonucleases. Figure 4B displays the most successful modified backbone linkage used so far, phosphorothioate (PS), in which a sulfur atom replaces one non-linking phosphate oxygen and which partially protects against nucleolytic activity. In addition, phosphorothioate modification has been shown to increase pharmacokinetics and cellular uptake[34-36]. Indeed, the first ASO drug introduced to the market was the full 21 nt full phosphorothioate oligodeoxynucleotide Vitravene, which has the sequence 5'-GCG TTT GCT CTT CTT CTT GCG-3' targeting the CMV protein IE2 mRNA[37]. Several other phosphodiester linkage modifications also confer nuclease resistance, for instance, methylphosphonate, phosphoramidate [Figure 4], and mesylphosphoramidate[38,39].

It should be noted that each PS substitution introduces a chiral phosphate with two stereoisomers, the Sp and Rp forms. The Sp diastereoisomers are more resistant to nucleases and should be preferred for a blocker ASO. In addition, the Rp diastereoisomers display a higher binding affinity and induce more efficient RNase H cleavage, which is more favorable for an mRNA degradation strategy. It has been shown that precise localization of Rp and Sp PS links could optimize the desired function and increase selectivity by decreasing off-target mismatch binding[29].

While phosphorothioate links confer improved relative nuclease resistance and metabolic stability, the protection is not complete. Moreover, PS links decrease ASO and siRNA binding affinity to the complementary mRNA. This can be corrected by introducing modifications to the ribose moiety. Modifications on the 2' carbon, such as 2'-fluoro, 2'-O-methyl or 2'-O-metoxyethyl are extensively used for both ASOs and siRNAs [Figure 4C]. Another way to increase nuclease resistance is by introducing constraints in the ribose ring, such as in the bicyclic locked nucleic acids (LNA) or in the constrained ethyl locked nucleic acids (cet-LNA).

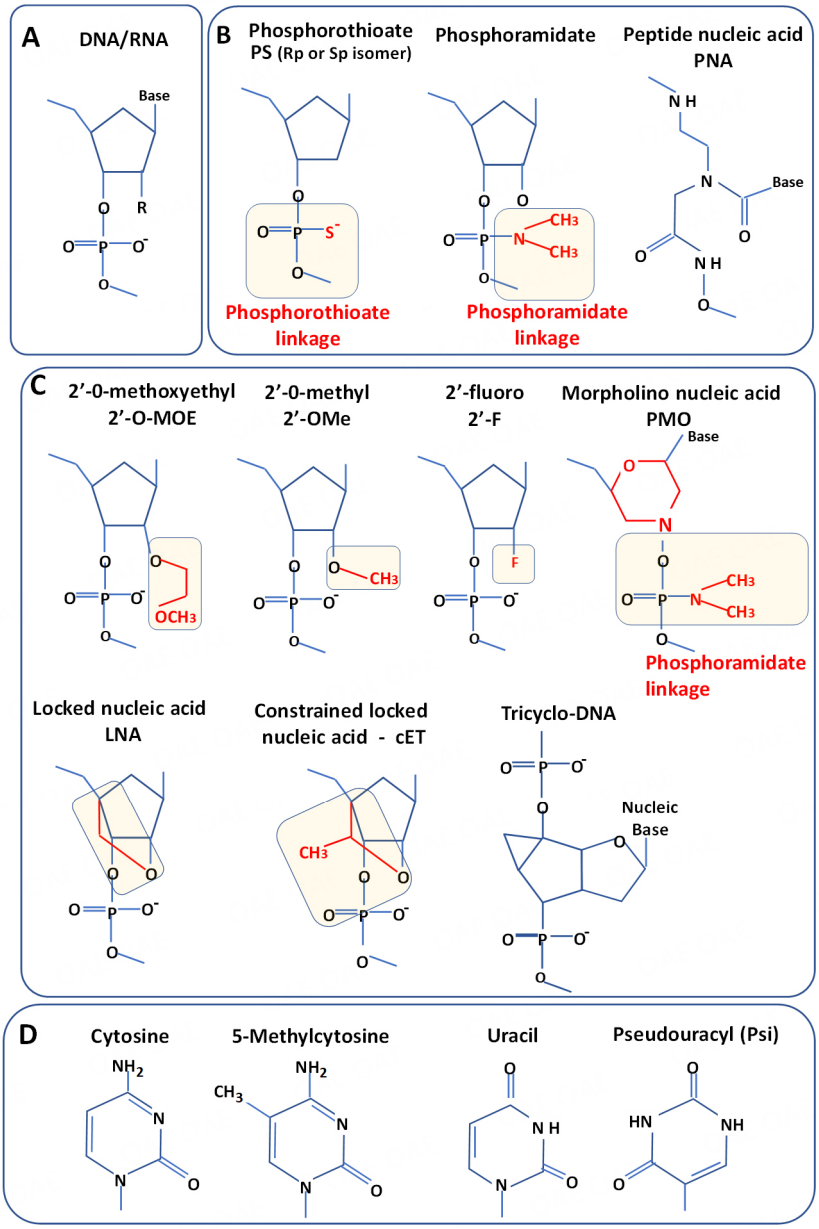


Figure 4. Most popular nucleotide analogs used in ASO and siRNA drugs. A: Natural DNA (R = H) and RNA (R = OH) units; B: linkage analogs. C: Ribose analogs; D: Base analogs. 5-methyl cytosine is the only one having been used in clinically approved drugs at the present time. Pseudouridine (Psi) is not used in RNA drugs but in mRNA vaccines, because it suppresses the innate immune response against double-stranded RNA stretch.

Other quite original structures, which are further modified from natural ribose or deoxyribose rings, have demonstrated their value in increasing metabolic stability while maintaining a high binding affinity toward mRNA. These are the morpholino, tricyclo-DNA, or PNA analogs [Figure 4C]. Morpholinos nucleic acids (commonly designated as PMOs) are methylenemorpholine rings linked through phosphorodiamidate groups instead of phosphates[40]. They display a strong affinity for mRNA leading to an efficient steric blocking effect[41]. Preclinical and clinical studies have shown that PMOs demonstrate improved efficacy, excellent kinetic behavior, biological stability, and a good safety profile[42]. Tricyclo-DNA oligomers display strong antisense and exon-skipping activity[43-44]. PNAs are peptide nucleic acids where the ribose-

phosphodiester backbone has been replaced by the peptide linkage analog N-(2-aminoethyl)-glycine units, which allows using convenient peptide synthesis technology[45]. However, it has been reported that PNAs had the disadvantage of rapid kidney elimination[46].

Finally, base modifications have been introduced, such as the base analog 5-methylcytosine, because this increases nuclease resistance while reducing innate immune response [Figure 4D]. However, the risk of genomic incorporation of these non-natural bases has hampered up to now their clinical use, except for 5-methyl cytosine, while pseudouridine (Psi) is not used in RNA drugs but in mRNA vaccines to alleviate the innate immune response against double-stranded RNA stretches.

More chemical refinements have been proposed to improve the pharmacokinetics and blocking properties of ASOs. For instance, it was found that a mixture of LNA with 2'O-methyl and 2'F nucleotide together with a PS backbone was most efficient in inhibiting miRNAs[47]. Such mixed oligomers are called "mixmers" and are schematized in Figure 5.

Other means to improve ASOs bioavailability to the desired tissue and cells imply nanoparticle encapsulation (see section *Type 1 myotonic dystrophy: different ASO modes of action*), or covalent coupling to a penetration enhancer or to a targeting moiety. Coupling morpholino nucleic acids to a peptide rich in alanine and the cationic amino-acid arginine has been reported to increase tissue delivery and the efficacy of exon skipping or exon restoration in models of Duchenne dystrophy and spinal muscular atrophy[48,49]. Linking ASO to a fatty acid chain showed promising results in a spinal muscular atrophy model[50]. The use of triantennary N-acetyl-galactosamine (GalNac) for targeting and high-performance delivery to liver hepatocytes via the asialoglycoprotein receptor (ASGPR) represents one of the most successful strategies, both for ASOs and siRNAs. It has led to impressive therapeutic achievements[51-54] (see below *Tri-GalNac siRNA Vutrisiran for transthyretin hereditary amyloidosis treatment*). Attempts to deliver ASOs through the intestine and blood-brain barrier have been reported[55-56].

Enhanced bioavailability and nuclease resistance are sufficient conditions for achieving the distinct ASOs therapeutic mechanisms of cation illustrated in Figure 1: steric block of mRNA translation, microRNA inhibition (antagomir effect), exon skipping, and exon restoration. Several FDA-approved drugs demonstrate the success of these chemical modifications for treating rare diseases, which will be further detailed below for muscular and neuromuscular diseases.

ASO chemical optimization for RNase H - induced mRNA cleavage

The necessity to maintain RNase H activity represents a major constraint that limits the use of many of the chemical modifications presented above. Phosphorothioate linkages are compatible with RNase H activity. Inversely, methylphosphonate substitution must be finely optimized. In a typical model study, duplexes formed with deoxy oligonucleotides or phosphorothioate analogs were allowing mRNA cleavage by RNase H, whereas a duplex formed with an oligonucleotide containing six methylphosphonate deoxynucleosides alternating with normal deoxynucleotides was not permissive to RNase H attack. The mRNA susceptibility to cleavage by RNase H increased in parallel to a reduction in the number of methylphosphonate linkages[38].

Sugar modifications such as morpholino or LNA are not tolerated by RNase H. Uniformly modified 2'-deoxy-2'-fluoro phosphorothioate oligonucleotides led to antisense molecules with strong binding affinity, high selectivity for the RNA target, and stability towards nucleases, but they did not support RNase H activity on mRNA. However, the incorporation of a mixture of these modifications into "chimeric"

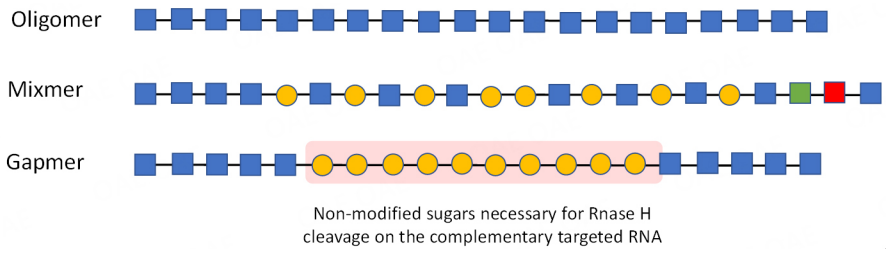


Figure 5. Different types of ASOs developed at the preclinical and clinical stages. Squares, circles, and assorted colors represent the diverse types of nucleotides displayed in Figure 4. The linkage between each nucleotide might also vary, for instance, the phosphorothioate Sp and Rp diastereoisomers. In a gapmer, the yellow circles represent non-modified nucleotides allowing cleavage by RNase H of the annealed complementary mRNA.

oligonucleotides has been shown to activate mammalian RNase H-mediated degradation[57]. Consequently, a sizable proportion of RNase H-dependent ASOs are "gapmers," in which a gap of 10 unmodified deoxyribose is flanked by 3' and 5' "wings" whose partial composition in chemically modified nucleotides leads to metabolic stability, enhanced binding to target, and cellular availability[Figure 5].

A large variety of gapmer geometries can be envisioned. A reduced gap size confers more precision in the cleavage zone and potentially contributes to allele specificity. Different flanking wings have been proposed to increase affinity and selectivity to the target RNA. However, care should be taken that the cleaved mRNA must dissociate to initiate another mRNA degradation. Thus, ASOs' affinity for the mRNA target must not be too high. For instance, with LNA-containing wings, an increased binding affinity has been reported, which leads to an optimal size of 12 to 15 nucleotides[58]. On the opposite, the clinically approved gapmer Inotersen for treating transthyretin amyloidosis is a fully phosphorothioate-modified ASO with five 2'-O-methoxyethyl ribonucleotides on each side, thus consisting of a 5-10-5 structure)[7]. In addition, a too strong affinity might induce off-target binding and RNase H cleavage of mismatched mRNAs (see section *Challenges faced by the synthetic ASO and siRNA technology*). Due to a high ASO concentration observed in the liver, off-target hepatic toxicity has been reported for 2'Fluoro or LNAs[59-61].

Chemical and delivery optimization of siRNAs

Contrary to ASOs which might be efficient as steric blockers with no concomitant nuclease activity, siRNAs efficacy strictly depends on argonaute 2 nucleolytic activity. As for RNase H-dependent ASOs, the modifications introduced for increasing metabolic stability are limited and must be finely optimized for the guide siRNA strand. More freedom for modifications is allowed on the passenger sense strand, which is not involved in the RISC-guided nuclease activity. An additional constraint to be considered is to limit the innate immune response induced by natural RNA duplexes, which has initially represented a major obstacle to the development of the technology.

A useful mapping of the functional domains of a typical siRNA gives guidance on where chemical diversity can be introduced [Figure 6A]. While most siRNA designs are based on a non-covalent two strands double helix [Figure 6A and 6B], corresponding to the presently clinically approved RNA drugs (see *Chemical and delivery optimization of siRNAs*), other concepts have been proposed and shown to possess promising properties at least at the preclinical level, such as di-siRNA composed of two double-helical siRNAs made of one double-sized passenger strand and two guide strands Figure 6C[62]. The geometry developed by the DIcerna company is that of a nicked hairpin [Figure 6D]. While the guide antisense strand is of 22 bases canonical length, the sense passenger strand is longer (36 bases), and it auto-hybridizes through a GC-rich sequence (GCAGCC hybridized to GGCUGC) to form a GAAA loop at its extremity. Hence, a nick of the

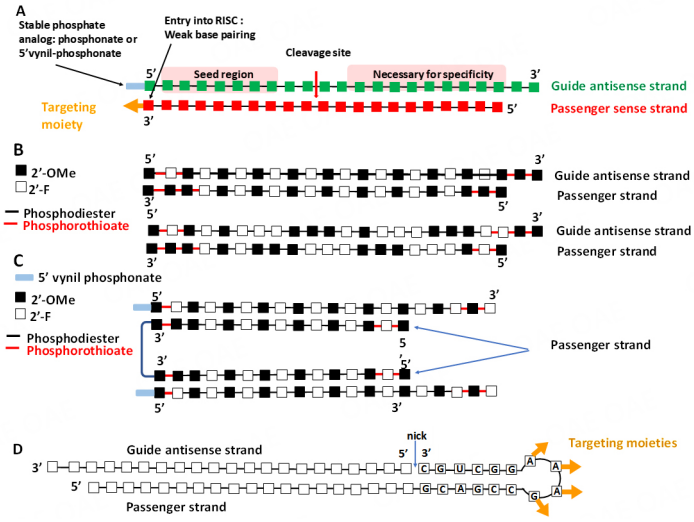


Figure 6. A: The siRNA functional regions. More modifications are tolerated on the passenger strand (red squares) which is not involved in argonaute 2-mediated mRNA cleavage. A targeting moiety can be covalently linked to the 3′ strand (orange arrow). A stable phosphonate resistant to dephosphorylation enzymes can be included instead of phosphate (blue rod) at the 5′end of the guide siRNA (green square). Both are necessary and compatible for binding the guide strand to the RISC complex. Fewer modifications are allowed on the seed region of the guide siRNA strand (nucleotides 2 to 8). An optimized guide strand must contain a flexible 5′ end (which can be obtained by lowering base pairing and facilitates capture by the RISC complex), a high affinity 'seed' region, which drives the initial base pairing between the guide strand and mRNA target, and a lower affinity 3′-region required for cleaved mRNA release. B: Frequently used siRNAs are made resistant to exonucleases by 2 to 3 terminal phosphorothioate linkages on both 3′ and 5′ terminals of each strand. Modified sugars 2′-OMe and 2′-fluoro have led to siRNAs highly resistant to nuclease, of improved bioavailability, and of high binding affinity to the RISC complex. This leads to an unprecedented duration of action which can reach up to 6 months or more after a single administration, due to the recycling mechanism displayed in Figures 4 and 5 inspired from[18,29]. C: Scheme of a di- siRNA[62,145]. D: In this presented geometry, the antisense strand is of 22 bases canonical length, while the sense passenger strand is much longer (36 bases), and it auto-hybridizes through a GC-rich sequence (GCAGCC hybridized to GGCUGC) to form a GAAA loop at its extremity.

hairpin structure is located between the sense and antisense strands[63,64]. Targeting moieties such as GalNac sugars have been linked to the GAAA loop, thus providing a high tetravalent sugar moiety for targeting liver hepatocytes.

The presence of a 5' phosphate on the siRNA guide strand is an essential factor for entry into the RISC complex and loading to the Ago2 nuclease. Stable phosphate analogs, such as phosphonate, have been introduced with a strong enhancing effect [Figure 6A][65]. A non-cleavable targeting moiety can be linked to the passenger strand. With a tri-antennary GalNac targeting head that binds to the hepatocyte asialoglycoprotein receptor, a dramatic increase in liver uptake and silencing efficiency has been observed[66-67], leading to several months silencing effect after a single dose. This represents one of the most exciting perspectives of siRNA therapeutics for liver-associated diseases[53] (see *Exon-skipping ASO for Duchenne muscular dystrophy*).

While 2'fluoro ribose modification is well tolerated by the RISC machinery, phosphodiester linkage substitution by phosphorothioate can only be introduced in a limited number, and this is outside the seed region and cleavage site. Similarly, sugar modifications such as 2'OMe or LNA are more tolerated on the passenger strand, and they are favored at the 5'end because they block passenger strand entry into the RISC complex and consequently favor RISC exclusive loading with the guide strand. Two typical popular siRNA geometries are displayed in Figure 6B. In the upper siRNA, 2'fluoro and 2'OMe are intercalated and face each other to obtain a canonical alpha helix geometry. In the second more widespread example, stretches of 3 consecutive 2'fluoro and 2'OMe nucleotides are present and frequently facing each other. Two to three

phosphorothioate linkages are introduced in the 5' and 3' ends of both strands, which ensures sufficient metabolic stability against exonucleases. Another backbone modification has been recently introduced in the form of a divalent siRNA, in which two passenger moieties are covalently linked. These di-siRNAs display a favorable distribution in the central nervous system and promising efficacy in neurodegenerative disease models in rodent and non-human primates[62].

While the maximum number of 2'fluoro must be controlled because of potential toxicity, other modifications have proven their utility, such as 5' carbon pyrimidines. Moreover, using a systematic iterative screening technology, it has been shown that optimizing the positioning of 2'-deoxy-2'-fluoro and 2'-O-methyl ribose across both strands enhanced metabolic stability. This could be obtained with a low 2'-deoxy-2'-fluoro content[68].

Numerous formulations have been proposed to improve siRNA pharmacokinetics, such as lipid nanoparticles (LNP), a detailed reviewing of which is out of the scope of the present review. Lipid nanoparticles have proven their efficacy in targeting the liver *in vivo*, leading to the clinically approved Patisiran siRNA drug in transthyretin amyloidosis[69,70]. While liver targeting is presently well mastered using either LNP or the GalNac technologies (see*Exon-skipping ASO for Duchenne muscular dystrophy*), challenges remain for other organs, particularly the brain. Nonetheless, progress is being made in terms of oral and ocular delivery[71-73] and intravenous (IV) delivery to inflammatory sites[74-76].

The following sections illustrate the above concepts by a selection of typical examples of ASOs and siRNAs approaches for treating muscular and neuromuscular disorders, and for which either marketing approval or very promising results have been obtained. Since any genetic disease caused by a dominant negative variant might benefit from an RNase H-dependent ASO or a siRNA approach, and since many others might benefit from anti-miRNA or splice modulation properties, this review can by no means be fully exhaustive concerning the ongoing preclinical studies.

Exon-skipping ASO for duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is caused by anomalies in the dystrophin gene located on the X chromosome (Xp21.2). Because the dystrophin gene is the largest known gene in the human genome, genetic variants and deletions occur at a higher frequency than in other genes, and DMD has one of the highest prevalence rates among rare diseases (about 6/100,000). Diagnosis is suspected based on the clinical picture, family history, and laboratory findings (serum creatine kinase being 100-200 times the normal level). Genetic testing is a critical tool for accurate DMD diagnosis (Orpha 98,896)[77].

DMD onset occurs in early childhood, and affected boys may show a delay in walking accompanied by speech and/or global developmental retardation. Autism and behavioral problems, such as ADHD (attention deficit hyperactivity disorder), anxiety, and obsessive-compulsive disorder, are common. Untreated DMD children rarely achieve the ability to run or jump. The condition progresses rapidly, and the child develops a waddling gait and a positive Gowers sign. Proximal muscles are affected first, then distal limb muscle. Climbing stairs becomes difficult and the child falls frequently. Loss of independent ambulation occurs between the ages of 6 and 13 years, the average being 9.5 years in non-steroid treated patients. Once ambulation is lost, joint contractures and scoliosis develop rapidly. Until recently, untreated patients might not survive over late teens to early twenties because of respiratory failure and/or cardiomyopathy, but life expectancy is increasing with adapted cardiac care and assisted ventilation[78].

DMD belongs to the larger group of rare genetic progressive muscular dystrophies called dystrophinopathies, which also include Becker muscular dystrophy (BMD) and a symptomatic form in female carriers. Dystrophinopathies present a spectrum of severity ranging from progressive skeletal and cardiac muscle wasting and weakness (DMD, BMD) to less severe muscle weakness or isolated cardiomyopathy affecting carrier females. At the mildest end of the spectrum exercise-induced muscle cramps and myoglobinuria may be the only feature, while at the severe end, there may be a complete loss of muscle function, cardiomyopathy, and respiratory failure. BMD presents a mild phenotype and a broad spectrum of clinical severity, with the onset of symptoms occurring from early childhood to as late as sixty. A very severe, rapidly progressive, X-linked dilated cardiomyopathy (code Orphanet 262) may also be caused by mutations in the dystrophin gene[77].

Muscle damage in DMD is caused by the complete absence of the cytoplasmic sarcolemmal protein dystrophin, which participates in a complex connecting muscle fiber cytoskeleton to the surrounding extracellular matrix through the cell membrane. Dystrophin protects myotubes' integrity during muscle contraction[79]. Dystrophin possesses a central rod domain of twenty-four spectrin-like repeats. Its primary muscular transcript measures about 2,100 kilobases. The mature mRNA, which is formed by the junction of seventy-nine exons, measures 14.0 kilobases and encodes a protein of 3,685 amino acid residues. Hence, intensive splicing is necessary for dystrophin biosynthesis[80].

Dystrophinopathies are allelic conditions caused by deletions, duplications, and mutations in the dystrophin gene. While DMD genetic variants are frameshift, BMD variants are in-frame. While severe DMD results from dystrophin complete absence, the moderate BMD form is only observed when one or several of the spectrin-liked repeats is missing. Deletions identified in DMD patients are shifting the translational open reading frame (ORF), thus resulting in a non-completed abnormal protein product of which the COOH terminal fragment is either non-functional or lost. On the opposite, BMD patients' deletions maintain the in-frame translational ORF for amino acids and predict a shorter protein of lower molecular weight. This indicates that the smaller protein product devoid of a certain number of internal spectrin-like domains remains semi-functional, resulting in the milder Becker clinical phenotype[81,82]. From this, it was predicted that, for some DMD patients, an in-frame skipping of the exon containing a nonsense mutation or deletion could have a therapeutic value. Figure 7 illustrates an exon skipping obtained by the binding of a steric blocker ASO targeting an intronic splice acceptor or donor site or an exonic splice enhancer site.

Given the fact that several thousands of variants have been reported for DMD patients, a search identified which in-frame exon skipping was favorable to treat a high proportion of patients. As shown in Figure 8, skipping exon 51 results in an in-frame skipping and leads to a partially functional protein in several deletion scenarios. Such partially internally shortened proteins would contain the following junctional exons, respectively: 50-53, 49-52, 49-52, 48-52, 47-52, 46-52, and 44-52. The deletions displayed in Figure 8 correspond to about 15 to 17% of the DMD population[83,84].

An elegant *in vivo* exon skipping proof of concept leading to the expression of a partially functional dystrophin molecule devoid of a certain number of spectrin-like domains was obtained by using an AAV viral vector delivering an antisense moiety born by a U7 or U1 small nuclear RNA[85,86]. The muscle force recovery was restored in these experiments. These very promising results prompted the development of synthetic steric blockers ASOs. It has led to the drug Eteplirsen (AVI-4658; Exondys 51), which has been approved by FDA but not by EMA, indicating that its efficacy is a matter of debate[87]. Eteplirsen is a thirty morpholino nucleotide oligomer delivered intravenously. Its sequence is CTCCAACATCAAGGAAGATGGCATTTCTAG. Other commercialized ASOs for DMD are skipping

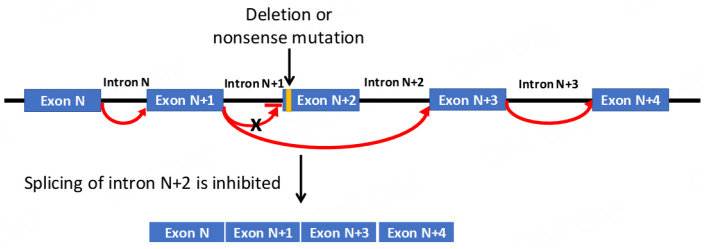


Figure 7. Therapeutic exon skipping. An ASO targeting and blocking an intronic splice acceptor or donor site or an exonic splice enhancer site enforces exon skipping. In the case of the picture, the ASO targets and binds to the acceptor site of intron N+1, resulting in the skipping of ExonN+2. The exons N+1 and N+3 must be in frame to ensure the expression of a protein devoid of only exon N+2, which might still be fully or partially functional.

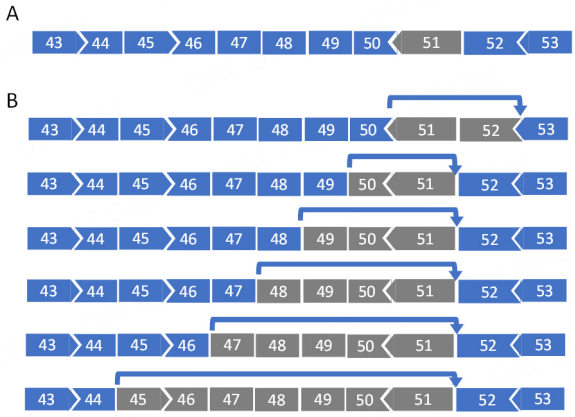


Figure 8. A: Exons 43 to 53 of the integral dystrophin gene. B: In grey, the group of exons whose concomitant deletion could, in theory, lead to a shortened functional or semi-functional dystrophin protein comprising only the protein encoded by the exons in blue, and allowing to skip mutated Exon 51.

exons 53 and 45, and at present times, there are 4 ASOs on the market for DMD, all being morpholinos. Other chemistries are in clinical development: 2'MOE-PS 20 mer ASO and peptide-conjugated ASO[88,89]. The clinical results obtained with Etiplisiren and other exon 51, exon 53, or exon 45 exclusion drugs (Golodirsen and Casimersen) have been reviewed recently[7,8].

Spinal muscular atrophy

Spinal muscular atrophy (SMA) is the second most common fatal, autosomal recessive disease of infants. It is a rare neuromuscular disease affecting 1/6000 children and is characterized by progressive general muscle waste. Spinal muscular atrophy is caused by mutations or deletions in the Survival Motoneuron 1 gene (SMN1)[90,91]. The gene for SMA was mapped to chromosome 5q13 within the telomeric region. The 20 kb gene encodes a 294 amino acids protein.

SMA occurs under different levels of severity. Less severe forms are classified as Type II, Type III, and Type IV, based on age of onset and ultimate motor disability. The most severe form is Type I (Werdnig-Hoffmann disease), which occurs in about 60% of the cases and is associated with quadriplegia, respiratory muscle paralysis, and mortality shortly after birth. Werdnig-Hoffmann patients are never able to sit by themselves and necessitate to be supported for their nutritional and ventilation function[92]. SMA patients who can sit but are unable to walk without help are classified as Type II. Milder type III SMA patients can be able to sit and walk, but they lose the walking capacity in adulthood[93]. Type IV patients have a normal

life expectancy but develop muscle weakness with time. Homozygous deletions or deleterious mutations in the SMN1 gene are present in all SMA patients.

SMN2 gene is a paralog of SMN1 which results from the duplication of the 5q13 region gene and has been identified in a more centromeric position. The SMN2 gene is unique to Homo Sapiens and differs from SMN1 in less than 20 nucleotides, but a functionally crucial difference is the existence of thymine instead of cytosine in exon 7[94]. This modification inactivates a splicing enhancer and, on the opposite, creates a splicing silencer. Consequently, exon7 is skipped by the splicing machinery in about 90% of maturated SMN2 mRNA, leading to an unstable non-functional shorter SMN2 protein [Figure 9]. Still, about 10% of the maturated SMN2 transcripts contain Exon 7 and lead to a functional SMN2 protein with identical neuroprotective properties as those of SMN1.

The SMN2 gene is located in an unstable chromosomal region and is consequently present under a variable copy number in the population, leading to variable production of functional SMN2 protein. The broad spectrum of the severity of the SMA disease has been correlated with the number of SMN2 copies, which is coherent with the assumption that more SMN2 copies might, at least partially, compensate for the absence of SMN1 protein. As schematized in Figure 9, the presence of multiple copies of the SMN2 gene partially alleviates the disease symptoms by allowing more functional SMN2 protein to be produced, with Type I patients generally possessing 2 SMN2 copies, Type II having 3 SMN2 copies, and Type III having 3-4 SMN2 gene copies. Thus, the number of SMN2 copies is clearly inversely correlated to SMA severity, but other genes have also been proposed to be involved in SMA severity[95,96].

The observation that SMN2 was a strong disease modifier established the basis for the quest to increase the production of a complete and functional SMN2 protein by antagonizing the exon 7 skipping reaction. Using a systematic study of minigene mutants with different deletions at the 5' end of intron 7, a novel inhibitory element located immediately downstream of the 5' splice site in intron 7 was identified, which was called intronic splicing silencer N1 (ISS-N1)[97]. A pragmatic approach consisting of screening a large number of overlapping ASOs targeting introns 6 and 7, as well as exon 7, identified several sites on the 3 sequences whose steric blocking would inhibit undesired exon 7 skipping and confirmed that ISS-N1 was the most promising target sequence for enforcing full SMN2 protein expression[98]. Thus, ASOs targeting a sequence at approximately 10 nucleotides downstream of the 5'splice site were further developed, leading to the drug Nusinersen.

Nusinersen (Spinraza™) was made available in 2016 as the first treatment for spinal amyotrophy. Remarkably, Nursinersen's discovery rationale was based on the above-described thorough elucidation of the molecular mechanism of SMN1 and SMN2 mRNA maturation. Nusinersen increases child survival and is administered by repeated intrathecal delivery (i.e., in the spinal cord). Nusinersen is an 18-nucleotide oligomer whose 5'-3' sequence is: UCACUUUCAUAAUGCUGG. It is a highly modified ASO having a full phosphorothioate backbone and 2'O-Me modified ribose to increase metabolic resistance and bioavailability. In addition, all pyrimidines are 5-methylated: uracil replaced by thymine, and cytosine replaced by 5'-methyl cytidyl. The Nusinersen IUPAC formula is detailed in Table 2.

Nusinersen displays high efficacy for promoting exon 7 recovery into SMN2 protein. It was shown that increasing SMN exclusively in peripheral tissues completely rescued muscle necrosis in mild SMA mice models and robustly extended survival in severe SMA mice, with significant improvements in vulnerable tissues and motor function[99]. However, CNS effects upon IV injection could only be observed in neonates but not in adult mice. It is known that the blood-brain barrier is permeant in neonates and is subsequently

Table 2. Nusinersen and inotersen IUPAC formula

Nusinersen condensed IUPAC formula:
Thy-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-Ade-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-Ade-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Ade-MeOEt(-2)Ribf-sP-Ade-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Gua-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Gua-MeOEt(-2)Ribf-sP-Gua-MeOEt(-2)Ribf
Nusinersen detailed IUPAC formula:
O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-cytidylyl-(3'- > 5')-O2'-(2-methoxyethyl)-P-thio-adenylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-cytidylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-cytidylyl-(3'- > 5')-O2'-(2-methoxyethyl)-P-thio-adenylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-P-thio-adenylyl-(3'- > 5')-O2'-(2-methoxyethyl)-P-thio-adenylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-P-thio-guanylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-cytidylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'->5')-O2'-(2-methoxyethyl)-P-thio-guanylyl-(3'- > 5')-O2'-(2-methoxyethyl)-guanosine
Inotersen compacted IUPAC formula:
Thy-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Gua-MeOEt(-2)Ribf-sP-dGuo-sP-dThd-sP-dThd-sP-dAdo-sP-m5Cyt-dRibf-sP-dAdo-sP-dThd-sP-dGuo-sP-dAdo-sP-dAdo-sP-Ade-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf

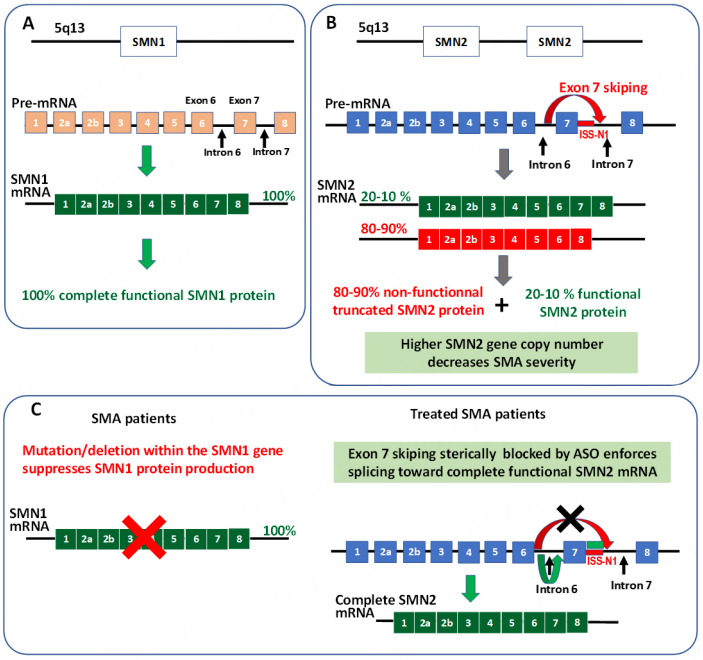


Figure 9. Summary of the genetic and molecular defects causing the several types of SMA severity and of treatment by a blocker ASO. A: In non-affected individuals, both SMN1 and SMN2 genes are functional wild-type. SMN1 pre-mRNA is transcribed into a complete mRNA corresponding to the 9 exons: 1,2a,2B,3,4,5, 6,7and 8. This leads to the production of a completely functional SMN1 protein, which does not occur in SMA patients. B: The SMN2 paralog gene contains several sequences which negatively impact the correct splicing and impair the incorporation of Exon 7 into the maturated SMN2 mRNA. The most important of these inhibitory sequences is the intronic splicing silencer N1 (ISS N1) located in 5′ of Intron 7. Therefore, only a 10 to 20 % minority of SMN2 transcripts contain all necessary exonic sequences for a functional SMN2 protein. From 80 to 90% of SMN2 transcripts are translated in a shortened non-functional SMN2 protein devoid of Exon 7, which is unstable. The presence of multiple copies of the SMN2 gene results in a higher quantity of functional SMN2 being produced and partially alleviates the disease severity. Type I patients generally possess 2 SMN2 copies, Type II having 3 SMN2 copies, and Type III and IV having 4 SMN2 gene copies. **C:** In SMA patients, a mutation/deletion induces the loss of SMN1 protein production. An ASO designed as a steric blocker of ISS N1 is highly efficient in increasing the level of fully competent SMN2 protein, and has been clinically validated as the Nusinersen drug.

closing itself, concomitantly with the expression in capillary endothelium of tight junctions and Glut1 glucose transporter. This suggested that the PS- 2'O-Me ASOs Nusinersen did not cross the blood-brain barrier. However, direct Nusinersen administration into the cerebrospinal fluid or the brain parenchyma

allowed to achieve reasonable distribution through the brain and spinal cord, the latter being an absolute requisite for SMA.

Acutely conceived clinical development allowed us to prove the clinical benefit of intrathecal Nusinersen in Type I SMA in only a few years. The results obtained on patients were so spectacular that FDA approval was obtained within about 3 months, which represents one of the fastest delays ever observed. Recent clinical data show that the earlier the treatment is given after birth, the best are the clinical results. This opened the way to systematic SMA newborn screening in various countries' legislation, which was not the case before. More details on the clinical advances and ongoing development of Nusinersen have been published elsewhere[5,7,8].

Multiple other candidate ASOs are being tested as splice modifiers, such as an LNA/DNA mixmer[100]. Remarkably, quite a short time after the revolutionary Nusinersen commercialization, two other approaches led to approved drugs against SMA. Zolgensma is a self-complementary AAV-9 vector able to cross the blood-brain barrier[101] carrying the SMN1 cDNA sequence, which showed positive results by systemic IV administration[102] and has been FDA- and EMA-approved. Ridisplam is an orally administered splicing modifier of SMN2 which increases the level of functional SMN2 protein. The Ridisplam large domain of application covers all types of SMA, and it has been approved in the USA, Japan, and Europe as a treatment that does not necessitate hospital intervention.

RNase H-dependent ASO inotersen for the treatment of hereditary transthyretin amyloidosis

Hereditary transthyretin amyloidosis (hATTR) is a gain-of-function genetic rare disease. It represents a paramount example of clinical success for RNA drugs inducing mRNA degradation because both ASO and siRNA drugs have reached clinical use. This disease represents the first indication of a clinically approved siRNA. Moreover, two siRNA drugs with different delivery principles have been approved for hATTR in a brief period of time, and more are on the way.

The transthyretin protein (TTR) is secreted by the liver, choroid plexus, and retinal pigment epithelium. Its important function is to transport the thyroid hormone thyroxine (T4) and retinol to the liver. TTR is a 55 kDa homotetramer, which is formed by the first association of dimers, followed by dimer-dimer binding. Hereditary transthyretin amyloidosis is a group of several dominant negative diseases related to variants in the TTR gene. More than 140 different TTR variants have been identified. In these rare hereditary diseases, mutant TTR misfolding leads to the formation of amyloid aggregates which accumulate in various tissues and cause various pathologies: hereditary transthyretin amyloidosis (hATTR), familial amyloid polyneuropathy (FAP) and familial amyloid cardiomyopathy (FAC)[103-105]. Cardiomyopathy results from myocardial infiltration of abnormal amyloid protein. Moreover, wild-type transthyretin amyloidosis (wtATTR) has been observed, which occurs mainly at a late age and is caused by the aggregation of normal transthyretin[106]. It affects older age patients carrying the wild-type TTR gene. In addition to polyneuropathy and cardiomyopathy, other transthyretin amyloidosis symptoms involve nephropathy and ocular pathology.

Before the introduction of RNA drugs, hATTR treatment involved liver transplantation and, more recently, the small molecule drugs Diflunisal and Tafamidis, which stabilize TTR in a non-aggregating form. In recent years, however, a very efficient treatment for hATTR with polyneuropathy has been obtained through RNA drugs decreasing liver TTR mRNA. Either RNases H-dependent ASOs such as Inotersen (Tegsedi) or Ago-dependent siRNA Patisiran (Onpattro) and Vutrisiran have successfully reached the market.

The ASO Inotersen (Tegsedi) is a 20-mer gapmer similar to that represented in Figure 5. It leads to the degradation of TTR mRNA, with minimal off-target effect. It is fully PS-modified (phosphorothioate backbone), with 5 ribose on both 5' and 3'ends carrying 2'MOE (2'O-methoxyethyl) moieties [Figure 4C]. The 10 central nucleotides have a natural ribose. The nucleotidic sequence of Inotersen is UCUUGGTTACATGAAAUCCC, with the nucleotides UCUUG and AUCCC carrying a 2'OMOE. As for Nusinersen, all cytosines and uracil of Inotersen are 5'-methylated (uracil replaced by thymine and cytosine by 5-methyl cytosine). The Inotersen IUPAC formula is detailed in Table 2.

After subcutaneous (SC) injection, Inotersen leads to a robust decreasing effect on the level of both variant and wild-type transthyretin. It clearly inhibits TTR production and slows down the progression of the disease[107] but does not seem to induce any reverse effect on already-formed amyloid aggregates[7]. Reported Inotersen adverse events include thrombocytopenia. This could be linked to the presence of phosphorothioate linkages, which have been shown to be a potent platelet activator[108,109], glomerulonephritis, and hepatic toxicity[110].

siRNA patisiran for the treatment of transthyretin hereditary amyloidosis

Patisiran is a siRNA. The antisense guide strand formula is A-U-G-G-A-A-Um-A-C-U-C-U-U-G-G-U-Um-A-C-dT-dT). It is complexed with the complementary passenger sense strand (G-Um-A-A-Cm-Cm-A-A-G-A-G-Um-A-Um-Um-Cm-Cm-A-Um-dT-dT (A, adenosine; C, cytidine; G, guanosine; U, uridine; Cm, 2'-O-methylcytidine; Um, 2'-O-methyluridine; dT, thymidine). Thus, Patisiran bears eleven 2'O-methylated pyrimidines, which increases its lipophilicity. All inter-ribose linkages are phosphodiesters[111].

To protect this phosphodiester siRNA from rapid degradation and kidney filtration, the siRNA active component of Patisiran is formulated into a lipid nanoparticle (LNP). In addition to this protecting effect, LNP also facilitates Patisiran delivery to the liver. It has long been known that some colloidal delivery systems, such as nanoparticles or liposomes, accumulate rapidly into the liver and spleen upon IV administration, at a ratio superior to 90%[74,112-115]. Nanoparticles or liposomes containing cationic and/or ionizable lipids thus represent a very favorable system for high-yield encapsulation of negatively charged siRNA and for preferential liver uptake, as previously demonstrated for DNA.

The LNP formulation which has been selected for Patisiran includes buffer components (disodium hydrogen phosphate, heptahydrate potassium dihydrogen phosphate, anhydrous sodium chloride), as well as the lipid DLin-MC3-DMA [(6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31¬ tetraen-19-yl-4-(dimethylamino) butanoate], an amine-containing ionizable lipid with a pKa of 6,4. In addition, the formulation comprises a neutral lipid distearoylphosphatidylcholine, cholesterol, and the PEGylated lipid DMG-PEG 2,000 [Figure 10A][111]. Each 1 mL Patisiran also contains 6.2 mg cholesterol USP, 13.0 mg DLin-MC3-DMA, 3.3 mg DSPC, and 1.6 mg α-(3'-{[1,2-di(myristyloxy)propanoxy] carbonylamino}propyl)-ω-methoxy, polyoxyethylene (PEG2000 C-DMG0). In such a typical LNP formulation, cholesterol is added to provide rigidity to the 40-100 nm nanoparticles, and the neutral lipid distearoylphosphatidylcholine attenuates the charge repulsion between the DLin-MC3-DMA cationic heads. In addition, the PEGylated lipid PEG2000 C-DMG0 ensures colloidal stability through the highly hydrated polyethyleneglycol chains forming a protecting shield around the nanoparticle.

The mechanism by which the LNPs are targeted to the liver hepatocytes is schematized in Figures 10B and 10C. It is caused by the natural binding of the plasma protein ApoE to the PEGylated LNP. Since liver hepatocytes express a high concentration of the ApoE receptor, LNPs decorated with ApoE bind to the hepatocyte surface [Figure 10B]. The resulting ApoE clustering induces internalization by a clathrin-

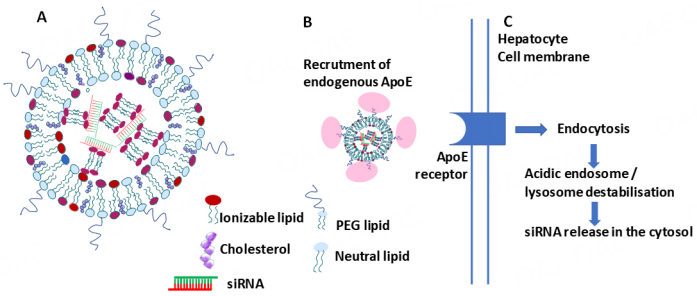


Figure 10. A: RNAi encapsulated in LNPs are protected from the actions of nucleases and targeted to the liver. LNPs are specifically targeted to the liver because they bind apolipoproteins E in the blood circulation and then bind to the ApoE-LDL receptor on the surface of the hepatocytes. B: LNP decorated with Apo E bind to Apo E receptor on the hepatocyte plasma membrane. C: DLin-MC3-DMA lipids at the LNP surface are neutral at pH 7. Inside the endosome, DLin-MC3-DMA becomes cationic, which favors either fusion with the endosome membrane and siRNA release or endosome breakage caused by the "proton pump" osmotic effect.

dependent endocytosis mechanism. Inside the endosome, it is postulated that the ionizable DLin-MC3-DMA lipid becomes cationic because of endosomal/lysosomal acidification. This induces endosome breakage, for which two different mechanisms have been proposed.

1) In the first one, the ionizable lipid captures and titrates the H+ ions taken up into the endosomes by the endolysosomal proton pump ATPase. This induces the sustained uptake of a large number of protons and chloride anions which diffuse into the endosomes to equilibrate the proton cationic charges. This "proton sponge" effect induces endosome swelling and breakage.

2) According to the alternative proposed mechanism, the ionizable DLin-MC3-DMA lipid at the LNP surface becomes cationic in the acidic endosomal compartment, leading to the fusion of the positively charged LNP surface with the negatively charged endosome membrane.

Both postulated mechanisms result in siRNA release into the cytosol and further interaction with the RISC complex.

Patisiran was granted orphan drug status, fast track designation, priority review, and breakthrough therapy designation due to its novel mechanism and the rarity of the condition it is treating. It was approved for medical use in USA ad EU in August 2018. However, with the development of other transthyretin amyloidosis treatments, such as Inotersen and Vutrisiran which is accumulated into the liver through a triGalNac N-acetylglycosamine targeting moiety, there is now intense competition between these different RNA drugs.

Tri-GalNac siRNA Vutrisiran for transthyretin hereditary amyloidosis treatment

Vutrisiran (AMVUTTRA™) is a subcutaneously administered TTR-specific siRNA developed for hATTR and wtATTR treatment. Vutrisiran was FDA approved in 2022 for the treatment of hATTR amyloidosis with polyneuropathy in adults, following fast-track designation in 2020 given the very promising efficacy of this RNA drug.

The Vutrisiran sequence was designed to bind to a conserved sequence on all TTR mRNA variants. Because Vutrisiran is administered as a naked siRNA, i.e., not associated with a lipid nanoparticle delivery system, the drug must be metabolically protected and targeted to hepatocytes. Nuclease resistance has been achieved by introducing much more modifications in Vutrisiran than in Patisiran. Indeed, while Patisiran contains

only eleven 2'O-Me nucleotides and only phosphodiester linkages, Vutrisiran is a heavily modified siRNA with 6 phosphorothioate linkages at the end of each strand, 35 nucleotides carrying a 2'OMe ribose and 9 nucleotides modified with a 2'F ribose. Thus, the two Vutrisiran strands (one of 21 nt and one of 23 nt) are fully modified[116,117]. Optimization of the number and respective positions of these modifications has been performed in view of reducing toxicity, particularly hepatotoxicity[19,46,54,116,117].

The most innovative feature of Vutrisiran is the functionalization of the sense passenger strand with a very efficient hepatocyte targeting moiety, the tri-N-acetyl-galactosamine (tri GalNac). The triGalNac head binds with high avidity to the ASGPR, a lectin that is present at a remarkably high density on the hepatocyte surface (500,000 ASGPR per cell). Binding to the ASGPR actively promotes the cellular uptake of the ligand upon clustering and aggregation of several receptors in coated pits. The ASGPR turnover is very fast since only about 5%-10% of ASGPR are permanently accessible at the cell plasma membrane. After endocytosis, ASGPR recycling occurs in ~15 min concomitantly with the release of the bound ligand in the acidified endosomal compartment, in a process analog to that of the transferrin receptor cycle. Hence, high ASGPR density on hepatocyte surface and fast turnover points Tri GalNac as an ideal targeting head candidate for any liver-targeted RNA drug[118]. The fact that more than 80% of an IV injected compound expressing galactose or galactosamine is taken up by the liver has been evidenced by SPECT or luminescent imaging techniques using lactosylated albumin[119,120].

As shown in Figure 11, the tri-GalNac is covalently linked to the 3' end of the siRNA sense strand, so as not to interfere with the binding of the complementary antisense to RISC. The improved metabolic stability of Vutrisiran and the very potent targeting efficacy of its tri-Gal-Nac lead to an exceptional intrinsic efficacy of Vutrisiran. This is demonstrated by the therapeutic efficacy of very low doses and the exceptionally long duration of action because a quarterly regimen is sufficient to achieve a similar therapeutic effect as Patisiran[121-125]. Both polyneuropathy, cardiomyopathy, and wt-TTR are envisioned as Vutrisiran indications.

As compared to the LNP formulation used for Patisiran, GalNac-siRNA conjugate allows simpler GMP preparation and storage conditions, and a more convenient administration protocol (SC versus IV). In addition, slow diffusion to capillaries through the extracellular conjunctive tissue creates a "depot" effect which, together with increased metabolic stability, allows less frequent administration. Remarkably, the last generation of GalNac siRNAs carrying a phosphate triglycan-end group allows a regimen as distant as twice a year for multiple indications requiring silencing of a liver-expressed protein. A one-year duration after a single dose has been reported for Cemdisiran, which has strong potential in the treatment of complement-mediated diseases such as paroxysmal nocturnal hemoglobinuria (PNH). This is a historically never achieved performance. Thus, the GalNac targeting technology represents actually the most promising approach for siRNAs, as illustrated by the fast development of Givosiran (FDA approved in 2019 for adults with acute hepatic porphyria), Lumasiran (FDA approved in 2020 for hyperoxaluria type 1), and Nedosiran (in phase III for primary hyperoxaluria[116,123]. Similarly, the GalNac targeting technology is also being pursued for ASO liver targeting[126,127].

Type 1 myotonic dystrophy: different ASO modes of action

Myotonic dystrophy (also called dystrophic myotonia) is a multisystemic disease with a frequency of 1/8000 worldwide. The most severe form (DM1) includes symptoms such as myotonia, muscle weakness, cardiac arrhythmias, cognitive dysfunction, and cataract. The genetic cause of DM1 comes from the repeated expansion of a CTG triplet motif in the DM Protein Kinase (DMPK) gene. Normal DMPK gene contains 5 to 37 repeats, while variant DM1 DMPK gene might contain up to thousands of CTG repeats, and the

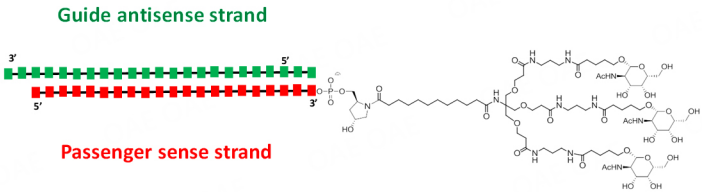


Figure 11. Tri-GalNac chemical group addressing RNAi to the hepatocyte asialoglycoprotein receptor ASGPR. The tri-GalNac is covalently linked to the 3′ end of the siRNA sense strand, so as not to interfere with capture by RISC of the complementary antisense strand necessary for mRNA cleavage.

number of repeats correlates with disease severity[128].

Variant DMPK mRNA form hairpin structures made from CUG repeats and assembling in well-characterized nuclear foci. The molecular mechanism of DM1 pathology results from the toxicity of these nuclear foci, which bind and sequester proteins of the family muscleblind-like (MBNL), thus interfering with their natural splicing functions[129,130]. The mis-splicing reactions due to insufficient availability of the MBNL protein in the nucleus induce a fetal-like pattern in adult DM1 cells, which concerns a multiplicity of proteins, such as muscle chlorine channel, insulin receptor, cardiac troponin T, bypass integrator 1, skeletal rapid troponin T, dystrophin (DMD) and cardiac sodium channel SCN5A. This spliceopathy leads to myotonia and other clinical symptoms[131].

ASO and siRNA strategies have been intensively studied on DM1 cellular models such as patient fibroblasts and mouse or drosophila models containing up to 1000 CTG triplets in DMPK gene, and in clinical trials with a gapmer ASO containing constrained ethyl (cEt) locked nucleic acid (LNA)[132,133]. A recent clinical trial was initiated in 2022 with DYNE-101, a muscle membrane antigen-binding fragment antibody (Fab) conjugated to an ASO to enable targeted muscle tissue delivery[134,135].

The multiple strategies displayed in Figure 12 illustrate that DM1 represents a model disease case where multiple RNA drug interventions can be envisioned, and this either separately or in combination. Since MBNL proteins are sequestered by poly CTG DMPK nuclear foci, the use of ASO or siRNA carrying a poly ACG triplet might lead to DMPK nuclear foci cleavage and degradation. Alternatively, an ASO can also function as a steric blocker of MBNL binding, thus releasing the necessary amount of MBNL for restoring adult phenotype splicing pattern [Figure 12A and B][130,131,136]. A second strategy is to target the miRNA (miR)-23b, which has been shown to negatively control MBNL expression. Thus, the administration of a blocker ASO complementary to (miR)23b would up-regulate MBNL expression and potentially compensate for the sequestration of MBNL by variant DMPK mRNA [Figure 12C and D][137]. Finally, since MBNL sequestering and lack of availability induces mis-splicing towards the fetal phenotype of various proteins, splice correctors ASOs, similar to the one described in Figures 2 and 9 for SMA, represent an appealing strategy to restore the expression of the adult form of the critical mis-spliced proteins [Figure 12E and F]. Such a downstream approach has been successfully reported using a morpholino ASO to restore the muscle chloride channel. This was achieved in electroporated muscle fibers[138] or using ultrasound-enhanced delivery of morpholino with bubble liposomes[139].

Allele-specific silencing for centronuclear myopathy

In gain-of-function dominant-negative disorders, an ideal strategy would be to specifically target for ASO or siRNA degradation the variant sequence on the pre-RNA and mRNA. However, this would require a personalized ASO or siRNA drug for each variant. This might not be feasible for several pharmacological

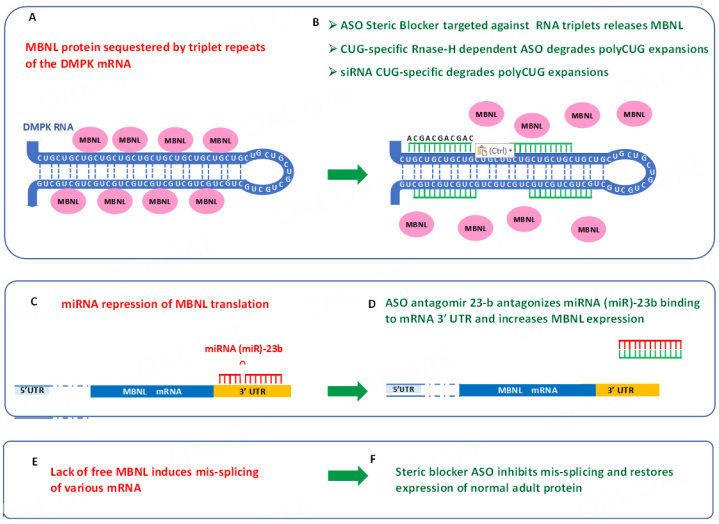


Figure 12. Different ASO- and siRNA-based strategies against Myotonic Dystrophy Type 1. A: MBNL proteins are sequestered by nuclear foci formed by the mRNA from the poly CTG DMPK variant. B: Poly ACG ASO or siRNA lead to DMPK mRNA cleavage and nuclear foci degradation. Alternatively, they can also function as a steric blocker of MBNL binding to the foci, thus releasing the necessary amount of MBNL to restore the adult phenotype splicing pattern. C, D: Since the miRNA (miR)-23b has been shown to repress MBNL expression, the administration of antagomir-23b antagonizes the miRNA (miR)23b which increases MBNL expression, thus compensating for the sequestration of MBNL by variant DMPK mRNA. E: The lack of free MBNL induces mis-splicing toward the fetal phenotype of various proteins. F: Splice correctors ASOs can restore the adult form of the mis-spliced proteins.

reasons including toxicity linked to the off-target effect of some of such specific ASO or siRNA, and cost considerations. In addition, this strategy is not usable for genetic diseases linked to gene duplication or to triplet repeat expansion such as Huntington's disease (CAG repeat expansion) or spinocerebellar ataxia subtypes (CAG or CTG repeats), in which the available variant sequences that could be targeted are present on both the wild-type and the mutated gene.

An ASO or siRNA targeting a non-variant sequence on the pathological gene is the most natural way to provide an RNA drug that could ideally treat all patients, independently of their specific variant genotype. However, this leads to complete repression of both the wild-type and dominant-negative variant alleles.

The above strategies cannot be applied if at least a minimal expression of the wild-type protein is required, and then allele-specific silencing must be sought[140]. Allele-specific silencing can be achieved by targeting one or several single nucleotide polymorphisms (SNP) associated with a variant allele in the patient population. This approach is only feasible if common specific SNPs can be identified in a high percentage of the various mutated alleles in the diseased population. This strategy has been followed in Huntington's disease (HD) and spinocerebellar ataxia[141,142].

Epidemiological studies on the HD patient population and targetable SNPs suggest that 80%-85% of HD patients could be treated with panels of 2 to 5 SNP heterozygosities, meaning that only the expanded variant allele possesses these 2 to 5 SNPs in these individuals[141,143,144]. Exciting recent preclinical results obtained with a brain-targeted di-siRNA have been reported with fully chemically modified, therapeutically translatable siRNAs targeting SNP heterozygosities specific to Huntingtin variants. It allowed a 50-fold discriminative power on the huntingtin variant genetic allele in a cell-based assay on human neurons derived from human Huntington chorea patient iPSCs. This optimized si-RNA was obtained by repeated targeted screening and chemical optimization. Selective silencing of the mutant huntingtin HTT allele

(> 85%) was detected throughout the brain in an HD mouse model with this technology[62,145].

Autosomal dominant centronuclear myopathy (AD-CNM, ORPHA 169 189) is a rare congenital myopathy characterized by numerous centrally placed nuclei on muscle biopsy. Clinical features are those of congenital myopathy: hypotonia, distal/proximal muscle weakness, rib cage deformities sometimes associated with respiratory insufficiency, ptosis, ophthalmoparesis, and weakness of the muscles of facial expression with dysmorphic facial features.

AD-CNM results from heterozygous mutations in the DNM2 gene, which encodes dynamin 2, and to date, 37 mutations (mainly missense) have been identified[146,147]. Dominant DNM2 mutations also cause rare cases of Charcot-Marie-Tooth peripheral neuropathy (CMT)[148] and hereditary spastic paraplegia[149]. The DNM2 protein belongs to the superfamily of large guanosine triphosphatases (GTPases) and is involved in endocytosis and intracellular vesicle trafficking through its role in the deformation of biological membranes, particularly in muscle cell T-tubule biogenesis[146,150]. The role of DNM2 as a regulator of actin and microtubule cytoskeletons has also been reported. A potential AD-CNM pathophysiological mechanism is the formation of abnormally stable polymers by mutant Dynamin 2[151].

A single copy of the wild-type allele in heterozygous knockout mice expressing 50% Dnm2 displays a healthy wild-type phenotype[152]. Moreover, homozygous DNM2 mutation reported in 3 consanguineous patients leads to a lethal congenital syndrome associating akinesia, joint contractures, hypotonia, and skeletal abnormalities, together with brain and retinal hemorrhages[153]. These and other data point to the necessity of an allele-specific siRNA drug targeting only the dynamin 2 variant, in order to maintain 50% of dynamin 2 production.

The proof of concept of silencing the variant mRNA without affecting the wild-type transcript has been obtained in a mouse model with an shRNA gene delivered by an AAV virus[146]. Complete rescue of the muscle phenotype was maintained for at least 1 year after a single injection of the shRNA AAV, leading to a maintained reduction of the variantDnm2 transcript. This study also suggested a new potential pathophysiological mechanism linked to mutant protein accumulation with age, which does not occur in wild-type animals, and which can be prevented by the shRNA treatment.

Further progress was obtained by screening a library of siRNAs targeted to specific heterozygous SNPs associated with the DNM2 dominant-negative variant gene in the AD-CNM patient population. About 75% of patients could be covered by four different siRNAs targeting the four SNPs most frequently associated in a heterozygote manner with the AD-CNM patient population.

One allele-specific siRNA strategy is now in a clinical trial for the treatment of pachyonychia congenital, a rare skin disorder linked to a dominant negative mutation in keratin 6a[154,155]. More considerations on the allele-specific siRNA technology have been developed recently[156].

Combined RNA drug silencing and replacement gene therapy for dominant-negative oculopharyngeal muscular dystrophy

For the gain-of-function disorders related to triplet expansion which have been discussed in the preceding section, an alternative approach has been proposed which does not require the presence of specific SNP on the heterozygote variant allele. The strategy is based on the simultaneous knockdown of both variant and wild-type endogenous mRNAs, together with the administration of a gene encoding the wild-type protein. The replacement gene sequence must be codon-optimized using the genetic code degeneration to carry

differences with the endogenous human gene, thus allowing it to be discriminated and untargeted by the therapeutic silencing ASO, siRNA, or shRNA. Additionally, codon optimization might also contribute to ensuring a high expression level of the wild-type protein.

Oculopharyngeal muscular dystrophy (OPMD) is a rare muscle disease characterized by an onset of weakness in the pharyngeal and eyelid muscles. Inherited in an autosomal dominant mode, the disease is found on all continents, with several clusters identified in Quebec and Israel. The clinical diagnosis is confirmed by a genetic test which in most cases shows an 11-18 expansion of GCA or GCG triplets in the gene of Poly(A) Binding Protein Nuclear 1 (PABPN1) on chromosome 14. The disease is caused by the extension of the polyalanine tract in the PABPN1 protein leading to the gain-of-function formation of intranuclear inclusions or aggregates in the muscle of OPMD patients, which are the hallmark of the disease[157]. Although PABPN1 is ubiquitously expressed and l contributes to control gene expression in all tissues, playing key roles in post-transcriptional processing of RNA, the PABPN1 variant pathological phenotype is restricted to a limited set of skeletal muscles affected in OPMD. The exact pathophysiological process leading to the localized pathology and the precise role of intranuclear aggregates are still unclear[158].

In a gene therapy approach, two AAV vectors were co-delivered, the first expressing a cassette including a triple shRNA under the control of RNA polymerase III promoter and the second expressing human codon-optimized PABPN1 under the control of a skeletal and cardiac muscle-specific promoter. This treatment significantly reduced the number of myonuclei containing PABPN1-positive insoluble intranuclear inclusions, showed significant improvements in several histopathological features (muscle regeneration, fibrosis, and muscle force), and almost completely normalized the transcriptome muscles of A17 mice to that of wild-type mice[159].

Subsequent work managed to condense the two AAV vectors into one and reported reversion of already established insoluble aggregates and partial muscle rescue from atrophy, which are both crucially important since, in most cases, OPMD patients already have an established disease when diagnosed. Also reported were the prevention of the formation of muscle fibrosis and stabilization of muscle strength to healthy muscle levels[160]. Recent progress towards a clinical trial described a unique single bifunctional construct under the control of a muscle-specific promoter for the co-expression of both the codon-optimized PABPN1 protein and two siRNAs against PABPN1 modeled into microRNA (miRNA) backbones. A single intramuscular injection of the AAV9 vector in a murine model resulted in the inhibition of mutant PABPN1 and in PABPN1 replacement, leading to restoration at a normal level of muscle strength and other muscle parameters[161].

Challenges faced by the synthetic ASO and siRNA technology

RNA drugs face several challenges and bottlenecks. The first one concerns pharmacodynamic/pharmacokinetic (PK/PD) properties. Except for the liver, delivery of RNA drugs to other organs and tissues is still not ideally performed. Lack of proper delivery of sufficient amount into muscles of the Eteplirsen morpholino ASO might be responsible for its suboptimal performance[7,87], and CNS delivery of RNA drugs still necessitates intrathecal, intraventricular or intracerebral administration, which is clinically demanding and associated with infection risk. For muscular and neuromuscular indications, a novel muscle-targeting platform obtained by conjugation of siRNAs with anti-CD71 Fab' fragment has been reported[134,162]. This conjugate led to one-month durable gene-silencing in the heart and skeletal muscle after IV administration in normal mice, and to significant gene-silencing when injected intramuscularly. In a mouse model of peripheral artery disease, the intramuscular administration of an anti-myostatin siRNA resulted in significant silencing of myostatin in muscle and led to the recovery of the running performance.

This technology is now in a clinical trial for a Type 1 myotonic dystrophy RNA drug[135].

Another PK/PD challenge paradoxically comes from the tremendous efficacy of the last generation of RNA drugs. The remarkable duration of the siRNA silencing effect after a single dose results from the siRNA metabolic stability and increased affinity to RISC, which have been brought by the optimized chemistries described in Figures 4 and 5. In addition, grafting functional groups such as 5'vynil phosphonate or methyl phosphonate facilitates guide strand interaction with the RISC complex [Figure 6]. In this way, the guide strand can remain stably bound to RISC and undergo sustained long-term recycling [Figure 3, left side]. The fact that a single administration can lead to 6 to 12 months of activity raises the necessity to find ways to terminate the treatment on demand, and to do this fast enough in case of severe adverse events. The stopping drug effect can be obtained by promoting guide strand dissociation from RISC. Such a reversal system has been described in the liver. It uses short synthetic high-affinity oligonucleotides complementary to the siRNA guide strand that can compete with RISC binding to the guide strand[163]. The authors reported that 9-mers with five locked nucleic acids (LNAs) have the highest potency across several targets to displace the guide strand from RISC and to stop the interference reaction. This 9-mer is targeted to the liver through a tri-GalNac moiety.

Concern must also be considered about the risk of potential toxicity effects of ASOs / siRNA. The first type of toxicity effects might result from direct interactions between the ASOs / siRNA and cellular proteins via a hybridization-independent mechanism. This potential toxicity has to be studied in a similar way than for any classical drug.

The hybridization of ASOs, siRNA/RISC complex, or of free single-strand siRNA to non-intended RNAs sharing some sequence homology with the targeted mRNA is called "off-target effect". For instance, ASO hepatotoxicity in mice has been described to be partly mediated via the RNase H-dependent degradation of off-target RNAs[164,165]. Some strategies are being developed in order to alleviate the ASO off-target effects. Optimization of the ASO nucleotide length by extending its size from a 14-mer to an 18-mer has been shown to reduce the number of off-target candidates, presumably by decreasing the number of matching with non-intended mRNAs[166].

siRNA might suppress the expression of unintended mRNAs with partially complementary sequences by a similar mechanism to that of miRNA-mediated RNA silencing. This siRNA-mediated off-target effect occurs mainly from similarities in the siRNA seed region (nucleotides 2-8). An in-depth analysis of which base pairing was responsible for the off-target effect involving a machine learning technique and using a random sampling procedure led to the conclusion that nucleotides 2-5 were mostly responsible for siRNA off-target effects on RNAi[167]. Enhanced stabilization chemistry has been recently proposed to substantially reduce siRNA seed-mediated binding to off-target transcripts while maintaining on-target activity[68].

Another cause of off-target silencing is improper strand selection by RISC. Passenger-strand silencing can be avoided by selecting siRNA sequences with high thermodynamic asymmetry or by chemically modifying the sense strand, as described above[168,169].

Abundant siRNAs or shRNA can overload the endogenous RNAi pathway leading to toxicity, which has been mostly reported in hepatocytes. Precise dosing must be carefully optimized to avoid this saturation of the RISC system and limit hepatotoxicity of short- and long-term clinical gene silencing by both approaches[169-171].

Other challenges for the future of synthetic ASO and siRNA drugs originate from the rapid advance of alternative techniques such as viral gene therapy for delivering a shRNA, which leads to the same effect as ASO and siRNA in terms of steric blocker for exon skipping or translation inhibition, and for inducing mRNA cleavage/degradation. Indeed, at the present time, most proofs of concept for RNA drugs have been obtained at the laboratory scale by specifically designed AAV or lentivirus vectors. However, this gene therapy approach is less flexible than synthetic ASOs and siRNA because no conditional expression system has been clinically approved at the present time, which renders hazardous the control of gene therapy treatment termination. Moreover, synthetic compounds might be preferable in terms of production costs, and finally, synthetic RNA drugs can be administered repeatedly without initiating any immune response, whereas distinct viral serotypes must be used for multiple-dose administration.

Genome editing is a rapidly evolving technology. Targeted DNA double-strand breaks (DSBs) using CRISPR-Cas9 have revolutionized genetic intervention by enabling efficient and accurate genome editing in a broad range of eukaryotic systems. Multiple applications are actively investigated, such as targeted knockout of dominant negative pathological genes or viral genomes (either integrated or episomal).

In 2021, a clinical trial involving the in vivo use of CRISPR-Cas9 in humans was disclosed[4]. The in vivo gene-editing therapeutic agent NTLA-2001 consists of lipid nanoparticles LNP containing a single guide RNA (sgRNA) that targets the human TTR gene. A human-codon-optimized mRNA sequence of Streptococcus pyogenes Cas9 protein was added to the LNP formulation. Preclinical results of IV NTLA-2001 were impressive, with a durable 95% drop in TTR concentration in monkeys. The genome-editing drug was then administered to patients with hereditary ATTR amyloidosis with polyneuropathy. The treatment was associated with only mild adverse events and led to a decrease in serum TTR protein concentration resulting from the targeted knockout of the TTR gene.

Multiple other genome editing preclinical studies are promising, for instance, in myotonic dystrophy targeting the DMPK gene [see Figure 12][171]. Since LNPs can efficiently deliver CRISPR-Cas9 to the liver, many ASO and siRNA indications might eventually also be treated by genome-editing technology. However, more time is necessary to assess the safety of this approach in the long term, especially concerning off-target effects, genotoxicity, and germ-line modification, since CRISPR-Cas9 makes cuts in the genomic DNA in contrary to ASOs and siRNA, which target mRNA. Also, genome editing represents an irreversible therapy through a one-shot knockout of the targeted gene. Thus, the pros and cons of this technology must be weighted for each specific indication in comparison to reversible synthetic RNA drugs.

CONCLUSION AND PERSPECTIVES

The examples detailed here illustrate the wide and versatile capacities of antisense therapies in muscular and neuromuscular disorders. A large additional number of these diseases could potentially benefit from RNA drugs. Without being exhaustive, one can mention several other diseases where specific silencing has shown benefit in preclinical models. In facioscapulohumeral muscular dystrophy (FSHD), the knockdown of the FSHD region gene 1 (FRG1) was achieved using miRNAs delivered using an AAV vector system[172]. The AAV2/9-mediated delivery of an shRNA targeting the Pmp22 mRNA and injected in the sciatic nerve prevented the development of pathological features in a rat model of Charcot-Marie-Tooth disease 1 A[173]. An allele-specific RNA interference using an AAV9 has been described in a Charcot-Marie-Tooth disease type 2D mouse model. RNAi sequences targeting the dominant mutant of glycyl-tRNA synthetase (GARS) mRNA, but not wild-type, were optimized and then packaged into AAV9 for in vivo delivery. This prevented neuropathy in mice treated at birth. However, delaying the treatment until after disease onset drastically reduced the benefit of gene therapy, and the therapeutic effect decreased with the delay in

treatment, which points to the value of early diagnosis[174]. In most cases, the proof of concept was obtained using shRNA delivered by an AAV vector because this is easily obtained at the laboratory level and because several AAV serotypes display suitable organ penetration and accessibility[100,175]. An AAV shRNA proof of concept then opens the way to the search for synthetic genetic RNA drugs.

Genetic pharmacology with synthetic RNA drugs, which is sometimes presented as belonging to the broader gene therapy field, has made remarkable progress after 20 years of relentless and intensive efforts to improve RNA drug performances. Two to three logs enhancement of their potency has led to recent extraordinary successes, such as being able to silence a pathological gene by a twice, or even once-a-year subcutaneous administration of a small amount of compound, which represents a historical performance never reached before by a chemical compound.

The chemical RNA drugs technology platform, whose chemistry is still continuously improving, is very versatile and can be applied to a large variety of therapeutic applications once the first proof of concept has been obtained, including for targeting previously non-druggable proteins. Of particular value for rare diseases is the possibility to tackle dominant negative gain-of-function genetic diseases, among them those linked to triplet expansion. Trinucleotide repeat diseases lead to either toxic RNA or toxic protein product, and they represent a widening class of rare diseases that is not restricted to the ones illustrated or mentioned in the present review: Type 1 myotonic dystrophy, centronuclear myopathy, Huntington chorea, and spinocerebellar ataxia[141-145], but also cover other expanding disease families[176,177].

The mRNA vaccine technology can be rapidly adapted to any new pathogen by administering pathogen-specific mRNA sequences through a unique delivery technology platform and could thus be rapidly applied to the COVID-19 pandemic spread. Similarly, RNA drug improvements are of general and extendable value, such as modifications in the phosphodiester linkage, sugars, and nucleic bases. However, it must be stressed that the number and respective position of the linkage, ribose, and base modifications need a tailor-made optimization that might be optimized for specific antisense sequences[68,145].

Tri-GalNac targeting has now demonstrated its extraordinary efficacy in delivering RNA drugs to the liver, and the majority of actual clinical trials concerning RNA drugs make a profit from this technology, not only for rare metabolic or neuromuscular disorders such as TTR, or blood disorders such as hemophilia but also for diseases concerning a much larger share of the population, such as hypercholesterolemia by targeting the PCSK9 gene[178], hypertension, diabetes or chronic hepatitis.

In parallel to this extension of RNA drugs application to highly prevalent diseases, the technology shows the potential to treat an increasing number of severely debilitating or life-threatening ultrarare diseases, which can affect worldly only 1 or a very limited number of patients. They are now referred to as "N-of-1" treatments. How this ultrarare diseases population could benefit from antisense RNA drugs is now raising increasing interest from scientists, GMP producers, and regulatory authorities[179]. Since there might be little or no commercial value for these unmet medical needs, patient advocacy groups, charities, and foundations are at the frontline of this challenge[180].

In 2018, the ASO Milasen was developed to treat a single 6-year-old patient with neuronal ceroid lipofuscinosis 7, a neurodegenerative lysosomal storage disorder originating from excessive accumulation of pigment lipofuscin in the body's tissues. The clinical trial result was published in 2019[181]. Remarkably, the molecular diagnosis of this fatal condition led to the rational design, testing, and manufacture of the splice-modulating antisense ASO tailored to this unique patient. Proof-of-concept experiments in cell lines from

the patient served as the basis for launching the N-of-1 clinical trial of Milasen within 1 year after first contact with the patient.

More N-of-1 ASOs drugs have been developed through accelerated regulatory pathways for specific forms of ataxia-telangiectasia and amyotrophic lateral sclerosis (ALS)[3]. It is worth mentioning that gene editing is also progressing to treat n-of-1 diseases, such as in the case of a rare mutation of Duchenne muscular dystrophy[182]. Treating N-of-1 diseases by personalized therapy, which was unthinkable a few years ago, is now becoming a reality with the availability of well mastered technological platforms which drastically reduce development duration and costs. This personalized medicine perspective for N-of-1 patients might represent the hallmark of the ongoing genetic drug revolution. However, this will necessitate solving the main obstacle to the generalization of RNA drug use, which consists in identifying efficient delivery methods to all tissues.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by AMMF - The Cholangiocarcinoma Charity and by Sapienza University of Rome-Fondi di Ateneo.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Kaufmann SH. Paul Ehrlich: founder of chemotherapy. *Nat Rev Drug Discov* 2008;7:373. DOI
2. Drews J. Paul ehrlich: magister mundi. *Nat Rev Drug Discov* 2004;3:797-801. DOI
3. Synofzik M, van Roon-Mom WMC, Marckmann G, et al. Preparing n-of-1 antisense oligonucleotide treatments for rare neurological diseases in europe: genetic, regulatory, and ethical perspectives. *Nucleic Acid Ther* 2022;32:83-94. DOI PubMed PMC
4. Gillmore JD, Gane E, Taubel J, et al. CRISPR-Cas9 *in vivo* gene editing for transthyretin amyloidosis. *N Engl J Med* 2021;385:493-502. DOI
5. Bennett CF, Krainer AR, Cleveland DW. Antisense oligonucleotide therapies for neurodegenerative diseases. *Annu Rev Neurosci* 2019;42:385-406. DOI PubMed PMC
6. Hu B, Weng Y, Xia XH, Liang XJ, Huang Y. Clinical advances of siRNA therapeutics. *J Gene Med* 2019;21:e3097. DOI PubMed
7. Holm A, Hansen SN, Klitgaard H, Kauppinen S. Clinical advances of RNA therapeutics for treatment of neurological and neuromuscular diseases. *RNA Biol* 2022;19:594-608. DOI PubMed PMC
8. Brunet de Courssou JB, Durr A, Adams D, Corvol JC, Mariani LL. Antisense therapies in neurological diseases. *Brain* 2022;145:816-31. DOI PubMed

9. Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc Natl Acad Sci USA* 1978;75:280-4. DOI PubMed PMC
10. Stephenson ML, Zamecnik PC. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci USA* 1978;75:285-8. DOI PubMed PMC
11. Sun P, Liu DZ, Jickling GC, Sharp FR, Yin KJ. MicroRNA-based therapeutics in central nervous system injuries. *J Cereb Blood Flow Metab* 2018;38:1125-48. DOI PubMed PMC
12. Gan W, Guan Z, Liu J, et al. R-loop-mediated genomic instability is caused by impairment of replication fork progression. *Genes Dev* 2011;25:2041-56. DOI PubMed PMC
13. Pang J, Guo Q, Lu Z. The catalytic mechanism, metal dependence, substrate specificity, and biodiversity of ribonuclease H. *Front Microbiol* 2022;13:1034811. DOI PubMed PMC
14. Nowotny M, Gaidamakov SA, Crouch RJ, Yang W. Crystal structures of RNase H bound to an RNA/DNA hybrid: substrate specificity and metal-dependent catalysis. *Cell* 2005;121:1005-16. DOI
15. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 1998;391:806-11. DOI PubMed
16. Montgomery MK, Xu S, Fire A. RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* 1998;95:15502-7. DOI PubMed PMC
17. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001; 411: 494-498. DOI PubMed
18. Bumcrot D, Manoharan M, Koteliansky V, Sah DW. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat Chem Biol* 2006;2:711-9. DOI PubMed PMC
19. Lisa J. Scherer LJ, Rossi JJ. Principles of RNAi trigger expression for gene therapy. Advanced Textbook on Gene Transfer, Gene Therapy and Genetic Pharmacology. 2014; pp53-72; Editor Scherman Daniel. Imperial College Press. ISBN: 9781786346872. DOI
20. Snead NM, Rossi JJ. Biogenesis and function of endogenous and exogenous siRNAs. *Wiley Interdiscip Rev RNA* 2010;1:117-31. DOI PubMed
21. Scherer L, Rossi JJ. RNAi applications in mammalian cells. *Biotechniques* 2004;36:557-61. DOI PubMed
22. Foster DJ, Brown CR, Shaikh S, et al. Advanced siRNA designs further improve *in vivo* performance of GalNAc-siRNA conjugates. *Mol Ther* 2018;26:708-17. DOI PubMed PMC
23. Gosselin NH, Schuck VJA, Barriere O, et al. Translational population-pharmacodynamic modeling of a novel long-acting siRNA therapy, inclisiran, for the treatment of hypercholesterolemia. *Clin Pharmacol Ther* 2023;113:328-38. DOI PubMed
24. Langlois MA, Boniface C, Wang G, et al. Cytoplasmic and nuclear retained DMPK mRNAs are targets for RNA interference in myotonic dystrophy cells. *J Biol Chem* 2005;280:16949-54. DOI
25. Sobczak K, Wheeler TM, Wang W, Thornton CA. RNA interference targeting CUG repeats in a mouse model of myotonic dystrophy. *Mol Ther* 2013;21:380-7. DOI PubMed PMC
26. Bisset DR, Stepniak-Konieczna EA, Zavaljevski M, et al. Therapeutic impact of systemic AAV-mediated RNA interference in a mouse model of myotonic dystrophy. *Hum Mol Genet* 2015;24:4971-83. DOI PubMed PMC
27. Gantier MP, Williams BR. The response of mammalian cells to double-stranded RNA. *Cytokine Growth Factor Rev* 2007;18:363-71. DOI PubMed PMC
28. Gantier MP, Sadler AJ, Williams BR. Fine-tuning of the innate immune response by microRNAs. *Immunol Cell Biol* 2007;85:458-62. DOI PubMed
29. Kleinman ME, Yamada K, Takeda A, et al. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature* 2008;452:591-7. DOI PubMed PMC
30. Cho WG, Albuquerque RJ, Kleinman ME, et al. Small interfering RNA-induced TLR3 activation inhibits blood and lymphatic vessel growth. *Proc Natl Acad Sci U S A* 2009;106:7137-42. DOI PubMed PMC
31. Bramsen JB, Pakula MM, Hansen TB, et al. A screen of chemical modifications identifies position-specific modification by UNA to most potently reduce siRNA off-target effects. *Nucleic Acids Res* 2010;38:5761-73. DOI PubMed PMC
32. Khvorova A, Watts JK. The chemical evolution of oligonucleotide therapies of clinical utility. *Nat Biotechnol* 2017;35:238-48. DOI PubMed PMC
33. Crooke ST, Liang XH, Baker BF, Crooke RM. Antisense technology: a review. *J Biol Chem* 2021;296:100416. DOI PubMed PMC
34. Koller E, Vincent TM, Chappell A, De S, Manoharan M, Bennett CF. Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes. *Nucleic Acids Res* 2011;39:4795-807. DOI PubMed PMC
35. Eckstein F. Phosphorothioates, essential components of therapeutic oligonucleotides. *Nucleic Acid Ther* 2014;24:374-87. DOI PubMed
36. Geary RS, Norris D, Yu R, Bennett CF. Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides. *Adv Drug Deliv Rev* 2015;87:46-51. DOI PubMed
37. Crooke ST. Vitravene-another piece in the mosaic. *Antisense Nucleic Acid Drug Dev* 1998;8:vii-viii. DOI PubMed
38. Furdon PJ, Dominski Z, Kole R. RNase H cleavage of RNA hybridized to oligonucleotides containing methylphosphonate, phosphorothioate and phosphodiester bonds. *Nucleic Acids Res* 1989;17:9193-204. DOI PubMed PMC
39. Zhang L, Liang XH, De Hoyos CL, et al. The combination of mesyl-phosphoramidate inter-nucleotide linkages and 2'-O-methyl in selected positions in the antisense oligonucleotide enhances the performance of RNaseH1 active PS-ASOs. *Nucleic Acid Ther*

2022;32:401-11. DOI PubMed PMC
40. Summerton J, Weller D. Morpholino antisense oligomers: design, preparation, and properties. *Antisense Nucleic Acid Drug Dev* 1997;7:187-95. DOI PubMed
41. Moulton JD. Using morpholinos to control gene expression. *Curr Protoc Nucleic Acid Chem* 2017;68:4.30.1-4.30.29. DOI PubMed PMC
42. Amantana A, Iversen PL. Pharmacokinetics and biodistribution of phosphorodiamidate morpholino antisense oligomers. *Curr Opin Pharmacol* 2005;5:550-5. DOI PubMed
43. Renneberg D, Bouliong E, Reber U, Schümperli D, Leumann CJ. Antisense properties of tricyclo-DNA. *Nucleic Acids Res* 2002;30:2751-7. DOI PubMed PMC
44. Goyenvalle A, Leumann C, Garcia L. Therapeutic potential of tricyclo-DNA antisense oligonucleotides. *J Neuromuscul Dis* 2016;3:157-67. DOI PubMed PMC
45. Nielsen PE, Egholm M, Berg RH, Buchardt O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* 1991;254:1497-500. DOI PubMed
46. McMahon BM, Mays D, Lipsky J, Stewart JA, Fauq A, Richelson E. Pharmacokinetics and tissue distribution of a peptide nucleic acid after intravenous administration. *Antisense Nucleic Acid Drug Dev* 2002;12:65-70. DOI PubMed
47. Lennox KA, Behlke MA. A direct comparison of anti-microRNA oligonucleotide potency. *Pharm Res* 2010;27:1788-99. DOI PubMed
48. Jearawiriyapaisarn N, Moulton HM, Buckley B, et al. Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice. *Mol Ther* 2008;16:1624-9. DOI PubMed PMC
49. Hammond SM, Hazell G, Shabanpoor F, et al. Systemic peptide-mediated oligonucleotide therapy improves long-term survival in spinal muscular atrophy. *Proc Natl Acad Sci U S A* 2016;113:10962-7. DOI PubMed PMC
50. Tajik-Ahmadabad B, Polyzos A, Separovic F, Shabanpoor F. Amphiphilic lipopeptide significantly enhances uptake of charge-neutral splice switching morpholino oligonucleotide in spinal muscular atrophy patient-derived fibroblasts. *Int J Pharm* 2017;532:21-8. DOI PubMed
51. Hangeland JJ, Flesher JE, Deamond SF, Lee YC, Ts'O PO, Frost JJ. Tissue distribution and metabolism of the [32P]-labeled oligodeoxynucleoside methylphosphonate-neoglycopeptide conjugate, [YEE(ah-GalNAc)3]-SMCC-AET-pUmpT7, in the mouse. *Antisense Nucleic Acid Drug Dev* 1997;7:141-9. DOI PubMed
52. Maier MA, Yannopoulos CG, Mohamed N, et al. Synthesis of antisense oligonucleotides conjugated to a multivalent carbohydrate cluster for cellular targeting. *Bioconjug Chem* 2003;14:18-29. DOI
53. Debacker AJ, Voutila J, Catley M, Blakey D, Habib N. Delivery of oligonucleotides to the liver with GalNAc: from research to registered therapeutic Drug. *Mol Ther* 2020;28:1759-71. DOI PubMed PMC
54. Schlegel MK, Janas MM, Jiang Y, et al. From bench to bedside: improving the clinical safety of GalNAc-siRNA conjugates using seed-pairing destabilization. *Nucleic Acids Res* 2022;50:6656-70. DOI PubMed PMC
55. Gennemark P, Walter K, Clemmensen N, et al. An oral antisense oligonucleotide for PCSK9 inhibition. *Sci Transl Med* 2021;13:eabe9117. DOI
56. Angeli E, Nguyen TT, Janin A, Bousquet G. How to make anticancer drugs cross the blood-brain barrier to treat brain metastases. *Int J Mol Sci* 2019;21:22. DOI PubMed PMC
57. Kawasaki AM, Casper MD, Freier SM, et al. Uniformly modified 2'-deoxy-2'-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets. *J Med Chem* 1993;36:831-41. DOI PubMed
58. Seth PP, Siwkowski A, Allerson CR, et al. Short antisense oligonucleotides with novel 2'-4' conformationaly restricted nucleoside analogues show improved potency without increased toxicity in animals. *J Med Chem* 2009;52:10-3. DOI PubMed
59. Shen W, De Hoyos CL, Sun H, et al. Acute hepatotoxicity of 2' fluoro-modified 5-10-5 gapmer phosphorothioate oligonucleotides in mice correlates with intracellular protein binding and the loss of DBHS proteins. *Nucleic Acids Res* 2018;46:2204-17. DOI PubMed PMC
60. Burel SA, Hart CE, Cauntay P, et al. Hepatotoxicity of high affinity gapmer antisense oligonucleotides is mediated by RNase H1 dependent promiscuous reduction of very long pre-mRNA transcripts. *Nucleic Acids Res* 2016;44:2093-109. DOI PubMed PMC
61. Kasuya T, Hori S, Watanabe A, et al. Ribonuclease H1-dependent hepatotoxicity caused by locked nucleic acid-modified gapmer antisense oligonucleotides. *Sci Rep* 2016;6:30377. DOI PubMed PMC
62. Alterman JF, Godinho BMDC, Hassler MR, et al. A divalent siRNA chemical scaffold for potent and sustained modulation of gene expression throughout the central nervous system. *Nat Biotechnol* 2019;37:884-94. DOI PubMed PMC
63. . Dicerna. Methods and compositions for the specific inhibition of transthyretin (TTR) by double-stranded RNA. US Pat; 2019. 1-240.
64. Hu B, Zhong L, Weng Y, et al. Therapeutic siRNA: state of the art. *Signal Transduct Target Ther* 2020;5:101. DOI PubMed PMC
65. Elkayam E, Parmar R, Brown CR, et al. siRNA carrying an (E)-vinylphosphonate moiety at the 5´ end of the guide strand augments gene silencing by enhanced binding to human Argonaute-2. *Nucleic Acids Res* 2017;45:3528-36. DOI PubMed PMC
66. Brown CR, Gupta S, Qin J, et al. Investigating the pharmacodynamic durability of GalNAc-siRNA conjugates. *Nucleic Acids Res* 2020;48:11827-44. DOI PubMed PMC
67. Kel'in AV, Zlatev I, Harp J, et al. Structural basis of duplex thermodynamic stability and enhanced nuclease resistance of 5'-C-Methyl Pyrimidine-Modified Oligonucleotides. *J Org Chem* 2016;81:2261-79. DOI PubMed

68. Schlegel MK, Janas MM, Jiang Y, et al. From bench to bedside: improving the clinical safety of GalNAc-siRNA conjugates using seed-pairing destabilization. *Nucleic Acids Res* 2022;50:6656-70. DOI PubMed PMC
69. Quan D, Obici L, Berk JL, et al. Impact of baseline polyneuropathy severity on patisiran treatment outcomes in the APOLLO trial. *Amyloid* 2023;30:49-58. DOI
70. Ranasinghe P, Addison ML, Dear JW, Webb DJ. Small interfering RNA: discovery, pharmacology and clinical development-an introductory review. *Br J Pharmacol* 2022. DOI PubMed
71. Wei Y, Li X, Lin J, et al. Oral Delivery of siRNA Using fluorinated, small-sized nanocapsules toward anti-inflammation treatment. *Adv Mater* 2023;35:e2206821. DOI PubMed
72. Busignies V, Arruda DC, Charrueau C, et al. Compression of Vectors for Small Interfering RNAs Delivery: Toward Oral Administration of siRNA Lipoplexes in Tablet Forms. *Mol Pharm* 2020;17:1159-69. DOI
73. Fattal E, Bochot A. Ocular delivery of nucleic acids: antisense oligonucleotides, aptamers and siRNA. *Adv Drug Deliv Rev* 2006;58:1203-23. DOI PubMed
74. Scherman D, Rousseau A, Bigey P, Escriou V. Genetic pharmacology: progresses in siRNA delivery and therapeutic applications. *Gene Ther* 2017;24:151-6. DOI PubMed
75. Khoury M, Escriou V, Courties G, et al. Efficient suppression of murine arthritis by combined anticytokine small interfering RNA lipoplexes. *Arthritis Rheum* 2008;58:2356-67. DOI
76. Courties G, Baron M, Presumey J, et al. Cytosolic phospholipase A2α gene silencing in the myeloid lineage alters development of Th1 responses and reduces disease severity in collagen-induced arthritis. *Arthritis Rheum* 2011;63:681-90. DOI
77. Available from: Orphanet: https://www.orpha.net/consor/cgi-bin/Disease.php?lng=EN [Last accessed on 29 May 2023].
78. Angelini G, Mura G, Messina G. Therapeutic approaches to preserve the musculature in duchenne muscular dystrophy: the importance of the secondary therapies. *Exp Cell Res* 2022;410:112968. DOI PubMed
79. Koenig M, Monaco AP, Kunkel LM. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* 1988;53:219-28. DOI PubMed
80. Tennyson CN, Klamut HJ, Worton RG. The human dystrophin gene requires 16 hours to be transcribed and is cotranscriptionally spliced. *Nat Genet* 1995;9:184-90. DOI PubMed
81. Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics* 1988;2:90-5. DOI PubMed
82. Aartsma-Rus A, Van Deutekom JC, Fokkema IF, Van Ommen GJ, Den Dunnen JT. Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle Nerve* 2006;34:135-44. DOI PubMed
83. Aartsma-Rus A, Fokkema I, Verschuuren J, et al. Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. *Hum Mutat* 2009;30:293-9. DOI
84. Neri M, Rossi R, Trabanelli C, et al. The genetic landscape of dystrophin mutations in Italy: a nationwide study. *Front Genet* 2020;11:131. DOI PubMed PMC
85. Goyenvalle A, Vulin A, Fougerousse F, et al. Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. *Science* 2004;306:1796-9. DOI
86. Denti MA, Rosa A, D'Antona G, et al. Chimeric adeno-associated virus/antisense U1 small nuclear RNA effectively rescues dystrophin synthesis and muscle function by local treatment of mdx mice. *Hum Gene Ther* 2006;17:565-74. DOI PubMed
87. Stein CA. Eteplirsen Approved for duchenne muscular dystrophy: the FDA faces a difficult choice. *Mol Ther* 2016;24:1884-5. DOI PubMed PMC
88. Flanigan KM, Voit T, Rosales XQ, et al. Pharmacokinetics and safety of single doses of drisapersen in non-ambulant subjects with Duchenne muscular dystrophy: results of a double-blind randomized clinical trial. *Neuromuscul Disord* 2014;24:16-24. DOI PubMed PMC
89. Deng J, Zhang J, Shi K, Liu Z. Drug development progress in duchenne muscular dystrophy. *Front Pharmacol* 2022;13:950651. DOI PubMed PMC
90. Melki J, Lefebvre S, Burglen L, et al. De novo and inherited deletions of the 5q13 region in spinal muscular atrophies. *Science* 1994;264:1474-7. DOI
91. Lefebvre S, Bürglen L, Reboullet S, et al. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* 1995;80:155-65. DOI
92. Finkel RS, McDermott MP, Kaufmann P, et al. Observational study of spinal muscular atrophy type I and implications for clinical trials. *Neurology* 2014;83:810-7. DOI PubMed PMC
93. Kaufmann P, McDermott MP, Darras BT, et al; Muscle Study Group (MSG); Pediatric Neuromuscular Clinical Research Network for Spinal Muscular Atrophy (PNCR). Prospective cohort study of spinal muscular atrophy types 2 and 3. *Neurology* 2012;79:1889-97. DOI PubMed PMC
94. Rochette CF, Gilbert N, Simard LR. SMN gene duplication and the emergence of the SMN2 gene occurred in distinct hominids: SMN2 is unique to Homo sapiens. *Hum Genet* 2001;108:255-66. DOI PubMed
95. Burlet P, Bürglen L, Clermont O, et al. Large scale deletions of the 5q13 region are specific to Werdnig-Hoffmann disease. *J Med Genet* 1996;33:281-3. DOI PubMed PMC
96. Wirth B, Brichta L, Schrank B, et al. Mildly affected patients with spinal muscular atrophy are partially protected by an increased

SMN2 copy number. *Hum Genet* 2006;119:422-8. DOI
97. Singh NK, Singh NN, Androphy EJ, Singh RN. Splicing of a critical exon of human Survival Motor Neuron is regulated by a unique silencer element located in the last intron. *Mol Cell Biol* 2006;26:1333-46. DOI PubMed PMC
98. Hua Y, Vickers TA, Okunola HL, Bennett CF, Krainer AR. Antisense masking of an hnRNP A1/A2 intronic splicing silencer corrects SMN2 splicing in transgenic mice. *Am J Hum Genet* 2008;82:834-48. DOI PubMed PMC
99. Hua Y, Liu YH, Sahashi K, Rigo F, Bennett CF, Krainer AR. Motor neuron cell-nonautonomous rescue of spinal muscular atrophy phenotypes in mild and severe transgenic mouse models. *Genes Dev* 2015;29:288-97. DOI PubMed PMC
100. Touznik A, Maruyama R, Hosoki K, Echigoya Y, Yokota T. LNA/DNA mixmer-based antisense oligonucleotides correct alternative splicing of the SMN2 gene and restore SMN protein expression in type 1 SMA fibroblasts. *Sci Rep* 2017;7:3672. DOI PubMed PMC
101. Benkhelifa-Ziyyat S, Besse A, Roda M, et al. Intramuscular scAAV9-SMN injection mediates widespread gene delivery to the spinal cord and decreases disease severity in SMA mice. *Mol Ther* 2013;21:282-90. DOI PubMed PMC
102. Mendell JR, Al-Zaidy SA, Rodino-Klapac LR, et al. Current clinical applications of *in vivo* gene therapy with AAVs. *Mol Ther* 2021;29:464-88. DOI PubMed PMC
103. ANDRADE C. A peculiar form of peripheral neuropathy: familiar atypical generalized amyloidosis with special involvement of the peripheral nerves. *Brain* 1952;75:408-27. DOI
104. Jacobson DR, Pastore RD, Yaghoubian R, et al. Variant-sequence transthyretin (isoleucine 122) in late-onset cardiac amyloidosis in black Americans. *N Engl J Med* 1997;336:466-73. DOI
105. Dyck PJB, Coelho T, Waddington Cruz M, et al. Neuropathy symptom and change: Inotersen treatment of hereditary transthyretin amyloidosis. *Muscle Nerve* 2020;62:509-15. DOI PubMed PMC
106. Westermark P, Sletten K, Johansson B, Cornwell GG 3rd. Fibril in senile systemic amyloidosis is derived from normal transthyretin. *Proc Natl Acad Sci U S A* 1990;87:2843-5. DOI PubMed PMC
107. Benson MD, Waddington-Cruz M, Berk JL, et al. Inotersen treatment for patients with hereditary transthyretin amyloidosis. *N Engl J Med* 2018;379:22-31. DOI PubMed
108. Sewing S, Roth AB, Winter M, et al. Assessing single-stranded oligonucleotide drug-induced effects in vitro reveals key risk factors for thrombocytopenia. *PLoS One* 2017;12:e0187574. DOI PubMed PMC
109. Flierl U, Nero TL, Lim B, et al. Phosphorothioate backbone modifications of nucleotide-based drugs are potent platelet activators. *J Exp Med* 2015;212:129-37. DOI PubMed PMC
110. Severi D, Palumbo G, Spina E, et al. A case of severe increase of liver enzymes in a ATTRv patient after one year of inotersen treatment. *Neurol Sci* 2023;44:1419-22. DOI PubMed PMC
111. Alnylam pharmaceuticals highlights of US prescribing information. Available from:https://www.alnylam.com/ [Last accessed on 29 May 2023].
112. Marimani MD, Ely A, Buff MC, et al. Inhibition of replication of hepatitis B virus in transgenic mice following administration of hepatotropic lipoplexes containing guanidinopropyl-modified siRNAs. *J Control Release* 2015;209:198-206. DOI
113. Kulkarni JA, Witzigmann D, Chen S, Cullis PR, van der Meel R. Lipid nanoparticle technology for clinical translation of sirna therapeutics. *Acc Chem Res* 2019;52:2435-44. DOI PubMed
114. van der Meel R, Chen S, Zaifman J, et al. Modular lipid nanoparticle platform technology for siRNA and lipophilic prodrug delivery. *Small* 2021;17:e2103025. DOI
115. Böttger R, Pauli G, Chao PH, et al. Lipid-based nanoparticle technologies for liver targeting. *Adv Drug Deliv Rev* 2020;154-155:79-101. DOI PubMed
116. Zhang MM, Bahal R, Rasmussen TP, Manautou JE, Zhong XB. The growth of siRNA-based therapeutics: updated clinical studies. *Biochem Pharmacol* 2021;189:114432. DOI PubMed PMC
117. Weng YH, Xiao HH, Zhang JC, Liang XJ, Huang YY. RNAi therapeutic and its innovative biotechnological evolution. *Biotechnol Adv* 2019;37:801-25. DOI
118. Springer AD, Dowdy SF. GalNAc-siRNA conjugates: leading the way for delivery of RNAi therapeutics. *Nucleic Acid Ther* 2018;28:109-18. DOI PubMed PMC
119. Chaumet-Riffaud P, Martinez-Duncker I, Marty AL, et al. Synthesis and application of lactosylated, 99mTc chelating albumin for measurement of liver function. *Bioconjug Chem* 2010;21:589-96. DOI PubMed
120. Salmon H, Gahoual R, Houzé P, et al. Europium labeled lactosylated albumin as a model workflow for the development of biotherapeutics. *Nanomedicine* 2019;18:21-30. DOI
121. Habtemariam BA, Karsten V, Attarwala H, et al. Single-dose pharmacokinetics and pharmacodynamics of transthyretin targeting N-acetylgalactosamine-small interfering ribonucleic acid conjugate, vutrisiran, in healthy subjects. *Clin Pharmacol Ther* 2021;109:372-82. DOI PubMed
122. Adams D, Tournev IL, Taylor MS, et al; HELIOS-A Collaborators. Efficacy and safety of vutrisiran for patients with hereditary transthyretin-mediated amyloidosis with polyneuropathy: a randomized clinical trial. *Amyloid* 2023;30:1-9. DOI
123. Ando Y, Adams D, Benson MD, et al. Guidelines and new directions in the therapy and monitoring of ATTRv amyloidosis. *Amyloid* 2022;29:143-55. DOI
124. Echaniz-Laguna A, Cauquil C, Labeyrie C, Adams D. Treating hereditary transthyretin amyloidosis: Present & future challenges. *Rev Neurol* 2023;179:30-4. DOI PubMed

125. Aimo A, Castiglione V, Rapezzi C, et al. RNA-targeting and gene editing therapies for transthyretin amyloidosis. *Nat Rev Cardiol* 2022;19:655-67. DOI
126. Tanowitz M, Hettrick L, Revenko A, Kinberger GA, Prakash TP, Seth PP. Asialoglycoprotein receptor 1 mediates productive uptake of N-acetylgalactosamine-conjugated and unconjugated phosphorothioate antisense oligonucleotides into liver hepatocytes. *Nucleic Acids Res* 2017;45:12388-400. DOI PubMed PMC
127. Kim Y, Jo M, Schmidt J, et al. Enhanced potency of GalNAc-conjugated antisense oligonucleotides in hepatocellular cancer models. *Mol Ther* 2019;27:1547-57. DOI PubMed PMC
128. Brook JD, McCurrach ME, Harley HG, et al. Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell* 1992;69:385. DOI PubMed
129. Konieczny P, Stepniak-Konieczna E, Sobczak K. MBNL proteins and their target RNAs, interaction and splicing regulation. *Nucleic Acids Res* 2014;42:10873-87. DOI PubMed PMC
130. Goodwin M, Mohan A, Batra R, et al. MBNL Sequestration by toxic RNAs and RNA misprocessing in the myotonic dystrophy brain. *Cell Rep* 2015;12:1159-68. DOI PubMed PMC
131. Overby SJ, Cerro-Herreros E, Llamusi B, Artero R. RNA-mediated therapies in myotonic dystrophy. *Drug Discov Today* 2018;23:2013-22. DOI PubMed
132. Lee JE, Bennett CF, Cooper TA. RNase H-mediated degradation of toxic RNA in myotonic dystrophy type 1. *Proc Natl Acad Sci USA* 2012;109:4221-6. DOI PubMed PMC
133. A safety and tolerability study of multiple doses of ISIS-DMPKRx in adults with myotonic dystrophy Type 1. Available from: https://clinicaltrials.gov/ct2/show/NCT02312011 [Last accessed on 29 May 2023].
134. Sugo T, Terada M, Oikawa T, et al. Development of antibody-siRNA conjugate targeted to cardiac and skeletal muscles. *J Control Release* 2016;237:1-13. DOI
135. Safety, tolerability, pharmacodynamic, efficacy, and pharmacokinetic study of dyne-101 in participants with myotonic dystrophy Type 1 (ACHIEVE). Available from: https://www.clinicaltrials.gov/ct2/show/NCT05481879 [Last accessed on 29 May 2023].
136. Nguyen Q, Yokota T. Degradation of Toxic RNA in myotonic dystrophy using gapmer antisense oligonucleotides. In: Yokota T, Maruyama R, editors. Gapmers. New York: Springer US; 2020. pp. 99-109. DOI
137. Cerro-Herreros E, González-Martínez I, Moreno-Cervera N, et al. Therapeutic potential of antagomiR-23b for treating myotonic dystrophy. *Mol Ther Nucleic Acids* 2020;21:837-49. DOI PubMed PMC
138. Wheeler TM, Lueck JD, Swanson MS, Dirksen RT, Thornton CA. Correction of ClC-1 splicing eliminates chloride channelopathy and myotonia in mouse models of myotonic dystrophy. *J Clin Invest* 2007;117:3952-7. DOI PubMed PMC
139. Negishi Y, Endo-takahashi Y, Ishiura S. Exon skipping by ultrasound-enhanced delivery of morpholino with bubble liposomes for myotonic dystrophy model mice. In: Yokota T, Maruyama R, editors. Exon Skipping and Inclusion Therapies. New York: Springer; 2018. pp. 481-7. DOI
140. Xia X, Zhou H, Huang Y, Xu Z. Allele-specific RNAi selectively silences mutant SOD1 and achieves significant therapeutic benefit in vivo. *Neurobiol Dis* 2006;23:578-86. DOI PubMed
141. Lombardi MS, Jaspers L, Spronkmans C, et al. A majority of Huntington's disease patients may be treatable by individualized allele-specific RNA interference. *Exp Neurol* 2009;217:312-9. DOI
142. Hauser S, Helm J, Kraft M, Korneck M, Hübener-Schmid J, Schöls L. Allele-specific targeting of mutant ataxin-3 by antisense oligonucleotides in SCA3-iPSC-derived neurons. *Mol Ther Nucleic Acids* 2022;27:99-108. DOI PubMed PMC
143. Pfister EL, Kennington L, Straubhaar J, et al. Five siRNAs targeting three SNPs may provide therapy for three-quarters of Huntington's disease patients. *Curr Biol* 2009;19:774-8. DOI PubMed PMC
144. Kay C, Collins JA, Caron NS, et al. A comprehensive haplotype-targeting strategy for allele-specific HTT suppression in huntington disease. *Am J Hum Genet* 2019;105:1112-25. DOI PubMed PMC
145. Conroy F, Miller R, Alterman JF, et al. Chemical engineering of therapeutic siRNAs for allele-specific gene silencing in Huntington's disease models. *Nat Commun* 2022;13:5802. DOI PubMed PMC
146. Trochet D, Prudhon B, Mekzine L, et al. Benefits of therapy by dynamin-2-mutant-specific silencing are maintained with time in a mouse model of dominant centronuclear myopathy. *Mol Ther Nucleic Acids* 2022;27:1179-90. DOI PubMed PMC
147. Dudhal S, Mekzine L, Prudhon B, et al. Development of versatile allele-specific siRNAs able to silence all the dominant dynamin 2 mutations. *Mol Ther Nucleic Acids* 2022;29:733-48. DOI PubMed PMC
148. Züchner S, Noureddine M, Kennerson M, et al. Mutations in the pleckstrin homology domain of dynamin 2 cause dominant intermediate charcot-marie-tooth disease. *Nat Genet* 2005;37:289-94. DOI
149. Sambuughin N, Goldfarb LG, Sivtseva TM, et al. Adult-onset autosomal dominant spastic paraplegia linked to a GTPase-effector domain mutation of dynamin 2. *BMC Neurol* 2015;15:223. DOI PubMed PMC
150. Fujise K, Noguchi S, Takeda T. Centronuclear myopathy caused by defective membrane remodelling of dynamin 2 and BIN1 variants. *Int J Mol Sci* 2022;23:6274. DOI PubMed PMC
151. Wang L, Barylko B, Byers C, Ross JA, Jameson DM, Albanesi JP. Dynamin 2 mutants linked to centronuclear myopathies form abnormally stable polymers. *J Biol Chem* 2010;285:22753-7. DOI PubMed PMC
152. Cowling BS, Chevremont T, Prokic I, et al. Reducing dynamin 2 expression rescues X-linked centronuclear myopathy. *J Clin Invest* 2014;124:1350-63. DOI PubMed PMC
153. Koutsopoulos OS, Kretz C, Weller CM, et al. Dynamin 2 homozygous mutation in humans with a lethal congenital syndrome. *Eur J*

Hum Genet 2013;21:637-42. DOI PubMed PMC
154. Study of TD101, a Small interfering RNA (siRNA) designed for treatment of pachyonychia congenita. Available from: https://clinicaltrials.gov/ct2/show/NCT00716014 [Last accessed on 29 May 2023].
155. Leachman SA, Hickerson RP, Schwartz ME, et al. First-in-human mutation-targeted siRNA phase Ib trial of an inherited skin disorder. *Mol Ther* 2010;18:442-6. DOI PubMed PMC
156. Trochet D, Prudhon B, Vassilopoulos S, Bitoun M. Therapy for dominant inherited diseases by allele-specific RNA interference: successes and pitfalls. *Curr Gene Ther* 2015;15:503-10. DOI PubMed
157. Roth F, Dhiab J, Boulinguiez A, et al. Assessment of PABPN1 nuclear inclusions on a large cohort of patients and in a human xenograft model of oculopharyngeal muscular dystrophy. *Acta Neuropathol* 2022;144:1157-70. DOI PubMed PMC
158. Banerjee A, Apponi LH, Pavlath GK, Corbett AH. PABPN1: molecular function and muscle disease. *FEBS J* 2013;280:4230-50. DOI PubMed PMC
159. Malerba A, Klein P, Bachtarzi H, et al. PABPN1 gene therapy for oculopharyngeal muscular dystrophy. *Nat Commun* 2017;8:14848. DOI PubMed PMC
160. Malerba A, Klein P, Lu-Nguyen N, et al. Established PABPN1 intranuclear inclusions in OPMD muscle can be efficiently reversed by AAV-mediated knockdown and replacement of mutant expanded PABPN1. *Hum Mol Genet* 2019;28:3301-8. DOI PubMed PMC
161. Strings-Ufombah V, Malerba A, Kao SC, et al. BB-301: a silence and replace AAV-based vector for the treatment of oculopharyngeal muscular dystrophy. *Mol Ther Nucleic Acids* 2021;24:67-78. DOI PubMed PMC
162. Cao W, Lia R, Pei X, et al. Antibody–siRNA conjugates (ARC): emerging siRNA drug formulation. *Medicine in Drug Discovery* 2022;15:100128. DOI
163. Zlatev I, Castoreno A, Brown CR, et al. Reversal of siRNA-mediated gene silencing in vivo. *Nat Biotechnol* 2018;36:509-11. DOI
164. Burel SA, Hart CE, Cauntay P, et al. Hepatotoxicity of high affinity gapmer antisense oligonucleotides is mediated by RNase H1 dependent promiscuous reduction of very long pre-mRNA transcripts. *Nucleic Acids Res* 2016;44:2093-109. DOI PubMed PMC
165. Kasuya T, Hori S, Watanabe A, et al. Ribonuclease H1-dependent hepatotoxicity caused by locked nucleic acid-modified gapmer antisense oligonucleotides. *Sci Rep* 2016;6:30377. DOI PubMed PMC
166. Yasuhara H, Yoshida T, Sasaki K, Obika S, Inoue T. Reduction of off-target effects of gapmer antisense oligonucleotides by oligonucleotide extension. *Mol Diagn Ther* 2022;26:117-27. DOI PubMed PMC
167. Kobayashi Y, Tian S, Ui-Tei K. The siRNA off-target effect is determined by base-pairing stabilities of two different regions with opposite effects. *Genes* 2022;13:319. DOI PubMed PMC
168. Kanasty RL, Whitehead KA, Vegas AJ, Anderson DG. Action and reaction: the biological response to siRNA and its delivery vehicles. *Mol Ther* 2012;20:513-24. DOI PubMed PMC
169. Alagia A, Eritja R. siRNA and RNAi optimization. *Wiley Interdiscip Rev RNA* 2016;7:316-29. DOI PubMed
170. Grimm D, Wang L, Lee JS, et al. Argonaute proteins are key determinants of RNAi efficacy, toxicity, and persistence in the adult mouse liver. *J Clin Invest* 2010;120:3106-19. DOI PubMed PMC
171. Cardinali B, Provenzano C, Izzo M, et al. Time-controlled and muscle-specific CRISPR/Cas9-mediated deletion of CTG-repeat expansion in the DMPK gene. *Mol Ther Nucleic Acids* 2022;27:184-99. DOI PubMed PMC
172. Wallace LM, Garwick-Coppens SE, Tupler R, Harper SQ. RNA interference improves myopathic phenotypes in mice over-expressing FSHD region gene 1 (FRG1). *Mol Ther* 2011;19:2048-54. DOI PubMed PMC
173. Gautier B, Hajjar H, Soares S, et al. AAV2/9-mediated silencing of PMP22 prevents the development of pathological features in a rat model of Charcot-Marie-Tooth disease 1 A. *Nat Commun* 2021;12:2356. DOI PubMed PMC
174. Morelli KH, Griffin LB, Pyne NK, et al. Allele-specific RNA interference prevents neuropathy in charcot-marie-tooth disease type 2D mouse models. *J Clin Invest* 2019;129:5568-83. DOI PubMed PMC
175. Muraine L, Bensalah M, Dhiab J, et al. Transduction efficiency of adeno-associated virus serotypes after local injection in mouse and human skeletal muscle. *Hum Gene Ther* 2020;31:233-40. DOI PubMed PMC
176. Boivin M, Charlet-Berguerand N. Trinucleotide CGG repeat diseases: an expanding field of polyglycine proteins? *Front Genet* 2022;13:843014. DOI PubMed PMC
177. Glineburg MR, Todd PK, Charlet-Berguerand N, Sellier C. Repeat-associated non-AUG (RAN) translation and other molecular mechanisms in fragile X tremor ataxia syndrome. *Brain Res* 2018;1693:43-54. DOI PubMed PMC
178. German CA, Shapiro MD. Small interfering RNA therapeutic inclisiran: a new approach to targeting PCSK9. *BioDrugs* 2020;34:1-9. DOI PubMed
179. Lemaitre MM. Individualized antisense oligonucleotide therapies: how to approach the challenge of manufacturing these oligos from a chemistry, manufacturing, and control-regulatory standpoint. *Nucleic Acid Ther* 2022;32:101-10. DOI PubMed
180. Crooke ST. Meeting the needs of patients with ultrarare diseases. *Trends Mol Med* 2022;28:87-96. DOI PubMed
181. Kim J, Hu C, Moufawad El Achkar C, et al. Patient-customized oligonucleotide therapy for a rare genetic disease. *N Engl J Med* 2019;381:1644-52. DOI PubMed PMC
182. Treatment of a single patient with CRD-TMH-001. Available from: https://clinicaltrials.gov/ct2/show/NCT05514249 [Last accessed on 29 May 2023].

Persico *et al. Rare Dis Orphan Drugs J* 2023;2:13
DOI: 10.20517/rdodj.2023.08

Rare Disease and Orphan Drugs Journal

Original Article

Open Access

Coenzyme Q10, Vitamin E and Polyvitamin B: an exploratory double-blind randomized cross-over study in Phelan-McDermid Syndrome

Antonio M. Persico[1], Arianna Ricciardello[2], Francesca Cucinotta[3], Laura Turriziani[2], Giorgia Calabrese[2], Pasquale Tomaiuolo[2], Tiziana Di Bella[2], Fabiana Bellomo[2], Maria Boncoddo[2], Giada Turturo[2], Silvestro Mirabelli[2], Lisa Asta[1], Federico Banchelli[4], Riccardo Cuoghi Costantini[4], Roberto D'Amico[4]

[1]Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, & Child and Adolescent Neuropsychiatry Program, Modena University Hospital, Modena I-41125, Italy.
[2]Interdepartmental Program "Autism 0-90", "G. Martino" University Hospital, Messina I-98124, Italy.
[3]IRCCS Centro Neurolesi "Bonino-Pulejo", Messina I-98124, Italy.
[4]Department of Medical and Surgical Sciences, University of Modena and Reggio Emilia, & Unit of Statistical and Methodological Support to Clinical Research, Modena University Hospital, Modena I-41125, Italy.

Correspondence to: Prof. Antonio M. Persico, Child & Adolescent Neuropsychiatry, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, via Giuseppe Campi 287, Modena I-41125, Italy. E-mail antonio.persico@unimore.it

How to cite this article: Persico AM, Ricciardello A, Cucinotta F, Turriziani L, Calabrese G, Tomaiuolo P, Bella TD, Bellomo F, Boncoddo M, Turturo G, Mirabelli S, Asta L, Banchelli F, Costantini RC, D'Amico R. Coenzyme Q10, Vitamin E and Polyvitamin B: an exploratory double-blind randomized cross-over study in Phelan-McDermid Syndrome. *Rare Dis Orphan Drugs J* 2023;2:xx. https://dx.doi.org/10.20517/rdodj.2023.08

Received: 5 Mar 2023 **Revised:** 24 Jun 2023 **Accepted:** 6 Jul 2023 **Published:** 14 Jul 2023

Academic Editor: Daniel Scherman **Copy Editor:** Yanbing Bai **Production Editor:** Yanbing Bai

Abstract

Aim: Defective mitochondrial function and increased oxidative stress have been documented in Autism Spectrum Disorder (ASD) and Phelan-McDermid syndrome (PMS), one of the best-known monogenic forms of ASD. The purpose of this exploratory, double-blind, randomized cross-over trial (RCT) is to verify the efficacy and safety of a "metabolic support therapy" (MST) in PMS, while defining the experimental methodology most apt at maximizing sensitivity and reliability.

Methods: A total of 31 PMS patients completed 4 months of Coenzyme Q10 (50/100 mg b.i.d.) + vitamin E (30/60 mg/d) + polyvitamin B ("active compound") *vs.* 4 months of only Vitamins E and B ("active comparator"). To explore their sensitivity and reliability, four primary outcome measures were used: VABS, CARS, CGI-I, and VAS. Secondary outcome measures span adaptive behaviors, social cognition, autism, problem behaviors, quality

of life (QoL), communication, and comorbidities.

Results: CoQ10+vit. E and B yielded significantly greater improvement in several measures of cognition and adaptive functioning, motor skills, and stereotypic behaviors compared to vit. E and B only. Maternal QoL was especially improved in the presence of CoQ10 ($P < 0.004$). Time x Treatment interactions in CGI-I and VAS "restricted interests" scores support positive contributions also by vitamins E and B. Side effects, including hyperactivity, insomnia, and irritability, were mild, rare, and did not differ between treatment periods.

Conclusion: MST may produce small-to-moderate improvement, especially in motor skills, social motivation, adaptive behaviors, responsiveness to environmental stimulation, and stereotypic behaviors in up to approximately 70% of PMS patients. A targeted confirmatory RCT contrasting Q10+vit E and B *vs.* inactive placebo is now warranted.

Keywords: 22q13 deletion syndrome, autism, autism spectrum disorder, coenzyme Q10, Phelan-McDermid syndrome, *SHANK3*, vitamin B, vitamin E.

INTRODUCTION

Phelan-McDermid syndrome (PMS, OMIM#606232) is a rare genetic neurodevelopmental disorder mainly characterized by global developmental delay, severe deficits in expressive language, and intellectual disability (ID)[1-4]. The prevalence of PMS has been estimated in the range of 2.5-10 cases per million births[5]. However, the prevalence of PMS is likely to be underestimated and over 3,000 individuals self-identified as PMS patients worldwide are registered in the "Phelan-McDermid Syndrome International Registry" of the PMS Foundation (https://www.pmsf.org/international/)[5]. Autism Spectrum Disorder is present in as many as 30%-80% of PMS patients, while at least 0.5%-2.0% of autistic individuals carry a genetic abnormality related to PMS in chr. 22q13[6-8]. A wide range of other neurological and behavioral signs/symptoms may also be present, such as muscle hypotonia and deficits in motor coordination, seizures, structural brain abnormalities, gastrointestinal symptoms, renal malformations, and minor dysmorphic features[1-4,7,8]. Recently, other psychiatric disorders were found to be associated with PMS, including catatonia, mood, and psychotic disorders[9]. Lasting regressions in communication, self-care, and motor function beginning within 3 years of the onset of psychiatric illness were found to exert a very negative impact on clinical prognosis[10].

The pathophysiology of PMS is due to a spectrum of genetic anomalies in the terminal region of the long arm of chromosome 22, ranging from disruptive single-nucleotide variants to ring chromosomes and large deletions affecting multiple genes[2]. *SHANK3* (OMIM *606230), located in the distal portion of the long arm of chromosome 22, is the strongest candidate gene to determine PMS[3,11]; however, genotype-phenotype correlations in PMS are complex, as even interstitial deletions sparing *SHANK3* have been described in some PMS patients[12]. The size of PMS-causing deletions can be extremely variable, ranging from 0.22 to 9.22 Mb[8], and a multitude of genes distributed along this terminal region may contribute to phenotypic heterogeneity and clinical severity[13]. Furthermore, rare autosomal recessive forms of mitochondrial disorders have been found to emerge from the coincidence of a large PMS-causing deletion on one chr. 22q13 allele and of a missense mutation or small deletion not detectable by array-CGH located on the other allele[14]. The larger chr.22 segment deleted in PMS hosts several genes involved in mitochondrial function (*SCO2, CHKB, TYMP, TRMU, NDUFA6, SAMM50, SULT4A1*)[13,15]. Abnormalities in electron transport chain (ETC) complex activity have been detected in 30/51 (59%) individuals with PMS[15], supporting the hypothesis that mitochondrial dysfunction could contribute to the pathological process and phenotypic expression of PMS in many patients.

Behavioral and psychological/physical treatments are considered the first line intervention, because there is no pharmacological compound yet proven effective in ameliorating the core signs/symptoms of PMS[16]. Pharmacological treatment strategies adopted to improve psychiatric comorbidities and sleep disorders are not different from those prescribed in the general population[17], but responses to traditional psychiatric intervention are variable and sensitivity to the development of side effects is seemingly high[9].

Coenzyme Q10 (CoQ10, ubiquinone) is a lipid-soluble quinone present in the highest concentrations in brain, heart, kidney, and liver tissue[18]; tissue levels reflect the extent of metabolic activity and energy of each tissue and organ[19]. CoQ10 can be found in three distinct redox states: oxidized (ubiquinone), semiquinone (ubisemiquinone), and reduced (ubiquinol)[18]. It is synthesized by human cells and can also be partly obtained from dietary sources (on average 5 mg/day)[20]. In the latter case, Q10 ubiquinone is reduced to Q10 ubiquinol in the liver following gut absorption. Both ubiquinone and ubiquinol are currently available as dietary supplements[21]. CoQ10 plays an important role in supporting energy production in living cells[19], and orally administered CoQ10, in the presence of partial tissue deficiency, can boost electron transfer and ATP synthesis[22-24]. More specifically, CoQ10 is present in two intracellular pools, a protein-bound pool (30%) and a free-cytosolic pool[21]. The former plays a pivotal role in the electron chain transport system responsible for the synthesis of ATP, whereby CoQ10 transfers electrons from complex I and II to complex III by shuttling in its redox cycle[21]. Through this mechanism, Q10 supplementation can boost energy levels and reduce the risk of mitochondrial damage due to excessive oxidative stress[21-25]. The cytosolic pool participates in other metabolic processes, most importantly playing an additional antioxidant role by protecting lipids and other cell components, while also restoring reduced levels of other antioxidants[21]. Overall, increased energy production and antioxidant capacity are predicted to limit the damage generated by the neuroinflammation and excitotoxicity well documented in ASD brains, ultimately leading to excessive neuritic pruning and/or cell apoptosis[26,27]. Interestingly, serum coenzyme Q10 levels were found to interact with genetic predisposition to a variety of neuronal diseases in a genome-wide association study[28]. Moreover, converging evidence strongly supports its neuroprotective effects[22,29,30] and numerous disease processes can benefit from oral administration of CoQ10, including neurodegenerative diseases[24,31-34].

In an attempt to transfer this knowledge "from bench to bedside", we designed a "metabolic support therapy" (MST) encompassing Q10 ubiquinol or CoQ10 (50-100 mg b.i.d.) + vit. E (30-60 mg/die) + poly vitamin B (B50 complex, 1/2 or 1 caps/die), under the hypothesis that its metabolic, antioxidant and neurochemical effects could at least partly improve the clinical signs/symptoms of neurodevelopmental disorders. Recently, we performed a retrospective chart review reporting small-to-moderate improvement in cognitive and adaptive functioning, social interaction and motor coordination in 45/59 (76.3%) patients with different neurodevelopmental disorders, with excellent tolerability[35]. These results were especially promising in five PMS patients, all displaying some measurable degree of clinical response[35].

Based on the evidence summarized above, we have now designed and performed an exploratory 32-week randomized, double-blind, placebo-controlled, cross-over trial (RCT), with (A) the primary aim to evaluate the efficacy and safety of MST in PMS patients and to begin dissecting the selective contribution of CoQ10 to efficacy and tolerability, as compared to vitamins alone; (B) the secondary aim to identify a panel of psychodiagnostic tools endowed with sufficient sensitivity to reliably detect a small-to-moderate effect size in a severely-compromised patient population. This information will then be used to design a confirmatory RCT applying a targeted strategy.

METHODS

Study design

This double-blind, randomized cross-over, placebo-controlled study assessed the efficacy and safety of an MST with CoQ10, vitamin E and complex-B vitamins in PMS. All procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration. The Ethical Committee of the University of Messina (Messina, Italy) approved the study (prot. n. 15/18, approved on May 2nd, 2018). The parents of all patients enrolled in this study were informed about potential adverse reactions. Written informed consent was obtained from all caregivers for their child's participation. This clinical trial adheres to CONSORT guidelines and has been registered at https://clinicaltrials.gov/ with NCT n. 04312152.

The experimental design of this trial is displayed in Figure 1. Briefly, the total duration of the trial was 8 months. Each patient received an active compound [CoQ10 + Vit. E + polyvit. B] for 4 months and active comparator [Vit. E + polyvit. B] for 4 months. Half of the sample received the active compound during period I (months 1-4) and the active comparator during period II (months 5-8), while the remaining half assumed the active comparator during period I and active compound during period II [Figure 1]. More specifically, following a medical screening visit, enrolled patients were double-blindly randomized according to a permuted two-block design, to receive either active compound or active comparator (Period I) and then were switched to the other treatment condition (Period II); each treatment period lasted for 4 months. Initially eligible subjects underwent a complete clinical and psychometric assessment (T0), which was repeated at the end of each 4-month treatment period [T1 and T2 in Figure 1]. Three investigators (L.T., G.C., T.DB.), never involved in clinical assessments nor in direct contact with patients and caregivers, were in charge of treatment allocation. To monitor compliance, unused capsules were retrieved and counted at each time point. One investigator (A.R.), not involved in clinical assessments, interacted with caregivers as needed throughout the study, for any medical need and for issues regarding the trial itself. Families were requested to refrain from commenting on their experience and purported treatment efficacy on social media.

Participants

Inclusion and Exclusion Criteria

Participants were required to hold a diagnosis of PMS due to a genetically documented deletion of human chromosome 22q13.3 or a disruptive mutation in the *SHANK3* gene. Other inclusion criteria were: (1) age between 2 and 40 years old; (2) Children's Global Assessment Scale (CGAS) score between 45 and 59 at baseline; (3) medication regimen stable for at least three months prior to enrolment and kept constant throughout the 8-month duration of the trial; (4) behavioral intervention started at least 3 months prior to enrolment and kept unchanged throughout the 8-month duration of the trial; (5) written informed consent provided by both parents or a legally authorized patient representative; (6) parents and guardian able to understand and comply with the experimental protocol.

Potential participants were excluded in the presence of any of the following: (1) known genetic syndromes other than PMS (for example, Rett syndrome, fragile-X syndrome, *etc.*); (2) serious medical illnesses (chronic renal disease, severe liver disease, cardiovascular disorders, uncontrolled hypertension with systolic pressure values > 170 mm Hg and diastolic pressure > 100 mm Hg, malignant tumors, HIV infection); (3) history of acute cerebrovascular disease; (4) history of stomach bleeding or active peptic ulcer; (5) documented allergies, hypersensitivity or intolerance to one of the excipients of the experimental compound or comparative product; (6) treatment with anticoagulants. Seizures were not a cause of exclusion and were categorized as “frequent” if occurring more often than once every 6 months, “rare” if occurring at a lower frequency, or otherwise “absent”.

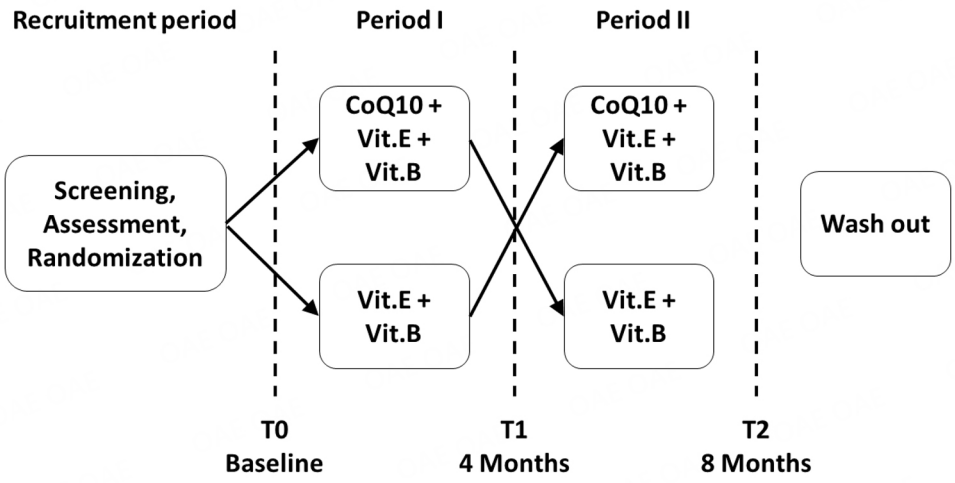


Figure 1. Experimental design of the trial. Patients were assessed at baseline (T0), 4 months (T1), and 8 months (T2), to monitor the safety and efficacy of active compound [CoQ10 + Vit. E+ Polyvit. B] *vs.* active comparator [Vit. E+ Polyvit. B].

Trial interruption criteria include (1) the onset of a severe medical condition during the trial; (2) changes in psychopharmacological or behavioral treatment lasting longer than two weeks during the 8-month duration of the trial; (3) medical conditions requiring anticoagulant treatment started during the trial.

Patient sample

Sixty-six PMS patients were screened for eligibility at the Interdepartmental Program "Autism 0-90" of the "G. Martino" University Hospital in Messina, Italy, between March 2019 and September 2021. The CONSORT flow-chart of the study is depicted in Figure 2. Five patients did not meet inclusion criteria, five already received open treatment with CoQ10/ubiquinol and vitamins[34], and twenty-one refused participation. The remaining 35 PMS patients were recruited. Comorbid neurodevelopmental disorders were diagnosed based on DSM-5 criteria[36]. The total sample was stratified by sex and randomly assigned to receive the active compound [CoQ10+VitE+VitB] either during the first or the second administration period. Two patients, one per group, dropped out due to non-compliance; hence a total of 33 PMS patients completed the entire trial and were included in the statistical analysis [Figure 2].

Intervention

The active compound encompasses CoQ10 (50-100 mg b.i.d.), vitamin E (30-60 mg b.i.d.), and B-group vitamins, including nicotinamide, dexpanthenol, riboflavin-5'- sodium phosphate, inositol, pyridoxine hydrochloride, and cyanocobalamin, while active comparator is devoid of CoQ10 and includes vitamins E and B only [Table 1]. Active compound and active comparator were overencapsulated to preserve blinding and administered orally twice daily (BID) during the trial. If patients were unable to swallow capsules, parents were allowed to open the capsules and immediately administer their contents in a small quantity of juice or soft drink. Two different doses were prescribed based on body weight below or above 20 kg [Table 1]. Vitamins and minerals were dosed considering the maximum daily intake allowed in food supplements by the Italian Ministry of Health (Italian Ministry of Health, Directorate-General for Hygiene and Food Safety and Nutrition, 2021) and on the basis of our prior retrospective analysis[35].

Patient assessment and outcome measures

At baseline, each patient underwent a comprehensive medical evaluation, including physical and neurological examination, routine hematology and blood chemistry. We collected family, developmental,

Table 1. Composition of the active compound. The active comparator is devoid of CoQ10 and contains all the remaining components

Compounds	Daily dose by patient weight	
	≤ 20 kg	> 20 kg
CoQ10	50 mg	100 mg
Vitamin E	30 mg	60 mg
Vitamin B1(thiamine)	1.05 mg	2.10 mg
Vitamin B2 (riboflavin HCl)	1.2 mg	2.4 mg
Vitamin B3 (Niacin)	9 mg	18 mg
Vitamin B5 (Pantothenic acid)	4.5 mg	9 mg
Vitamin B6 (Pyridoxine)	1.5 mg	3 mg
Vitamin B12 (Cyanocobalamine)	4.5 µg	9 µg
Folic acid	0.1 mg	0.2 mg
Biotin	0.1125 mg	0.225 mg

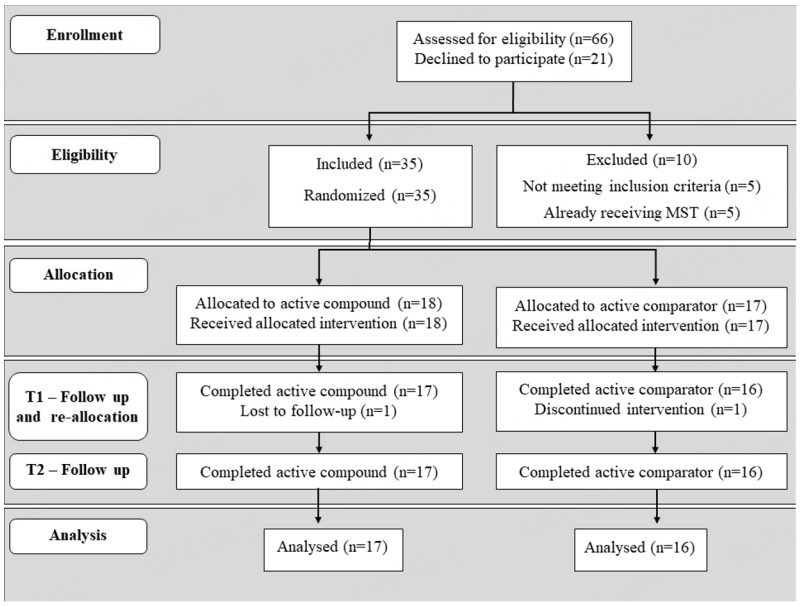


Figure 2. CONSORT flow-chart. Sixty-six PMS patients were assessed for eligibility. Thirty-five of them were recruited and randomized into groups receiving either active compound (*n* = 18) or active comparator (*n* = 17) during the first 4 months. Thirty-three patients completed the entire study, whereas one patient dropped out of each treatment group during the first 4-month period.

educational, medical and psychiatric history. A detailed mental status examination was also performed, applying DSM-5 diagnostic criteria[36]. The complete battery of neuropsychological and behavioral assessments performed at T0, T1, and T2 is summarized in Table 2.

Intellectual/developmental level was assessed using the Griffiths Developmental Rating Scales III, Wechsler Intelligence Scale for Children - Fourth Edition (WISC-IV), or the Leiter III scale, depending on age and expressive language development. Independent evaluators (B.F., B.M., T.G.), blind to treatment conditions and safety data, performed neuropsychological and behavioral testing. Given the exploratory nature of this RCT and the lack of preliminary data on sensitivity to change-over-time of psychodiagnostic scales in PMS patients, multiple primary and secondary measures were applied, selected among those previously used in individuals with PMS, ASD, intellectual disability, or displaying greater sensitivity in RCTs of fragile X syndrome[1,4,37-39].

Table 2. Clinical and psychodiagnostic assessment performed at each time point

	Clinical Assessment	Psychodiagnostic assessment
T0 Baseline	√ Physical and neurological examination √ Family, developmental, educational, medical and psychiatric history √ Auxological parameters (height, weight, head circumference) √ Routine hematology and blood chemistry	√ **Questionnaires:** SRS, RBS-R, ABC, CPRS, CBCL, SSP, QOLA, WHOQOL, SCQ √ **Clinical scales:** VAS, CGAS and CGI-S (clinician ratings); VAS (parental ratings) √ **Test:** ADOS2; ADI-R; GMDS-III or cognitive test; VABS-II; CARS2
T1 4 months	√ Physical and neurological examination √ Anamnestic data (Period I) √ Measures of therapy adherence √ Drug safety monitoring	√ **Questionnaires:** SRS, RBS-R, ABC, CPRS, CBCL, SSP, QOLA, WHOQOL, SCQ √ **Clinical scales:** VAS, CGAS, and CGI-I (clinician ratings); VAS and CGI-I (parental ratings) √ **Test:** VABS-II; CARS2
T2 8 months	√ Physical and neurological examination √ Anamnestic data (Period II) √ Measures of therapy adherence √ Drug safety monitoring	√ **Questionnaires:** SRS, RBS-R, ABC, CPRS, CBCL, SSP, QOLA, WHOQOL, SCQ √ **Clinical scales:** VAS, CGAS, and CGI-I (clinician ratings); VAS and CGI-I (parental ratings) √ **Test:** VABS-II; CARS2

VAS: Visual Analog Scale; CGAS: Children's Global Assessment Scale; CGI-I: Clinical Global Impression-Improvement; SRS: Social Responsiveness Scale, RBS-R: Repetitive Behaviors Scale - Revised; ABC: Aberrant Behavior Checklist; CPRS: Conners' Parent Rating Scale-Revised, CBCL: Child Behavior Checklist, SSP: Short Sensory Profile, QOLA: The Quality of Life in Autism Questionnaire; WHOQOL: The World Health Organization's Quality of Life Questionnaire; SCQ: Social Communication Questionnaire; GMDS-III: Griffiths Developmental Rating Scales; VABS-II: Vineland Adaptive Behavior Scale; CARS2: Childhood Autism Rating Scale 2.

Primary outcome measures include:

A. Vineland Adaptive Behaviour Scales-II (VABS-II)[40], a standardized semi-structured parental interview to measure adaptive functioning in real life. Standard scores have a mean of 100 and a standard deviation of 15.

B. Childhood Autism Rating Scale 2 (CARS2)[41], for trained clinicians to rate the presence and severity of signs and symptoms of ASD by direct observation of the child. Scores range from 15 to 60 (< 30, non-autistic; 30-36.5, mild to moderate autism; 37-60, severe autism).

C. Clinical Global Impression - Severity/Improvement scale (CGI-S/I)[42], a 7-point scale for the clinician to quantify illness severity (CGI-S) at baseline, patient improvement/worsening (CGI-I) at follow-up, and treatment side effects.

D. Visual Analog Scales (VAS)[43,44], spanning 16 items listed in Table 3, and ranging from 0 = "no impact" to 10 = "severe impact". Items include all core ASD symptoms based on DSM-5 criteria, fine and gross motor function, as well as an additional set of behaviors reflecting different aspects of social cognition (imitation, play, joint attention, enjoyment in shared play). The latter variables were chosen for their relevance in neurodevelopmental disorders and based on our prior observations in patients receiving an open treatment with MST[35]. VAS was completed both by clinicians and parents at each time point.

Secondary Outcome Measures include:

A. Children's Global Assessment Scale (CGAS)[45], which provides a global measure of the level of functioning in children and adolescents. The measure provides a single rating on a 0-100 scale, with higher scores indicating better functioning.

Table 3. Visual Analog Scales (VAS) used to assess core autism symptoms based on DSM-5 criteria for Autism Spectrum Disorder[36], and additional behaviors of clinical relevance. Each item ranges from 0 (symptom absent) to 10 (extreme severity)

ITEMS	DEFINITION
Socio-emotional skills	Deficits in socio-emotional reciprocity, ranging, for example, from abnormal social approach and failure of normal back-and-forth conversation, to reduced sharing of interests, emotions, or affect, to failure to initiate or respond to social interactions
Non-verbal communication behaviors	Deficits in nonverbal communication behaviors used for social interaction, ranging from poorly integrated verbal and nonverbal communication to abnormalities in eye contact and body language or deficits in understanding and use of gestures
Verbal communication behaviors - expressive language	Deficits in expressive language and verbal communication behaviors used for social interaction
Verbal communicative behaviors - receptive language	Deficits in receptive language and verbal communication behaviors used for social interaction
Theory of mind	Deficits in developing, maintaining, and understanding relationships, ranging, for example, from difficulties adjusting behavior to suit various social contexts, to difficulties in sharing imaginative play or in making friends, to absence of interest in peers
Stereotyped or repetitive motor movements	Stereotyped or repetitive motor movements, use of objects, or speech
Sameness	Insistence on sameness, inflexible adherence to routines, or ritualized patterns of verbal or nonverbal behavior
Restricted interests	Highly restricted, fixated interests that are abnormal in intensity and/or focus
Hold an object in hand	Strong attachment or concern for objects which are often held in the hand for a long time or continuously
Sensory issues	Hyper- or hypo-reactivity to sensory input or unusual interest in sensory aspects of the environment
Imitation	Deficit of imitation with objects and/or imitation of actions and/or verbal imitation.
Play	Quality of spontaneous play (i.e., functional/imitation play, symbolic/representational play)
Fine-motor skills	Motor control deficits in small movements of the hands and fingers, as well as in small muscles of the face and mouth (tongue)
Gross-motor skills	Motor control deficits in global movements, such as pushing and manipulating large objects, rolling around, climbing, and jumping.
Joint attention	Lack of ability to coordinate attention with another person, i.e., you point at an object and see if the child looks in that direction and then turns his/her gaze back towards you.
Enjoyment in shared play	Lack of participation in play activities with others, employing exchanges of glances and smiles.

B. Social Responsiveness Scale (SRS)[46], a 65-item questionnaire used to assess social impairment, communication deficits, and repetitive behaviors in children and adolescents 4-18 years old. Autism is severe, moderate, or mild when T-scores are > 75, 66-75, or 60-65, respectively. Scores < 60 are not clinically significant.

C. Repetitive Behaviors Scale - Revised (RBS-R)[47], a 44-item parental questionnaire used to assess repetitive behaviors in children 6-17 years old. Each behavior is rated on a 0-3 point scale to measure increasing symptom severity, as does the overall global score ranging from 0-100.

D. Aberrant Behaviour Checklist (ABC)[48], a 58-item parental questionnaire used to assess problem behaviors rated on a 0-3 point scale to measure increasing severity.

E. Short Sensory Profile (SSP)[49], a 38-item parental questionnaire assessing sensory processing, sensory modulation, and behavioral/emotional responses. The overall score ranges from 0 to 190, with lower scores reflecting greater symptom severity.

F. Conners' Parent Rating Scale-Revised (CPRS-R)[50], a 48-item parental rating scale used to evaluate the presence and intensity of hyperactivity/inattention, impulsivity, and externalizing behaviors. Each item is rated on a 0-3 point scale to reflect increasing symptom severity.

G. Child Behaviour Checklist (CBCL)[51,52], which provides parental ratings for 20 and 120 items regarding competence and behavioral problems, respectively, in youth aged 6-18 years old. Each item is scored 0-2 to reflect symptom severity or frequency. Standard scores are scaled so that 50 is average for the youth's age and gender, with a standard deviation of 10 points.

H. The Quality of Life in Autism Questionnaire (QOL-A)[53], which measures the parental quality of life either broadly (part A, 28 items, score range 28-140) or specifically related to the ASD affecting their offspring (part B, 20 items, score range 20-100). The total score range is 48-240, with higher scores indicating better quality of life.

I. The World Health Organization's Quality of Life Questionnaire (WHOQOL)[54], which is used to assess parental quality of life in four domains: physical, psychological, social, and environmental. The score can range from 15 to 105, with a higher score indicating better quality of life.

Safety and Adverse Events

Adverse Events (AEs) were monitored during the study by extensive clinical evaluation at each time point, including physical and neurological examination, routine hematology and blood chemistry. Moreover, an investigator (A.R.) blind to treatment and not involved in patient assessments was available to interact with families by phone throughout the study for prompt detection and management of potential adverse effects in-between assessments. The AEs were documented for severity, duration, and management.

Statistical analyses

The statistical analysis was carried out using repeated measures mixed models. The dependent variables were the post-treatment outcome values, whereas the independent variables were treatment (active compound *vs.* active comparator), time (Period II *vs.* Period I), and sequence (the active compound in the first period *vs.* active compound in the second period). A random intercept term was used in the model to account for repeated measurements over the same individuals. Results for numerical variables were reported as the mean difference (MD) from a linear mixed model, whereas for binary or ordinal categorical variables as the odds ratio (OR) from a logistic mixed model or as the cumulative odds ratio (cOR) from an ordinal logistic mixed model, respectively. To describe the effect of treatment in Period I and Period II, standard linear, logistic, and ordinal logistic models were used, with post-treatment values as the dependent variable and treatment as the independent variable. All linear models were also adjusted for pre-treatment outcome values. Uncertainty in estimates was described using 95% confidence intervals (CI). Given the exploratory nature of this study, nominal P-values are presented and statistical significance is set at nominal $P < 0.05$ without controlling for multiple comparisons. Effect modification related to age (considering two subgroups: < 9 and ≥ 9 years) was assessed by significance testing of the treatment x age interaction term in a separate model for each primary outcome variable reaching nominal significance. Analyses were performed using the R 3.6.3 statistical software[55].

RESULTS

The study was completed by 33 patients whose demographic, genetic, and clinical characteristics are summarized in Table 4. The sample was randomized to receive the active compound either during the first (n = 18, 51.4%) or during the second administration period (n = 17, 48.6%). Almost all patients displayed

Table 4. Demographic and clinical characteristics of the sample (*n* = 33)

Characteristics	*n*	Mean ± S.E.M. (range) or %
Age in yrs	33	11.87 ± 0.33 (3-37)
• 0-9 yrs	19	57.6%
• 10-18 yrs	9	27.3%
• ≥ 19 yrs	5	15.2%
Sex		
• M	17	51.5%
• F	16	48.5%
• M : F ratio	1.06 : 1	
Genetics features:		
• chr 22q13.3 deletion	26	78.8%
• SHANK3 mutation	4	12.1%
• Ring chromosome 22	3	9.1%
• Mosaicism	2	6.1%
DSM-5 Diagnosis:	32	97.0%
• Global Developmental Delay (GDD) or Intellectual Disability (ID)	19	57.6%
• GDD or ID + Autism Spectrum Disorder	13	39.4%
IQ[†]:		
• > 70	3	9.1%
• ≤ 70	30	90.9%
CGI-Severity score[‡]:		
• Borderline ill	2	6.1%
• Mildly ill	1	3.0%
• Moderately ill	11	33.3%
• Markedly ill	14	42.4%
• Severely ill	5	15.2%
Behavioral regression history:		
• Present	10	30.3%
• Absent	23	69.7%
Bipolar Disorder:		
• Present	7	21.2%
• Absent	26	78.8%
Epilepsy:		
• Frequent	1	3.0%
• Rare	5	15.2%
• Absent	27	81.8%
Developmental Coordination Disorder:		
• Gross-motor skills	33	100.0%
• Fine-motor skills	30	90.9%
Expressive language (single words):		
• Normal development (< 18 mo)	2	6.1%
• Developmental delay (≥ 18 mo)	11	33.3%
• Lost after normal development	1	3.0%
• Lost after developmental delay	3	9.1%
• Never acquired	16	48.5%
Mean VABS scores (Adaptive skills)[§]:		
• Communication	33	41.8 ± 3.1 (19-72)
• Daily living skills	33	49.0 ± 3.1 (21-81)
• Socialization	33	52.4 ± 2.8 (20-79)
• Motor skills	10	58.2 ± 3.1 (37-72)

• Composite score	33	47.0 ± 2.7 (19-72)
Ongoing psychopharmacological treatment:		
• Antiseizure medications	5	15.2%
• Atypical Antipsychotics	3	9.1%
• Lithium carbonate	1	3.0%
• Melatonin	3	9.1%
• Dietary supplements	1	3.0%

[†]IQ: Intelligence Quotient, [‡]CGI: Clinical Global Impression, [§]VABS: Vineland Adaptive Behavior Scale.

Intellectual Disability (ID) or Global Developmental Delay (GDD), either alone (n = 19, 57.6%) or associated with Autism Spectrum Disorder (ASD) (n = 13, 39.4%). All 33 patients also displayed Developmental Motor Coordination Disorder, while 7 (21.2%) have a history of Bipolar Disorder. Severe Language Developmental Disorder was present in 31 (93.9%) patients. Thirteen (42.9%) patients were taking psychopharmacological treatment or nutraceuticals at baseline (T0), which were kept constant throughout the study [Table 4].

Regarding primary outcome measures, several measures reached nominal significance in favor of the active compound [CoQ10 + Vit. E + polyvitamin B] producing greater improvement than the active comparator [Vit. E + polyvitamin B] [Table 5A for summary statistics and Figure 3, depicting single patients]. Specifically, greater positive responses were recorded mainly in:

(a) Motor skill deficits, with VABS motor skill displaying a sizable improvement (MD +6.81, 95%CI: +1.89; +11.72, P = 0.0147). This statistic includes only 10/33 patients whose age falls within the range for VABS motor skill (0-6 years), but it is paralleled by non-significant improvements in VAS gross- and fine-motor skills collected for all 33 patients, reaching a P = 0.058 in clinician-scored VAS for fine-motor skills. These results are also consistent with clinical observation of widespread small-to-moderate improvement, especially in fine-motor function. This positive effect was surprisingly most evident in three highly compromised patients initially wheelchair-bound due to very large deletions spanning from *SHANK3* to *TCF20* and beyond*:* all three became able to hold up and use fork and spoon during meals; for one, it became feasible to sit up and undertake sessions of mild horse-assisted intervention.

(b) "Enjoyment in shared play", i.e., participation in play activities with others, employing exchanges of glances and smiles (VAS, P = 0.0199). This variable exemplifies one of the benefits most consistently reported by parents, namely increased responsiveness to environmental stimulation and greater social motivation;

(c) Repetitive behaviors were significantly reduced according to parents (VAS, P = 0.0198), with a similar trend also observed by clinicians (VAS, P = 0.0770). This observation describes a consistent subgroup of patients and not a small subset because at T0, parents reported the presence of motor stereotypies in 27 (81.8%) patients, and during the intake visit, motor stereotypic behaviors were directly observed by the clinician in 15 (45.5%) patients.

No effect modification by age was detected for enjoyment in shared play (P = 0.2896) and repetitive behaviors (med P = 0.5297, par P = 0.5656), whereas the number of observations for VABS motor skills was not sufficient to allow reliable testing of age effects.

Table 5. Primary outcome measures reaching nominal significance, indicating (A) greater improvement with active compound [CoQ10 + Vit. E + polyvit. B] *vs.* active comparator [Vit. E + polyvit. B], or (B) Time × Treatment interactions with active compound displaying greater efficacy when administered during period II, but not during period I (also see Figure 4, A and B).

	Subscale		MD or cOR	95% CI		Nominal *P*-value
A	VABS Motor Skills		+6.81	+1.89	+11.72	0.0147
	VAS Stereotyped or repetitive motor movements (rated by parents)		-0.79	-1.45	-0.13	0.0198
	VAS Stereotyped or repetitive motor movements (rated by clinicians)		-0.97	-2.06	0.12	0.0770
	VAS Enjoyment in shared play (rated by parents)		-0.54	-0.99	-0.09	0.0199
B	VAS Restricted Interest (rated by parents)	- T1	+0.42	-0.18	+1.01	0.1701
		- T2	-1.40	-2,76	-0.04	0.0434
	CGI-Improvement	- T1	+0.78	+0.21	+2.94	0.7150
		- T2	+4.55	+1.12	+20.00	0.0336

MD: mean difference; cOR: cumulative odds ratio.

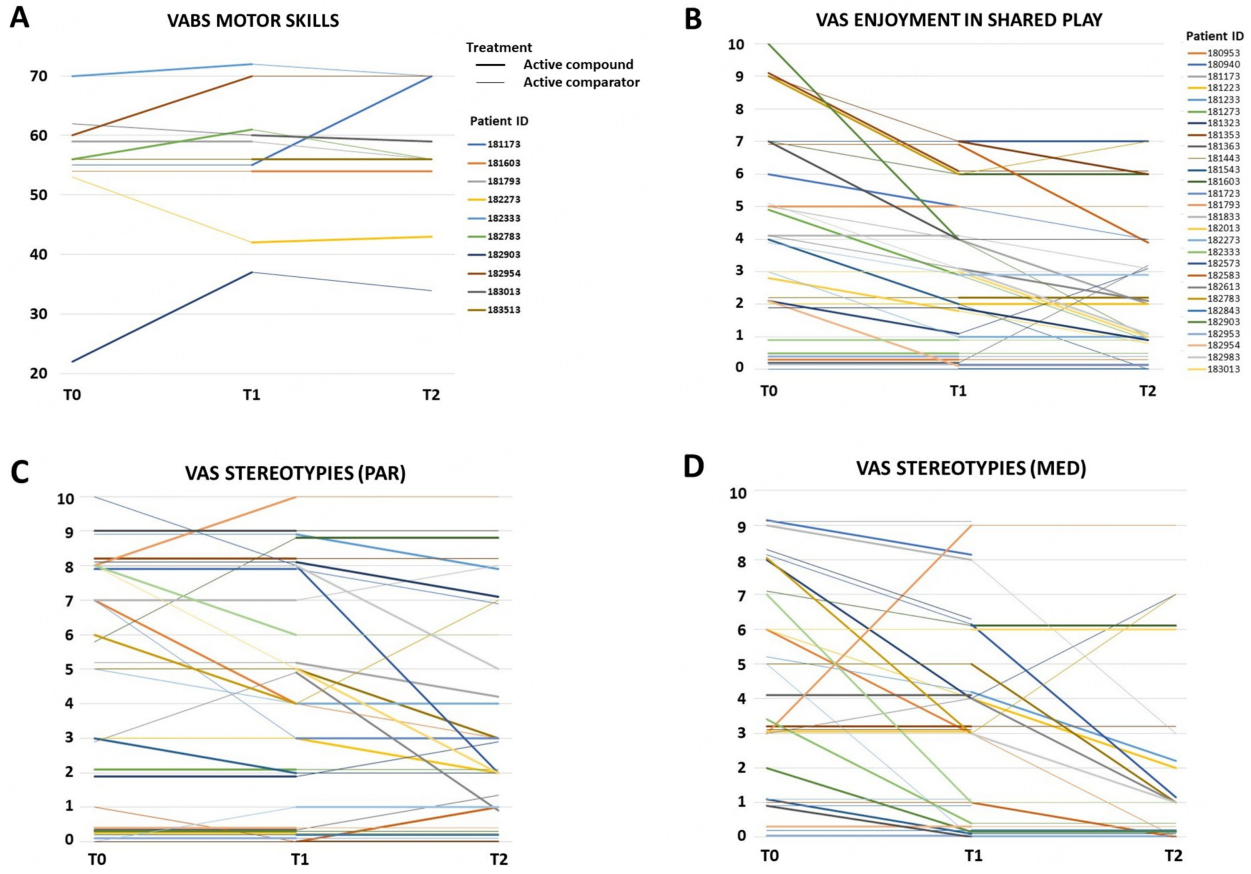


Figure 3. Single-patient line graphs for primary outcome measures reaching significantly greater improvement with active compound (thick lines) *vs.* active comparator (thin lines) [see Table 5A]: (A) VABS Motor Skills scores; (B) VAS Enjoyment in shared play; (C) VAS Stereotyped behaviors (parent-rated); (D) VAS Stereotyped behaviors (clinician-rated). The patient legend of panel B also refers to panels C and D. Higher scores in panel A indicate better motor skills; in panels B-D, higher scores indicate greater symptom severity.

CGI-I scores were consistent with both active compound and active comparator producing overall improvement in a sizable number of cases, with a slight, non-significant advantage for active compound. However, CGI-I scores and parent-rated VAS "Restricted Interests" scores yielded significant Time × Treatment interactions [Table 5B, Figure 4]. In fact, both variables displayed no difference when the active compound was administered during the first four months (Period I), whereas significantly greater efficacy was observed when active compound was administered during Period II, following four months of administration of vitamins E and B complex [Table 5B, Figure 4]. Finally, secondary outcome measures revealed a very significant positive influence of active compound on parental quality of life, with over four

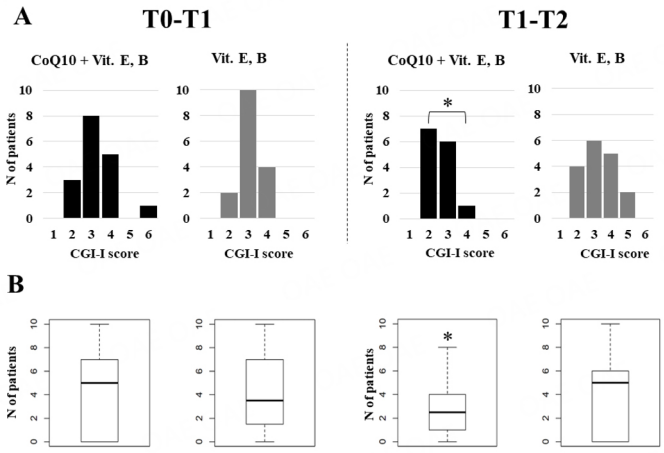


Figure 4. Time x Treatment interaction for changes in (A) CGI-Improvement rated by clinicians; and (B) VAS "Restricted interests" rated by parents. For both variables, active compound produces significantly greater improvement when administered during Period II, but not during Period I. CGI-I scores: 1 = Very much improved, 2 = Much improved, 3 = Minimally improved, 4 = No change, 5 = Minimally worse, 6 = Very much worse.*P<0.05.

Table 6. Number of patients reporting side effects

	Hyperactivity + sleep disturbance	Hyperactivity + increased appetite	Increased appetite and/ or weight gain	Irritability	TOTAL
CoQ10 + Vit. E + polyvit. B	2	0	0	1	3
Vit. E + polyvit. B	1	1	1	1	4

points of mean increase in maternal WHOQOL scores (MD +4.72, 95%CI: +1.34; +8.11, P = 0.0079), and paternal scores displaying a similar trend (MD +3.61, 95%CI: -0.40; +7.63, P = 0.0755). None of the single WHOQOL items reached statistical significance. Subjective parental reports describe their child as becoming more alert, more responsive to parental requests possibly due to improved receptive language skills, displaying a better mood and greater social motivation, and overall better adapted to family life and in school.

Side effects were rare, minor, transient and did not significantly differ between active compound and active comparator [Table 6]. The most frequent were hyperactivity and sleep disturbances, irritability, and increased appetite. Among six patients with a history of seizures [Table 4], none displayed a clinically meaningful change in seizure frequency and/or severity during either treatment period compared to baseline. No serious adverse event occurred and no participant had to stop treatment because of intolerable side effects.

DISCUSSION

This is the first clinical trial on the effects of an MST, encompassing CoQ10, Vitamin E, and Polyvitamin B, in Phelan-McDermid Syndrome (PMS). In addition to being a relatively frequent cause of intellectual disability, PMS represents one of the most important syndromic forms of ASD. In fact, our sample includes 13 (39.4%) patients who also satisfy DSM-5 diagnostic criteria for ASD, in agreement with the 30%-80% range reported in the Literature[6-8]. The present study represents a timely follow-up of our recent retrospective chart review, documenting the potential efficacy of MST in a variety of neurodevelopmental disorders, and includes five PMS patients who, surprisingly, all display some degree of clinical

improvement[35]. Despite the exploratory nature of this RCT, the comparison between full MST *vs.* "vitamins only" provides further evidence pointing towards the beneficial effects of CoQ10 as part of MST in PMS. Clinical improvement in our PMS cohort appears to mostly occur in the domains of motor skills, cognition, responsiveness to environmental stimuli (adaptive functioning), and stereotypic behaviors [Table 5]. Given the severity and pathophysiology of this genetic syndrome and of neurodevelopmental disorders in general, it is not surprising that most responders to MST appear "minimally improved", with only some patients occasionally appearing "much improved". We did not detect significant effects on language and communication skills, in contrast to our retrospective study[35]. This discrepancy may reflect the more severe language impairment present in PMS compared to other neurodevelopmental disorders, with its documented structural underpinnings[11,56]. Importantly, the positive influence recorded here on parental quality of life with MST compared to vitamins only, especially for mothers, provides converging support to MST efficacy from a different point of observation. Finally, the extreme safety of this therapy is documented by the rare, mild, and easily manageable side effects reported by parents, equally distributed between the active compound and active comparator [Table 6].

Mitochondrial function plays a pivotal role in brain development and central nervous system (CNS) function. Most brain energy supply is generated by mitochondrial oxidative phosphorylation, and each different neuronal cell type presents local mitochondrial adaptation processes reflecting functional heterogeneity[57]. In addition to energy production, mitochondria are involved in neurogenesis and apoptosis, neuronal migration and differentiation, and neuroplasticity by regulating redox homeostasis, protein and lipid mediators, and intracellular calcium levels[58]. Therefore, it is not surprising that mitochondrial dysfunction and oxidative stress appear to be involved in the pathogenesis of many neurodevelopmental disorders and that antioxidants produce clinical improvement in many patients[59-62]. More specifically, Autism Spectrum Disorder (ASD) is frequently diagnosed in PMS patients and often represents their first clinical diagnosis, formulated long before genetic testing[6-8]. ASD represents a wide collection of multiple genetic and/or epigenetic disorders (the "autisms"), sharing socio-communication deficits, repetitive behaviors, insistence on sameness and abnormal sensory processing as their clinical expression end-point[63]. Enhanced oxidative stress and mitochondrial dysfunction represent one of the most replicated abnormalities detected both systemically and in the Central Nervous System (CNS) of autistic individuals[64]. Targeted and untargeted studies of post-mortem brains, plasma, CSF, urines, and peripheral blood mononuclear cells (PBMCs) consistently documented significant oxidative damage to proteins, lipids and nucleotides, reductions in GSH/GSSG ratio, reduced activity levels of redox protection enzymes, including superoxide dismutase 2 (SOD2), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutamate-cysteine ligase, reduced activity of respiratory chain complex I and complex IV due to mitochondrial oxidative damage[65-73]. Abnormalities in these redox parameters have been found to be significantly correlated with the severity of autistic behaviors[74,75] and gastrointestinal symptoms[76,77], which are frequent among autistic children[78]. Not surprisingly, the activity of complex I and IV is significantly reduced in as many as 59% of PMS patients, whose clinical features often overlap with those of mitochondrial diseases[79]. In addition to non-specific impairment of energy metabolism, PMS-causing chr. 22q11.3 deletions often span genes playing relevant roles in mitochondrial function[13]. Although oxidative stress response may represent an evolutionarily preserved strategy aimed at boosting resilience[80] and usually represents the consequence and not the primary cause of ASD-associated genetic syndromes[65], reduced ATP production and excessive oxidative damage can potentially exacerbate the dysfunction caused directly by ASD-causing genetic or epigenetic defects, thereby imposing an additional

burden. Importantly, redox abnormalities have also been detected in young autistic children and are not correlated with age[69]. Therefore, enhanced oxidative stress and mitochondrial dysfunction represent a "trait-dependent" characteristic present in a consistent number of PMS patients and autistic individuals, regardless of their age and their specific underlying pathogenetic underpinnings. Since it is not yet possible to directly correct the genetic alteration underlying PMS or its major downstream consequences, sustaining mitochondrial function while controlling redox imbalance may represent a viable indirect therapeutic approach, potentially able to ameliorate cognition and adaptive functioning, motor skills and stereotypic behaviors in many patients, albeit with small-to-moderate effects and with large interindividual differences.

This is the first clinical trial to assess the efficacy and safety of CoQ10, vit. E and polyvit. B in PMS. In reference to ASD, three RCTs have evaluated the effects of over 30 supplements including CoQ10, multiple vitamins, minerals such as lithium, N-acetylcysteine, choline, acetyl-L-carnitine and inositol in 20, 55, and 67 autistic children and adults, respectively[81-83]. Several blood biomarkers were measured, including nutritional (vitamins, minerals, and aminoacids) and metabolic parameters (oxidative stress, methylation, sulfation, glutathione, and neurotransmitters). At baseline, the ASD sample showed significantly higher levels of oxidative stress compared to typically developing controls. After 3 months of treatment, these parameters were either improved or even normalized. Additionally, a behavioral improvement was recorded for several symptoms, as well as in mean Parental Global Impressions-Revised scores, especially for the hyperactivity, oppositional and receptive language subscales[82], and nonverbal intellectual ability[83]. These domains largely overlap with the areas of greatest improvement reported in our retrospective chart review of neurodevelopmental patients[35]. However, the administration of over thirty active compounds hampers the possibility of detecting with reasonable certainty specific contributions by CoQ10 to the observed improvement in biochemical and behavioral parameters. In contrast, more focused RCTs demonstrated a sizable reduction in autistic symptoms following 30 wks of high-dose Vitamin C (110 mg/kg)[84] or after high-dose Vitamin B6 (500-1,000 mg)[85]. To our knowledge, only one RCT has selectively supplemented 78 ASD children with placebo, 30 or 60 mg/day of CoQ10 (n = 26 per sample), recording with the higher CoQ10 dose an improvement in several peripheral redox parameters, paired with a mild reduction ASD severity, sleep disorders and gastrointestinal symptoms[86]. This RCT differs from the present study in experimental design (case-control *vs.* cross-over), duration (3 mo *vs.* 4 mo of active compound/comparator), CoQ10 dosage (not based on body weight or age in ref. 86), and in the composition of active compound and comparator (inactive placebo in ref. 86, vitamins E and B here). Nonetheless, its clinical outcome appears convergent with our past[35] and present observations.

From a methodological standpoint, multiple features of this RCT define its exploratory nature. On the one hand, they may represent the limitations of the present study, but on the other hand, they will enable investigators to precisely design targeted confirmatory trials in neurodevelopmental disorders. These points can be summarized as follows:

(A) Initially, this study was undertaken assuming that vitamins would have been entirely inactive on the severe clinical picture of PMS. However, this turned out not to be the case. On the one hand, the Time x Treatment interactions observed on CGI-I and VAS “Restricted Interests” scores demonstrate that in many patients, a four-month course of vitamin E and B seemingly paves the path to a greater response to CoQ10 [Figure 4]. On the other hand, a slight but detectable amelioration was recorded after the administration of vitamin E and polyvitamin B to most patients [Figure 4A]. In some cases, the convergent observation by parents, clinicians, and therapists blind to the treatment of a "much improved" therapeutic effect by vitamins E and B is unlikely to represent a placebo effect in a severe syndrome such as PMS. For the above-mentioned reasons, contrary to its initial expectation, this study has become an "active comparator-

controlled", randomized, double-blind, cross-over trial, as it would be inaccurate to define it as "placebo-controlled"[87].

(B) It is not customary to apply as many as four different primary outcome measures in an RCT. This strategy was chosen to verify which outcome measures would display the greatest sensitivity in detecting clinical change over time. The low sensitivity of psychodiagnostic scales and questionnaires in detecting clinically meaningful changes not large enough to bring patients outside the ASD spectrum has been one of the major hurdles encountered in experimental pharmacology of genetic syndromes such as Fragile X[39]. Based on our results, clinician-rated CGI-I and parent-rated VAS appear to be the most sensitive measures, followed by the VABS. For example, the outcome of RBS-R and VAS for stereotypic behaviors appears inconsistent. However, mean pre-/post-treatment changes in VAS for stereotypic behaviors were -0.79 and -0.97 for parent- and clinician-rated scores, respectively [Table 5]. VAS ranges from 0 to 10, whereas RBS-R is essentially a Likert scale ranging from 0 to 3. It is not surprising that the RBS-R may not be sufficiently sensitive to change in this context. Nonetheless, care must be placed in ensuring that CGI-I and VAS be administered by the same clinician throughout the study to ensure reliability (notice that also "parent-rated VAS" were explained and presented to parents by the same clinician and were not self-administered). The CARS instead failed to detect changes recorded by these other scales, likely due to difficulty in ensuring reliability at 4-month time intervals even when administered by the same clinician. In reference to secondary measures, we find especially promising that maternal quality of life improves by an average of +4.72 points at the WHOQOL, reaching the highest nominal P-value recorded in this study ($P = 0.0079$). This improvement with CoQ10 over vitamins alone is confirmed by the similar trend observed in fathers (see Results). This effect size is not at all marginal, considering that a 6-point improvement in maternal WHOQOL scores is produced by the complete normalization of child sleep patterns (Persico AM, Cucinotta F, *et al.*, manuscript in preparation).

(C) Placebo effects in behavioral pharmacology can be large enough to obscure the therapeutic effects exerted by the active compound. This is another critical issue, considering that the therapeutic effect size of MST can be realistically expected in the small-to-moderate range[35]. Several strategies potentially able to minimize the size of placebo effects were applied. Clinicians involved in medical visits and psychodiagnostic testing were screened from interactions with parents outside of T0, T1, and T2. Throughout the study, families were free to contact a dedicated investigator not involved in experimental measures whenever necessary. Using this strategy, the number of contacts between families and experimenters was nil or limited to extremely rare and pre-filtered major issues. Participating families were also generally compliant with our request not to comment on the study on social media. Overall, these strategies appeared to yield satisfactory results, although the lack of an inactive placebo here does not allow us to reliably quantify the placebo effect size and estimate their efficacy.

(D) The duration of RCTs is often dictated more by the available budget and other practical considerations than by preliminary efficacy and safety data. The Time x Treatment interactions depicted in Figure 4 suggest that, while 4 months represent a time interval of sufficient duration to produce therapeutic effects, a slightly longer trial duration, if possible, may yield an even greater therapeutic effect size and/or improvements in more areas.

(E) Cross-over studies typically include a one-week wash-out period between the "active compound" and the "active comparator" periods [Figure 1]. This strategy was not applied here under the hypothesis that neurodevelopmental plasticity changes, and not acute pharmacological effects, would underlie behavioral changes. Neuroplastic effects should be expected to carry over for weeks and may be more reliably detected

by searching for differential effects, depending on whether the active compound is administered during the first or the second period, rather than expecting one week of wash-out to truly bring all CNS parameters back to baseline. Our results provide some support to this line of thought; nonetheless, a one-week wash-out period could be applied in future studies.

(F) Our sample size was dictated by the availability of cases affected with a rare disorder, like PMS. It was sufficient to yield nominal significance for several relevant variables but not enough for a superiority trial design, given the small-to-moderate therapeutic effect (i.e., 0.2-0.3). Future studies should benefit from a greater gap in efficacy between MST and a truly inactive placebo, but given these premises, a sample size of $n = 50$ appears to be the minimum requirement for a cross-over study of MST. Multicenter studies may be necessary if single rare disorders are targeted.

(G) The vast majority of patients recruited for this study were unable to swallow capsules. In these patients, both active compound and active comparator were administered by opening capsules and dissolving their content in a small quantity of juice or soft drink. We allowed this procedure, because we were indeed aware that severe swallowing difficulties are frequent in PMS patients[88] and also because, in our retrospective study[35], we did not observe a loss of efficacy following this protocol. Nonetheless, we cannot entirely exclude that differences in drug administration strategy may have contributed to the interindividual variability in response recorded in the present trial.

In summary, this study provides further promising evidence of the positive effects of CoQ10 in PMS when administered in association with Vitamin E and Polyvitamin B, which also appear to provide independent synergistic contributions. Mild-to-moderate improvement was recorded in 24/31 (77.4%) PMS patients and concerned primarily with the domains of cognition, responsiveness to environmental stimuli and adaptive functioning, motor skills, and stereotypic behaviors. In addition, the low incidence and mildness of side effects encourage further studies of this therapeutic approach, given the severity of PMS and the lack of available treatments. From a different perspective, the small but tangible relief provided to parental quality of life by the addition of CoQ10 to vitamins appears to be an important added value in a severe genetic syndrome with a major impact on the daily living of family members[89] and on parental stress[90]. Defining the most sensitive and reliable outcome measures represents a major hurdle in the experimental psychopharmacology of neurodevelopmental disorders[91]. This trial has been instrumental in defining the outcome measures most sensitive to small-to-moderate clinical change in this severely-affected population, the optimal duration, and many other methodological aspects of the experimental design, setting the stage for confirmatory targeted RCTs for PMS, ASD, and other neurodevelopmental genetic syndromes. These studies will also benefit from the assessment of biochemical parameters of oxidative stress to explore whether and to what extent they predict clinical response.

DECLARATIONS

Acknowledgments

The authors wish to acknowledge all the families and patients who participated in this study, and the Italian Association for Phelan-McDermid Syndrome (AISPHEM) for partially funding this project. The authors also acknowledge Dr. Marilena Briguglio for contributing to the medical visits during her clinical activity.

Authors' contributions

Conceptualization, methodology, project coordination and administration: Persico AM
Patient randomization and treatment allocation: Turriziani L, Calabrese G, Di Bella T

Medical visits at T0, T1, and T2: Persico AM, Cucinotta F
Psychological testing at T0, T1, and T2: Boncoddo M, Bellomo F, Turturo G
Response to medical requests from patients and families: Ricciardello A
Data curation: Ricciardello A, Turriziani L, Calabrese G, Tomaiuolo P, Di Bella T, Cucinotta F, Mirabelli S, Persico AM
Statistical analysis: Banchelli F, Cuoghi Costantini R, Asta L, D'Amico R
Writing - original draft preparation: Cucinotta F, Ricciardello A, Turriziani L, Calabrese G, Tomaiuolo P, Di Bella T
Writing - review and editing: Persico AM, Asta L
Read and agreed to the published version of the manuscript: Persico AP, Ricciardello A, Cucinotta F, Turriziani L, Calabrese G, Tomaiuolo P, Di Bella T, Bellomo F, Boncoddo M, Turturo G, Mirabelli S, Asta L, Banchelli F, Cuoghi Costantini R, D'Amico R

Availability of data and materials
All relevant data is contained within the article. The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author/s.

Financial support and sponsorship
This work was partly funded by the Italian Association for Phelan-McDermid Syndrome (AISPHEM).

Conflicts of interest
All authors declared that there are no conflicts of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Ethical approval and consent to participate
The study was conducted in accordance with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration. The study protocol was approved by the Ethical Committee of the University of Messina (Messina, Italy) (prot. n. 15/18 approved on 06/18/2018). Written informed consent was obtained from all caregivers for their own and their children's participation.

Consent for publication
Not applicable.

REFERENCES

1. Phelan MC, Rogers RC, Saul RA, et al. 22q13 deletion syndrome. *Am J Med Genet* 2001;101:91-9. DOI
2. Phelan K, Rogers RC, Boccuto L. Phelan-McDermid syndrome. In M. P. Adam (Eds.) et. al., GeneReviews®. University of Washington, Seattle; 2005. Available from: https://www.ncbi.nlm.nih.gov/books/NBK1198/ [Last accessed on 10 Jul 2023]
3. Phelan K, McDermid HE. The 22q13.3 deletion syndrome (Phelan-McDermid Syndrome). *Mol Syndromol* 2012;2:186-201. DOI PubMed PMC
4. Kolevzon A, Angarita B, Bush L, et al. Phelan-McDermid syndrome: a review of the literature and practice parameters for medical assessment and monitoring. *J Neurodev Disord* 2014;6:39. DOI PubMed PMC
5. National Organization for Rare Disorders. Phelan-cDermid sndrome. Available from: https://rarediseases.org/rare-diseases/phelan-mcdermid-syndrome/#affected-populations [Last accessed on 7 Jul 2023].
6. Betancur C, Buxbaum JD. SHANK3 haploinsufficiency: a "common" but underdiagnosed highly penetrant monogenic cause of autism spectrum disorders. *Mol Autism* 2013;4:17. DOI PubMed PMC

7. Soorya L, Kolevzon A, Zweifach J, et al. Prospective investigation of autism and genotype-phenotype correlations in 22q13 deletion syndrome and SHANK3 deficiency. *Mol Autism* 2013;4:18. DOI PubMed PMC
8. Sarasua SM, Boccuto L, Sharp JL, et al. Clinical and genomic evaluation of 201 patients with Phelan-McDermid syndrome. *Hum Genet* 2014;133:847-59. DOI
9. Kohlenberg TM, Trelles MP, McLarney B, Betancur C, Thurm A, Kolevzon A. Psychiatric illness and regression in individuals with Phelan-McDermid syndrome. *J Neurodev Disord* 2020;12:7. DOI PubMed PMC
10. Kolevzon A, Delaby E, Berry-Kravis E, Buxbaum JD, Betancur C. Neuropsychiatric decompensation in adolescents and adults with Phelan-McDermid syndrome: a systematic review of the literature. *Mol Autism* 2019;10:50. DOI PubMed PMC
11. De Rubeis S, Siper PM, Durkin A, et al. Delineation of the genetic and clinical spectrum of Phelan-McDermid syndrome caused by SHANK3 point mutations. *Mol Autism* 2018;9:31. DOI PubMed PMC
12. Disciglio V, Lo Rizzo C, Mencarelli MA, et al. Interstitial 22q13 deletions not involving SHANK3 gene: a new contiguous gene syndrome. *Am J Med Genet A* 2014;164A:1666-76. DOI PubMed
13. Ricciardello A, Tomaiuolo P, Persico AM. Genotype-phenotype correlation in Phelan-McDermid syndrome: A comprehensive review of chromosome 22q13 deleted genes. *Am J Med Genet A* 2021;185:2211-33. DOI PubMed PMC
14. Vondráčková A, Veselá K, Kratochvílová H, et al. Large copy number variations in combination with point mutations in the TYMP and SCO2 genes found in two patients with mitochondrial disorders. *Eur J Hum Genet* 2014;22:431-4. DOI PubMed PMC
15. Frye RE. Mitochondrial disease in 22q13 duplication syndrome. *J Child Neurol* 2012;27:942-9. DOI PubMed
16. Costales JL, Kolevzon A. Phelan-McDermid sndrome and SHANK3: iplications for teatment. *Neurotherapeutics* 2015;12:620-30. DOI PubMed PMC
17. Verhoeven WMA, Egger JIM, de Leeuw N. A longitudinal perspective on the pharmacotherapy of 24 adult patients with Phelan-McDermid syndrome. *Eur J Med Genet* 2020;63:103751. DOI
18. Wang Y, Hekimi S. Understanding ubiquinone. *Trends Cell Biol* 2016;26:367-78. DOI PubMed
19. Mantle D, Dybring A. Bioavailability of cenzyme Q(10): an overview of the absorption process and subsequent metabolism. *Antioxidants* 2020;9:386. DOI PubMed PMC
20. Weber C, Bysted A, Hłlmer G. The coenzyme Q10 content of the average Danish diet. *Int J Vitam Nutr Res* 1997;67:123-9. DOI PubMed
21. Hargreaves IP. Coenzyme Q10 as a therapy for mitochondrial disease. *Int J Biochem Cell Biol* 2014;49:105e111. DOI PubMed
22. Matthews RT, Yang L, Browne S, Baik M, Beal MF. Coenzyme Q10 administration increases brain mitochondrial concentrations and exerts neuroprotective effects. *Proc Natl Acad Sci USA* 1998;95:8892-7. DOI PubMed PMC
23. Rötig A, Appelkvist EL, Geromel V, et al. Quinone-responsive multiple respiratory-chain dysfunction due to widespread coenzyme Q10 deficiency. *Lancet* 2000;356:391-5. DOI
24. Beal MF. Mitochondrial dysfunction and oxidative damage in Alzheimer's and Parkinson's diseases and coenzyme Q10 as a potential treatment. *J Bioenerg Biomembr* 2004;36:381-6. DOI PubMed
25. Papucci L, Schiavone N, Witort E, et al. Coenzyme q10 prevents apoptosis by inhibiting mitochondrial depolarization independently of its free radical scavenging property. *J Biol Chem* 2003;278:28220-8. DOI
26. Kalayci M, Unal MM, Gul S, et al. Effect of coenzyme Q10 on ischemia and neuronal damage in an experimental traumatic brain-injury model in rats. *BMC Neuroscience* 2011;12:75. DOI PubMed PMC
27. Kumari S, Mehta SL, Milledge GZ, Huang X, Li H, Li PA. Ubisol-Q10 prevents glutamate-induced cell death by blocking mitochondrial fragmentation and permeability transition pore opening. *Int J Biol Sci* 2016; 12:688-700. DOI PubMed PMC
28. Degenhardt F, Niklowitz P, Szymczak S, et al. Genome-wide association study of serum coenzyme Q10 levels identifies susceptibility loci linked to neuronal diseases. *Hum Mol Genet* 2016;25:2881-91. DOI
29. Somayajulu M, McCarthy S, Hung M, Sikorska M, Borowy-Borowski H, Pandey S. Role of mitochondria in neuronal cell death induced by oxidative stress; neuroprotection by Coenzyme Q10. *Neurobiol Dis* 2005;18:618-27. DOI PubMed
30. Moon Y, Lee KH, Park JH, Geum D, Kim K. Mitochondrial membrane depolarization and the selective death of dopaminergic neurons by rotenone: protective effect of coenzyme Q10. *J Neurochem* 2005;93:1199-208. DOI
31. Hochberg J, Fortes da Silva FB. Latissimus dorsi myocutaneous flap to restore elbow flexion and axillary burn contracture: a report on two pediatric patients. *J Pediatr Orthop* 1982;2:565-8. DOI PubMed
32. Müller T, Büttner T, Gholipour AF, Kuhn W. Coenzyme Q10 supplementation provides mild symptomatic benefit in patients with Parkinson's disease. *Neurosci Lett* 2003;341:201-4. DOI PubMed
33. Shults CW, Beal MF, Fontaine D, Nakano K, Haas RH. Absorption, tolerability, and effects on mitochondrial activity of oral coenzyme Q10 in parkinsonian patients. *Neurology* 1998;50:793-5. DOI PubMed
34. Cooper O, Seo H, Andrabi S, et al. Pharmacological rescue of mitochondrial deficits in iPSC-derived neural cells from patients with familial Parkinson's disease. *Sci Transl Med* 2012;4:141ra90. DOI PubMed PMC
35. Cucinotta F, Ricciardello A, Turriziani L, et al. Efficacy and safety of Q10 ubiquinol with vitamins b and e in neurodevelopmental disorders: a retrospective chart review. *Front Psychiatry* 2022;13:829516. DOI PubMed PMC
36. Psychiatric Association. Diagnostic and statistical manual of mental disorders. *Med Health Care Phil* 2014;17:241-244. DOI PubMed
37. Soorya L, Leon J, Trelles MP, Thurm A. Framework for assessing individuals with rare genetic disorders associated with profound intellectual and multiple disabilities (PIMD): the example of Phelan McDermid Syndrome. *Clin Neuropsychol* 2018;32:1226-55. DOI PubMed PMC

38. Braconnier ML, Siper PM. Neuropsychological assessment in autism spectrum disorder. *Curr Psychiatry Rep* 2021;23:63. DOI PubMed PMC
39. Jacquemont S, Berry-Kravis E, Hagerman R, et al. The challenges of clinical trials in fragile X syndrome. *Psychopharmacology* 2014;231:1237-50. DOI PubMed PMC
40. Sparrow SS, Cicchetti DV, Balla DA. Vineland adaptive behavior scales: second edition (Vineland II), survey interview form/caregiver rating form. Livonia, MN: Pearson Assessments; 2005. DOI
41. Vaughan CA. Test Review: E. Schopler, M. E. Van Bourgondien, G. J. Wellman, & S. R. Love Childhood Autism Rating Scale (2nd ed.). Los Angeles, CA: Western Psychological Services, 2010. *J Psychoeduc Assess* 2011;29:489-93. DOI
42. Guy W. Clinical global impressions ECDEU assessment manual for psychopharmacology. Rockville, MD: National Institute for Mental Health; 1976. Available from: https://ia600306.us.archive.org/35/items/ecdeuassessmentm1933guyw/ecdeuassessmentm1933guyw.pdf [Last accessed on 7 Jul2023].
43. Guyatt GH, Townsend M, Berman LB, Keller JL. A comparison of likert and visual analogue scales for measuring change in function. *J Chronic Dis* 1987;40:1129-33. DOI
44. Jaeschke R, Singer J, Guyatt GH. A comparison of seven-point and visual analogue scales. Data from a randomized trial. *Control Clin Trials* 1990;11:43-51. DOI PubMed
45. Shaffer D, Gould MS, Brasic J, et al. A children's global assessment scale (CGAS). *Arch Gen Psychiatry* 1983;40:1228-31. DOI
46. Constantino JN, Davis SA, Todd RD, et al. Validation of a brief quantitative measure of autistic traits: comparison of the social responsiveness scale with the autism diagnostic interview-revised. *J Autism Dev Disord* 2003;33:427-33. DOI
47. Lam KS, Aman MG. The repetitive behavior scale-revised: independent validation in individuals with autism spectrum disorders. *J Autism Dev Disord* 2007;37:855-66. DOI PubMed
48. Aman MG, Singh NN, Stewart AW, Field CJ. The aberrant behavior checklist: a behavior rating scale for the assessment of treatment effects. *Am J Ment Defic* 1985;89:485-91. PMID: 3993694. DOI PubMed
49. Tomchek SD, Dunn W. Sensory processing in children with and without autism: a comparative study using the short sensory profile. *Am J Occup Ther* 2007;61:190-200. DOI PubMed
50. Conners CK, Sitarenios G, Parker JD, Epstein JN. The revised Conners' Parent Rating Scale (CPRS-R): factor structure, reliability, and criterion validity. *J Abnorm Child Psychol* 1998;26:257-68. DOI PubMed
51. Achenbach TM, Rescorla LA. Manual for the ASEBA preschool forms and profiles. Burlington, VT: University of Vermont, Research Center for Children, Youth, and Families; 2000. Available from: https://www.researchgate.net/publication/243771256_Manual_for_the_ASEBA_Preschool_forms_profiles [Last accessed on 7 Jul 2023].
52. Achenbach TM, Rescorla LA. Manual for the ASEBA school-age forms and profiles. Burlington, VT: University of Vermont, Research Center for Children, Youth, and Families; 2001. Available from: https://store.aseba.org/MANUAL-FOR-THE-ASEBA-SCHOOL-AGE-FORMS-PROFILES/productinfo/505/ [Last accessed on 7 Jul2023].
53. McConachie H, Mason D, Parr JR, Garland D, Wilson C, Rodgers J. Enhancing the validity of a quality of life measure for autistic people. *J Autism Dev Disord* 2018;48:1596-611. DOI PubMed PMC
54. The WHOQOL Group. Development of the World Health Organization WHOQOL-BREF quality of life assessment. *Psychol Med* 1998;28:551-8. DOI PubMed
55. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Wien, Austria; 2020.
56. Ponson L, Gomot M, Blanc R, et al. 22q13 deletion syndrome: communication disorder or autism? *Transl Psychiatry* 2018;8:146. DOI PubMed PMC
57. Kann O, Kovács R. Mitochondria and neuronal activity. *Am J Physiol Cell Physiol* 2007;292:C641-57. DOI PubMed
58. Cheng A, Hou Y, Mattson MP. Mitochondria and neuroplasticity. *ASN Neuro* 2010;2:e00045. DOI PubMed PMC
59. Carmeli E, Imam B, Bachar A, Merrick J. Inflammation and oxidative stress as biomarkers of premature aging in persons with intellectual disability. *Res Dev Disabil* 2012;33:369-75. DOI PubMed
60. Valenti D, de Bari L, De Filippis B, Henrion-Caude A, Vacca RA. Mitochondrial dysfunction as a central actor in intellectual disability-related diseases: an overview of Down syndrome, autism, Fragile X and Rett syndrome. *Neurosci Biobehav Rev* 2014;46 Pt 2:202-17. DOI PubMed
61. Alvarez-Mora MI, Rodriguez-Revenga L, Madrigal I, Guitart-Mampel M, Garrabou G, Milà M. Impaired mitochondrial function and dynamics in the pathogenesis of FXTAS. *Mol Neurobiol* 2017;54:6896-902. DOI PubMed
62. Liu J, Wang L, Zhan S, Xia Y, Wang L. Coenzyme Q10 for Parkinson's disease. cochrane database of systematic reviews. *Chichester: John Wiley & Sons, Ltd* 1996. DOI
63. Persico AM, Cucinotta F, Ricciardello A, Turriziani L. Autisms. Neurodevelopmental disorders. Elsevier; 2020. pp. 35-77. DOI
64. Rossignol DA, Frye RE. Evidence linking oxidative stress, mitochondrial dysfunction, and inflammation in the brain of individuals with autism. *Front Physiol* 2014; 5:150. DOI PubMed PMC
65. Palmieri L, Persico AM. Mitochondrial dysfunction in autism spectrum disorders: cause or effect? *Biochim Biophys Acta* 2010; 1797:1130-7. DOI PubMed
66. Palmieri L, Papaleo V, Porcelli V, et al. Altered calcium homeostasis in autism-spectrum disorders: evidence from biochemical and genetic studies of the mitochondrial aspartate/glutamate carrier AGC1. *Mol Psychiatry* 2010; 15:38-52. DOI

67. Chauhan A, Audhya T, Chauhan V. Brain region-specific glutathione redox imbalance in autism. *Neurochem Res* 2012; 37:1681-9. DOI PubMed
68. Rose S, Melnyk S, Pavliv O, et al. Evidence of oxidative damage and inflammation associated with low glutathione redox status in the autism brain. *Transl Psychiatry* 2012; 2:e134. DOI PubMed PMC
69. Frustaci A, Neri M, Cesario A, et al. Oxidative stress-related biomarkers in autism: systematic review and meta-analyses. *Free Radic Biol Med* 2012; 52:2128-41. DOI
70. Tang G, Gutierrez Rios P, Kuo SH, et al. Mitochondrial abnormalities in temporal lobe of autistic brain. *Neurobiol Dis* 2013; 54:349-61. DOI PubMed PMC
71. Gu F, Chauhan V, Chauhan A. Impaired synthesis and antioxidant defense of glutathione in the cerebellum of autistic subjects: alterations in the activities and protein expression of glutathione-related enzymes. *Free Radic Biol Med* 2013; 65:488-96. DOI PubMed
72. El-Ansary A, Bjørklund G, Chirumbolo S, Alnakhli OM. Predictive value of selected biomarkers related to metabolism and oxidative stress in children with autism spectrum disorder. *Metab Brain Dis* 2017; 32:1209-21. DOI PubMed
73. Yenkoyan K, Harutyunyan H, Harutyunyan A. A certain role of SOD/CAT imbalance in pathogenesis of autism spectrum disorders. *Free Radic Biol Med* 2018; 123:85-95. DOI
74. Adams JB, Baral M, Geis E, et al. The severity of autism is associated with toxic metal body burden and red blood cell glutathione levels. *J Toxicol* 2009; 2009:532640. DOI PubMed PMC
75. Ghezzo A, Visconti P, Abruzzo PM, et al. Oxidative stress and erythrocyte membrane alterations in children with autism: correlation with clinical features. *PLoS One* 2013;8:e66418. DOI PubMed PMC
76. Gorrindo P, Lane CJ, Lee EB, McLaughlin B, Levitt P. Enrichment of elevated plasma F2t-isoprostane levels in individuals with autism who are stratified by presence of gastrointestinal dysfunction. *PLoS One* 2013;8:e68444. DOI PubMed PMC
77. Rose S, Bennuri SC, Murray KF, Buie T, Winter H, Frye RE. Mitochondrial dysfunction in the gastrointestinal mucosa of children with autism: a blinded case-control study. *PLoS One* 2017;12:e0186377. DOI PubMed PMC
78. McElhanon BO, McCracken C, Karpen S, Sharp WG. Gastrointestinal symptoms in autism spectrum disorder: a meta-analysis. *Pediatrics* 2014;133:872-83. DOI PubMed
79. Frye RE, Cox D, Slattery J, et al. Mitochondrial dysfunction may explain symptom variation in phelan-mcdermid syndrome. *Sci Rep* 2016;6:19544. DOI PubMed PMC
80. Naviaux RK. Oxidative shielding or oxidative stress? *J Pharmacol Exp Ther* 2012;342:608-18. DOI PubMed
81. Adams JB, Holloway C. Pilot study of a moderate dose multivitamin/mineral supplement for children with autistic spectrum disorder. *J Altern Complement Med* 2004;10:1033-9. DOI PubMed
82. Adams JB, Audhya T, McDonough-Means S, et al. Effect of a vitamin/mineral supplement on children and adults with autism. *BMC Pediatr* 2011;11:111. DOI PubMed PMC
83. Adams JB, Audhya T, Geis E, et al. Comprehensive nutritional and dietary intervention for autism spectrum disorder-a randomized, controlled 12-month trial. *Nutrients* 2018;10:369. DOI PubMed PMC
84. Dolske MC, Spollen J, McKay S, Lancashire E, Tolbert L. A preliminary trial of ascorbic acid as supplemental therapy for autism. *Prog Neuropsych Biol Psychiatry* 1993;17:765-74. DOI
85. Adams JB, George F, Audhya T. Abnormally high plasma levels of vitamin B6 in children with autism not taking supplements compared to controls not taking supplements. *J Altern Complement Med* 2006;12:59-63. DOI
86. Mousavinejad E, Ghaffari MA, Riahi F, Hajmohammadi M, Tiznobeyk Z, Mousavinejad M. Coenzyme Q(10) supplementation reduces oxidative stress and decreases antioxidant enzyme activity in children with autism spectrum disorders. *Psychiatry Res* 2018;265:62-9. DOI PubMed
87. Eldridge SM, Chan CL, Campbell MJ, et al; PAFS consensus group. CONSORT 2010 statement: extension to randomised pilot and feasibility trials. *Pilot Feasibility Stud* 2016;2:64. DOI PubMed PMC
88. Matuleviciene A, Siauryte K, Kuiper E, Grabrucker AM; European Phelan-McDermid syndrome guideline consortium. Consensus recommendations on chewing, swallowing and gastrointestinal problems in Phelan-McDermid syndrome. *Eur J Med Genet* 2023;66:104763. DOI PubMed
89. Serrada-Tejeda S, Martínez-Piédrola RM, Máximo-Bocanegra N, Sánchez-Herrera-Baeza P, Pérez-de-Heredia-Torres M. Descriptive analysis of adaptive behavior in Phelan-McDermid syndrome and autism spectrum disorder. *Front Neurosci* 2022;16:893003. DOI PubMed PMC
90. Droogmans G, Vergaelen E, Van Buggenhout G, Swillen A. Stressed parents, happy parents. An assessment of parenting stress and family quality of life in families with a child with Phelan-McDermid syndrome. *J Appl Res Intellect Disabil* 2021;34:1076-88. DOI PubMed
91. Jacob S, Anagnostou E, Hollander E, et al. Large multicenter randomized trials in autism: key insights gained from the balovaptan clinical development program. *Mol Autism* 2022;13:25. DOI PubMed PMC

Stojkovska Docevska *et al. Rare Dis Orphan Drugs J* 2023;2:14
DOI: 10.20517/rdodj.2023.09

Rare Disease and Orphan Drugs Journal

Review

Open Access

Cathepsin C: structure, function, and pharmacological targeting

Milena Stojkovska Docevska, Marko Novinec

Department of Chemistry and Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Ljubljana SI-1000, Slovenia.

Correspondence to: Dr. Marko Novinec, Department of Chemistry and Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Vecna pot 113, Ljubljana SI-1000, Slovenia. E-mail: marko.novinec@fkkt.uni-lj.si

How to cite this article: Stojkovska Docevska M, Novinec M. Cathepsin C: structure, function, and pharmacological targeting. *Rare Dis Orphan Drugs J* 2023;2:14. https://dx.doi.org/10.20517/rdodj.2023.09

Received: 16 Mar 2023 **First Decision:** 29 Jun 2023 **Revised:** 14 Jul 2023 **Accepted:** 20 Jul 2023 **Published:** 27 Jul 2023

Academic Editors: Daniel Scherman, Bridget Bax **Copy Editor:** Dan Zhang **Production Editor:** Dan Zhang

Abstract

Cathepsin C is a papain-like cysteine peptidase known primarily for its involvement in the activation of serine peptidases in neutrophils and other immune cells. Its critical role in this process qualifies cathepsin C as a target for the treatment of inflammatory diseases, and its most advanced inhibitor, brensocatib (Insmed), is currently in phase 3 clinical trials for the treatment of non-cystic fibrosis bronchiectasis. Beyond neutrophils, its importance is highlighted by loss-of-function mutations that cause the recessively inherited Papillon-Lefèvre syndrome. At the molecular level, cathepsin C has several structural and functional features that set it apart from other members of the family and enable its selective targeting. It possesses dipeptidyl-peptidase activity (its other common name is dipeptidyl-peptidase I) due to the presence of an additional exclusion domain that also controls its stepwise tetramerization during maturation. In this review article, we summarize the current state of the art regarding the biochemical properties of cathepsin C, its physiological and pathological roles in neutrophils and beyond, and recent advances in the development and evaluation of cathepsin C inhibitors.

Keywords: Dipeptidyl-peptidase I, DPPI, cysteine cathepsin, bronchiectasis, brensocatib

INTRODUCTION

Cathepsin C (also known as dipeptidyl-peptidase I, EC 3.4.14.1) was among the first peptidases identified in

animal tissues[1]. It belongs to the papain-like family of cysteine peptidases (PLPs), which is classified as family C1, clan CA in the MEROPS database of peptidases, inhibitors, and substrates[2]. It is found in animals and several other eukaryote lineages[3,4]. In humans, it is encoded by the *CATC* gene located at chromosomal location 11q14.2. It is ubiquitously expressed in humans and other mammals[5] and is a unique peptidase both in terms of its biochemical properties and its specific physiological and pathological functions[3]. It plays a pivotal role in the activation of effector serine peptidases in immune cells such as neutrophils, cytotoxic T lymphocytes, and others. This led to the establishment of cathepsin C as a promising target for the treatment of inflammatory diseases that are characterized by excessive activation of cells of the immune system[6]. Numerous more or less successful inhibitors of cathepsin C have been synthesized and evaluated to date[6]. The most advanced inhibitor, brensocatib (Insmed, Inc, USA), is currently undergoing a phase 3 clinical trial for the treatment of non-cystic fibrosis bronchiectasis[7]. Furthermore, cathepsin C is well-known for the fact that mutations in the *CATC* gene cause the recessive hereditary diseases called Papillon-Lefèvre syndrome and Haim-Munk syndrome[8-10], highlighting the systemic effects of cathepsin C deficiency. Symptoms include early periodontitis, pathological thickening of the skin on the palms and feet, and increased susceptibility to infection[11]. Apart from these, many other (patho)physiological functions have been attributed to cathepsin C in recent decades.

In this review, we summarize the current state of the art on the structural and functional properties of cathepsin C, its processing and trafficking in the cell, and its roles in human physiology and pathology. As this review is part of the special issue "Neutrophil Serine Proteases in Rare Diseases", the primary focus is on its roles in the immune system, particularly in neutrophils. In the end, we also provide an overview of current and emerging strategies for the pharmacological targeting of cathepsin C in human disease.

STRUCTURE AND FUNCTION OF CATHEPSIN C

Many expert reviews on the structural and functional properties of PLPs in general are available, e.g., references[3,12], and the reader is advised to refer to these for more in-depth information on this topic. Herein, we are focusing on cathepsin C and its specific structural and functional properties. Like other members of the PLP family, cathepsin C is synthesized as an inactive precursor called procathepsin C. Its active site contains a Cys-His catalytic dyad that forms a thiolate-imidazolium ion pair (residues Cys234 and His381 according to procathepsin C numbering). What distinguishes it from other PLPs is the additional exclusion domain, the fold of which is similar to that of metalloprotease inhibitors[13]. The exclusion domain is located at the N-terminus of procathepsin C and is separated from the catalytic peptidase domain by the propeptide [Figure 1A]. Upon activation, the propeptide (residues Ala111 through His206) is removed, leaving the exclusion domain non-covalently bound to the peptidase domain, which in turn is cleaved into a heavy chain and a light chain by cleavage between Arg370 and Asp371[13]. As its name suggests, the crucial functional role of the exclusion domain is that it sterically hinders access to the active site beyond site S2 [Figure 1B], making cathepsin C a dipeptidyl-peptidase, i.e., an exopeptidase that cleaves dipeptides of the N-termini of its substrates. Moreover, the side chain of residue Asp1 interacts with the N-terminus of the substrate and stabilizes its binding[13]. The selectivity of the S2 binding site, which is the primary specificity determinant of PLPs, is dominated by two negative charges, namely the aforementioned side chain of residue Asp1 at the entrance of the pocket and a chloride ion at the bottom of a deep hydrophobic pocket [Figure 1C]. The latter is required for enzyme activity and is another distinguishing feature of cathepsin C that sets it apart from other PLPs[13,14]. In our recent evolutionary analysis of cathepsin C, we found that residue Asp1 is strictly conserved in all cathepsin C enzymes throughout the phylogenetic tree, suggesting that dipeptidyl-peptidase activity is the conserved enzymatic activity of these enzymes in all species[4]. The substrate binding sites S1, S1', and S2' do not have any special features compared to other members of the family[15]. Cathepsin C has a relatively broad substrate specificity[16-19] and usually acts by the stepwise

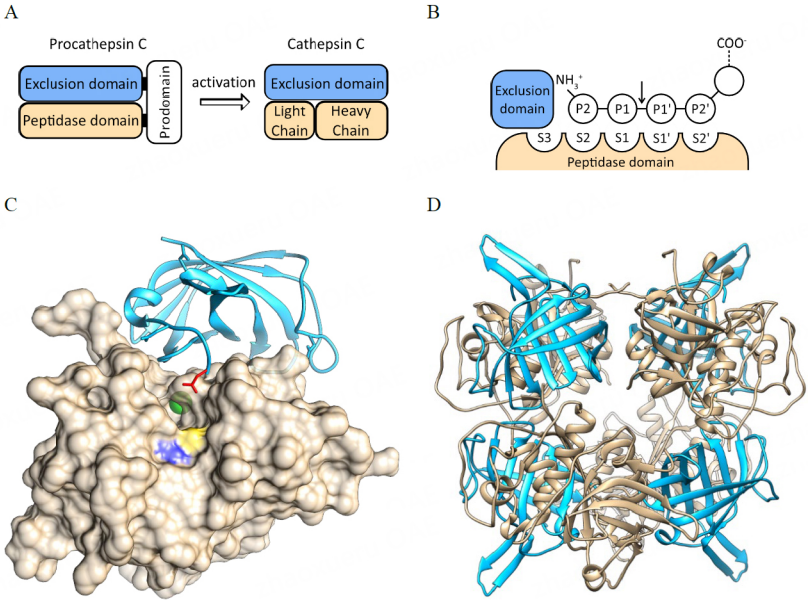


Figure 1. Structural characteristics of cathepsin C. (A) Schematic representation of the (pro)cathepsin C structure; (B) Schematic representation of the active site of papain-like peptidases adapted for cathepsin C. The substrate binding sites on the enzyme are labeled S3 through S2'. The substrate is presented with white circles corresponding to individual residues and residues P2 through P2' are labelled. The arrow denotes the scissile bond; (C) Three-dimensional structure of a cathepsin C subunit. The peptidase domain is shown in molecular surface representation and the exclusion domain in cartoon representation. Active site residues Cys234 and His381 are colored yellow and blue, respectively, Asp1 is shown as red sticks, and the chloride ion cofactor as a green sphere; (D) The cathepsin C tetramer shown in cartoon representation. Exclusion domains are colored blue and peptidase domains are colored tan, respectively. Coordinates were retrieved from the Protein Data Bank under accession code 1K3B.

removal of dipeptides from the N-terminus of the protein substrate. It stops only in three cases: when the N-terminal amino group is blocked, when a proline residue is adjacent to the cleavage site (P1 or P1' position), or when the N-terminal residues are lysine or arginine. Indeed, derivatives of positively charged and aromatic amino acids have been found to competitively inhibit cathepsin C[20]. Nevertheless, the enzyme shows specific preferences for certain residues at positions P1 and P2[16-18] and at positions P1' and P2'[19]. Due to its activity, most substrates used to measure cathepsin C activity *in vitro* are synthetic dipeptide derivatives. The first reported substrates were Gly-Phe-p-nitroanilide (Gly-Phe-pNA) and Gly-Phe-β-naphthylamide (Gly-Phe- βNA)[15]. Their 7-amido-4-methylcoumarin analog (Gly-Phe-AMC) is the most commonly used fluorogenic substrate in the published literature, although better substrates containing natural and unnatural amino acids, such as L-norleucine, L-4-benzoylphenylalanine or L-glutamic acid benzyl ester, have been identified by library screening[16-18]. In addition, highly specific internally quenched substrates have been developed for the detection of cathepsin C activity in biological samples[19]. Like most PLPs, cathepsin C shows optimal activity under slightly acidic pH conditions[21,22]. Unlike others, it also requires chloride (or other halides) ions for optimal activity below pH 7[14,23]. At neutral and basic pH, cathepsin C also exhibits transferase activity[24,25], but similar activity has been observed with other related and unrelated peptidases, e.g., papain[26]. While most cysteine cathepsins are monomers, mature human cathepsin C is a homotetramer organized as a dimer of dimers[13,22] [Figure 1D]. The exclusion domain plays an indispensable role in tetramer formation, as each copy interacts with the peptidase domains of two adjacent subunits. In the tetramer, the active sites of all subunits are fully exposed to the solvent, indicating that all four subunits can function simultaneously. Interestingly, distant cathepsin C homologs have been identified in unicellular eukaryotes, e.g., plasmodium, which were shown to be monomers[16]. We have recently conducted a phylogenetic and computational analysis of the evolution of cathepsin C and found that the tetrameric form is likely restricted to animal cathepsin C homologs[4].

PROCESSING AND ACTIVATION OF CATHEPSIN C

As mentioned in the previous section, cathepsin C is synthesized as an inactive single-chain precursor of ~ 60 kDa per subunit. At this stage, the unique exclusion domain presumably functions as an intramolecular chaperone that aids in the folding of the protein[27]. Evidence shows that human procathepsin C is a homodimer[28,29]. It contains four N-glycosylation sites, one in the heavy chain region of the peptidase domain (Asn252) and three in the exclusion domain (Asn5, Asn29, and Asn95), all of which are retained in the mature form of the enzyme. Proper N-glycosylation is critical for the proper processing and transport of procathepsin C[30], as well as for the assembly of the oligomeric structure, since recombinant non-glycosylated human cathepsin C produced in *E. coli* is a monomer[31]. There is also some interspecies variability in the number of N-glycosylation sites between mammals[4] and in the oligomeric states of cathepsin C orthologs, as rat cathepsin C was reported to be a dimer[32].

Procathepsin C is transported from the endoplasmic reticulum to lysosomes via the mannose-6-phosphate pathway[33,34]. Unlike other cysteine cathepsins, procathepsin C is incapable of autocatalytic processing and must be activated by other lysosomal peptidases. Cathepsins L and S were identified as the first cathepsin C activating peptidases[28]. The maturation of procathepsin C is a multistep process that involves the removal of the internal propeptide segment and the cleavage of the catalytic domain into a heavy chain and a light chain. Full processing is required to obtain the final active conformation[28]. However, it has been shown that cathepsins L and S are not required for the activation of procathepsin C in mice[35]. Similarly, a study on human neutrophil progenitor cell lines PLB-985 and HL60 revealed that complete inhibition of CatS was not sufficient to completely block the activation of procathepsin C, suggesting that other peptidases are involved in this process[36]. Recently, cathepsins F, K, and V were shown to activate procathepsin C *in vitro* via the same intermediate species as cathepsins L and S[29]. These results suggest that the maturation of procathepsin C is a redundant process that can be carried out *in vivo* by different peptidases in tissue-specific patterns.

The purpose of the tetrameric structure of cathepsin C, in contrast to the monomeric forms of other related peptidases, has remained largely unexplained. Olsen *et al.* suggested that the tetrameric structure merely stabilizes the interaction of the exclusion domain with the peptidase domain, thus maintaining the dipeptidyl-peptidase activity[37]. An early report also indicated that cathepsin C is an allosteric enzyme that exhibits pH- and cofactor-dependent cooperative effects[23]. However, these results were later disputed by others[14]. A partial inhibitor of cathepsin C, i.e., one that enables the enzyme to retain part of its activity in the enzyme/inhibitor complex, has been described that could presumably act via allosteric mechanisms at the level of individual subunits[31], a definitive answer to the question of cooperativity between subunits in cathepsin C is still pending.

The non-covalent interactions of the exclusion domain with the peptidase domain and between subunits are strong, and the tetramer does not dissociate in the presence of 2 M guanidinium chloride, but with further increased concentration, the oligomer is completely destroyed[38]. The recombinant peptidase domain alone has been shown to have similar endoproteolytic activity to other papain-like peptidases[39]. In addition, canine and rabbit cathepsins C have been shown to cleave substrates typical of PLP endopeptidases under certain conditions[40,41], suggesting that the exclusion domain can be removed.

PHYSIOLOGICAL AND PATHOLOGICAL FUNCTIONS OF CATHEPSIN C

As pointed out in the introduction, a major physiological role of cathepsin C is the activation of latent zymogens of effector serine peptidases in immune cells. These include four neutrophil serine peptidases (NSPs),i.e., neutrophil elastase, proteinase 3, cathepsin G[42], and the recently discovered neutrophil serine

protease 4[43], as well as granzymes in cytotoxic T lymphocytes, and chymases in mast cells. All belong to the chymotrypsin-like family, classified as clan PA, family S1, subfamily S1A in the MEROPS database of peptidases[2]. All are synthesized as preproenzymes and processed to inactive zymogens upon completion of synthesis and ER import. The zymogens contain an N-terminal propeptide of two amino acid residues and are converted to their active forms via a single cleavage by cathepsin C which removes the propeptide[44-46]. This causes a conformational change that results in the formation of a catalytically competent active site. The underlying molecular mechanism is well conserved in chymotrypsin-like peptidases[47] and was also studied at the molecular level for the activation of prochymase[48].

NSPs are synthesized at the promyelocyte stage. Proper timing of the N-terminal processing is essential for their activity and optimal storage in azurophil granules. Activation normally occurs after the sorting of zymogens to pregranule/granule compartments[49]. Garwicz *et al.* have shown that while the N-terminal propeptide is not strictly necessary for the sorting of neutrophil cathepsin G into the granules, premature activation of the zymogen is deleterious[50]. Similarly, abnormal processing in the absence of DPPI has been shown for granzyme A, resulting in products cleaved at alternative locations by unknown peptidases[46]. NSPs can be either stored in an active form in the granules or secreted from the cell as zymogens in varying proportions, depending on the individual peptidase[51]. Activated neutrophils secrete stored, active NSPs as soluble molecules or bound to chromatin in the form of neutrophil extracellular traps[52]. Once released into the extracellular environment, these pro-inflammatory peptidases can degrade various extracellular matrix components, resulting in tissue damage and chronic inflammation[53].

Some tissue studies have provided evidence that mast cells are the predominant cathepsin C-expressing cells in the non-inflamed airways of dogs[40] and mouse skin[54]. Studies in cultured cells suggested that some cathepsin C is packaged and secreted in serine protein-rich mature granules[55]. Whether cathepsin C, which is released into the extracellular environment rich in cystatins and other potential inhibitors, can also act outside the cell by cleaving extracellular proteins remains unclear, although some studies suggest it is capable of doing so[40,41]. The repertoire of mast-cell peptidases activated by cathepsin C includes cathepsin G and chymase[56-58]. Mast cells from cathepsin C-deficient mice express normal amounts of chymase, but it has the form of an unprocessed proenzyme[45]. The involvement of cathepsin C in the activation of tryptases is less clear[45,59,60]. Recent studies suggest that cathepsin C is sufficient, but not necessary, for the complete maturation of β-tryptases in human mast cells, and that one or more other cysteine cathepsins may take over this role[60]. Experiments in animal models support a role for cathepsin C in tryptase activation, as tryptase activity is reduced but not absent in cathepsin C-deficient mice[45]. In addition, studies in animal models indicate that cathepsin C in mast cells increases the likelihood of fatal sepsis, suggesting a role for cathepsin C in regulating interleukin-6 levels by regulating the production of tryptase and other interleukin-6-degrading peptidases[61]. Similarly, granzymes in cytotoxic T lymphocytes contain N-terminal pro-dipeptides (usually Gly-Glu or Glu-Glu)[62,63]. In these cells, cathepsin C is present in secretory granules[64], and several studies have shown that cathepsin C can activate these peptidases *in vitro*[65-67].

Roles in immunity other than activation of serine peptidases have also been described for cathepsin C. Evidence points towards its regulatory role in macrophage polarization into the M1 phenotype via an interplay with tumor necrosis factor α (TNFα), focal adhesion kinase (FAK) and the p38/mitogen-activated protein kinase (MAPK) pathway[68,69]. Similarly, cathepsin C was shown to promote microglia M1 polarization in the brain[70].

The crucial role of cathepsin C in the activation of peptidases in immune cells suggests that its activity is also an important factor in the development of pathological conditions associated with excessive activity of

these cells and their effector peptidases. These pathologies include various inflammatory and autoimmune diseases such as arthritis[71,72], asthma[40], abdominal aortic aneurysm[73], cystic fibrosis[36], pancreatitis[74], neuroinflammation[70,75] inflammatory lung diseases[36] including bronchiectasis[76], and indirectly chronic obstructive pulmonary disease[77]. Inhibition of cathepsin C activity in bone marrow was shown to be an effective method of silencing neutrophil serine peptidases, underscoring the importance of cathepsin C as a target for the treatment of these diseases[78], as discussed in more detail in the next section.

As expected for a ubiquitously expressed protein, physiological and pathological roles and substrates outside of the immune system have also been described for cathepsin C. The best known pathological examples are mutations in the *CATC* gene that cause the recessive hereditary diseases called Papillon-Lefèvre syndrome (PLS) and Haim-Munk syndrome (HMS)[8-10]. Symptoms of both diseases include early periodontitis, pathological thickening of the skin on the palms and feet, and increased susceptibility to infection, again highlighting the important role of cathepsin C in the immune system[11]. Neutrophil function is impaired but not completely abolished[79]. At the molecular level, mutations can result in truncated cathepsin C[9] or point mutations that cause loss of function[8,9]. At the cellular level, autophagy is impaired, and treatment of PLS patients with recombinant cathepsin C has been proposed as an approach to restore autophagic function[80].

Several studies pointed to the roles of cathepsin C in the systemic regulation of metabolism. The earliest study showed that cathepsin C inactivates the peptide hormone glucagon by sequential removal of dipeptides from the N-terminus[21]. Cathepsin C, in conjunction with plasma glutamate carboxypeptidase, was also shown to be involved in the extracellular processing of thyroglobulin, which is coupled with the release of the stress hormone thyroxin from the thyroid gland[81]. Furthermore, it has been shown to participate in lysosomal degradation of the digestive peptide hormone cholecystokinin together with tripeptidyl peptidase I (TPP-1)[82]. Due to their overlapping N-terminal exopeptidase activity, it was first suggested that cathepsin C may compensate for lack of TPP-1 activity and be used to alleviate the deleterious effects of TPP-1 loss-of-function mutations, which cause the lethal neurodegenerative lysosomal storage disorder classical late-infantile neuronal ceroid lipofuscinosis (CLN2)[82,83]. Unfortunately, studies using mouse models of the disease have invalidated this approach, as cathepsin C did not functionally compensate for the loss of TPP-I activity in the brain[84].

There is also increasing evidence that cathepsin C contributes to the progression of various cancers such as squamous cell carcinoma[54], hepatocellular carcinoma[85], gastric cancer[86], colorectal cancer[87], breast cancer[88], and so on. In these cancers, pathological cathepsin C activity originates either from tumor stromal cells[54] or directly from tumor cells[85-88], and contributes to tumorigenesis via diverse cellular and molecular mechanisms. In squamous cell carcinoma, increased activity of cathepsin C was observed in fibroblasts and bone marrow-derived cells of the tumor stroma, which promoted carcinogenesis[54]. In hepatocellular carcinoma, cathepsin C/TNFα/p38 MAPK interplay was observed similar to that contributing to macrophage M1 polarization[85], whereas in gastric and colorectal cancer, cathepsin C was associated with dysregulation of autophagy[86,87]. In breast cancer, cathepsin C was found to be secreted from tumor cells and to promote neutrophil recruitment during lung metastasis[88]. A detailed expert review on the indirect and direct roles of cathepsin C in cancer has been published recently[89].

In vitro, cathepsin C also plays a critical role in the permeabilization of the lysosomal membrane triggered by the lysosomotropic detergent L-leucyl-leucine methyl ester (LLOMe), resulting in the release of lysosomal hydrolases into the cytosol that ultimately triggers caspase-dependent apoptotic cell death. Cathepsin C has been proposed to mediate lysosomal membrane permeabilization by catalyzing the

polymerization of LLOMe accumulated in lysosomes into its membranolytic polymeric form[90].

In vivo, cathepsin C activity is regulated by endogenous proteinaceous inhibitors. Like other PLP, it is amendable to inhibition by inhibitors from the cystatin family[91,92]. Of note, it is also inhibited by cystatin F[93], which is expressed only in immune cells[94]. Interestingly, inhibition depends on the proteolytic processing of cystatin F to remove its N-terminal part, which cannot be accommodated into the active site of cathepsin C[93]. Recently, it was found that Glu-Lys and Gly-Glu dipeptides derived from the pro-region of granzymes A and B, respectively, inhibit the transferase activity of cathepsin C with IC_{50} values < 20 mM at pH 7.4 and peptidase activity at pH 5.5 with K_i values of 20 mM and 2.5 mM for Glu-Lys and Gly-Glu, respectively[95]. Although this finding is unlikely to be considered as a strategy for pharmacological targeting of cathepsin C, it is one of the few documented examples of product inhibition in this family of peptidases and provides additional insight into the diversity of cathepsin C regulation *in vivo*.

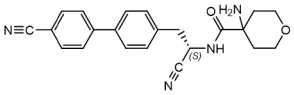
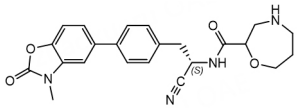
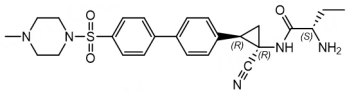
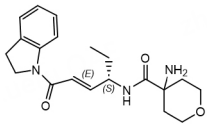
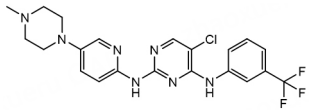
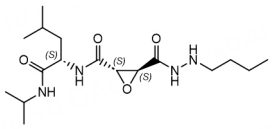
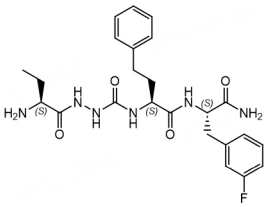
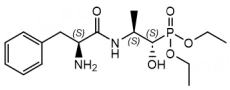
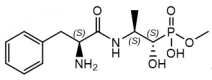
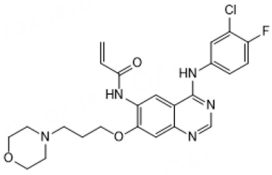
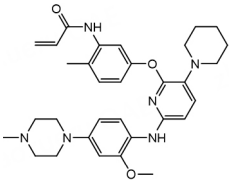
PHARMACOLOGICAL INHIBITION OF CATHEPSIN C

Because of its critical roles in the activation of neutrophil and other serine peptidases, cathepsin C is considered a promising target for the treatment of diseases associated with overactivity of cells producing and secreting these peptidases, such as chronic inflammatory diseases, autoimmune diseases, and so on. Cathepsin C is by no means an easy target in these diseases, as successful anti-cathepsin C therapies must achieve a sustained inhibitory effect directly in the bone marrow to prevent the maturation of NSPs and other serine peptidase zymogens in maturing immune cells[78,96]. Cathepsin C is also an emerging target for the treatment of various cancers[89]. An expert review dedicated specifically to the pharmacological targeting of cathepsin C has been published recently[6]. Here, we shall briefly summarize the current state-of-the-art in the inhibition of cathepsin C.

Most cathepsin C inhibitors are peptide-based and form covalent bonds to the catalytic residue Cys234 using reactive diazomethyl-ketone, nitrile, semicarbazide, or vinyl-sulfone groups (i.e., "warheads"). Some of the most advanced cathepsin C inhibitors to date are collected in Table 1. One of the earliest reported cathepsin C inhibitors was Gly-Phe-CHN_2[97], a substrate analog from a series of diazomethyl-ketone warhead-containing irreversible inhibitors (inactivators) of cysteine peptidases[98]. The high reactivity of the diazomethyl ketone group and instability in acidic media limited the use of such probes beyond *in vitro* experiments, as they are metabolically unstable and sometimes have deleterious side effects, while increased metabolic stability of such inhibitors was often accompanied by decreased inhibitory activity[99].

The first cathepsin C inhibitor selected as a candidate for *in vivo* studies was (S)-4-amino-N-(1-cyano-2-(4'-cyano-[1,1'-biphenyl]-4-yl)ethyl)tetrahydro-2H-pyran-4-carboxamide, a reversible inhibitor with a nitrile warhead developed by AstraZeneca (UK) and later named AZD5248[100] [Table 1]. The compound had pIC_{50} values of 9.1 ±0.1 for purified cathepsin C *in vitro* and 8.1 ± 0.1 in a cell-based assay[100]. The crystal structure of AZD5248 bound into the active site of cathepsin C showed that it interacts with the non-primed sites of the enzyme as well as the N-terminal part of the exclusion domain [Figure 2][100]. It effectively inhibited the activation of NSPs in rats[101] but showed aortic binding in quantitative whole-body autoradiography studies, which could lead to potential cardiovascular toxicity. At the molecular level, aortic binding has been associated with cross-reactivity of AZD5248 with aldehydes, resulting in cross-linking with elastin in the aortic wall[102]. Therefore, novel inhibitors based on AZD5248 were prepared to overcome aortic binding, including the most advanced inhibitor to date, AZD7986 ((S)-N-((S)-1-cyano-2-(4-(3-methyl-2-oxo-2,3-dihydrobenzo-[d]oxazol-5-yl)phenyl)ethyl)-1,4-oxazepane-2-carboxamide)[103]. Like AZD5248, AZD7986 is also a highly potent, reversible, and selective inhibitor of cathepsin C with pIC_{50} values of 8.4 for cathepsin C *in vitro* and cell-based assays[103]. It almost completely inhibited the activation of NSPs in primary $CD34^+$

Table 1. Selected synthetic inhibitors of human cathepsin C

Identifier	Structure	Warhead / Type of inhibition	Potency
AZD5248		Nitrile/reversible	pIC_{50} = 9.1 ± 0.1 (*in vitro*) pIC_{50} = 8.1 ± 0.1 (cell-based assay)
AZD7986		Nitrile/reversible	pIC_{50} = 8.4 ± 0.17 (*in vitro*) pIC_{50} = 6.84 ± 0.33 (cell-based assay)
IcatC		Nitrile/reversible	IC_{50} = 15 ± 1 nM
GSK2793660		α,β-unsaturated amide/irreversible	IC_{50} = 0.43-1 nM
Compound 54 in ref.[115]		Pyridine/reversible	IC_{50} = 57.4 ± 0.7 nM
Compound 11 in ref.[117]		Epoxide/irreversible	k_{inact} = (5.6 ± 1.7) × 10^4 M^{-1} s^{-1}
Compound 8 in ref.[121]		Semicarbazide/reversible	IC_{50} = 31 ± 3 nM K_i = 45 ± 2 nM
Compound 3c in ref.[122]		Phosphonate (non-covalent)/reversible	K_i = 23 ± 12 nM
Compound 4a in ref.[122]		Phosphonate (non-covalent)/reversible	K_i = 51 ± 16 nM
Canertinib		Acrylamide/irreversible	IC_{50} = 0.12 μM
Compound 22 in ref.[125]		Acrylamide/irreversible	IC_{50} = 17.3 ± 0.9 nM (*in vitro*) IC_{50} = 1-5 nM (cell-based assay)

neutrophil progenitor cells from human bone marrow with a pIC_{50} value of 6.84 ± 0.33 and had favorable predicted plasma stability and clearance rate[103].

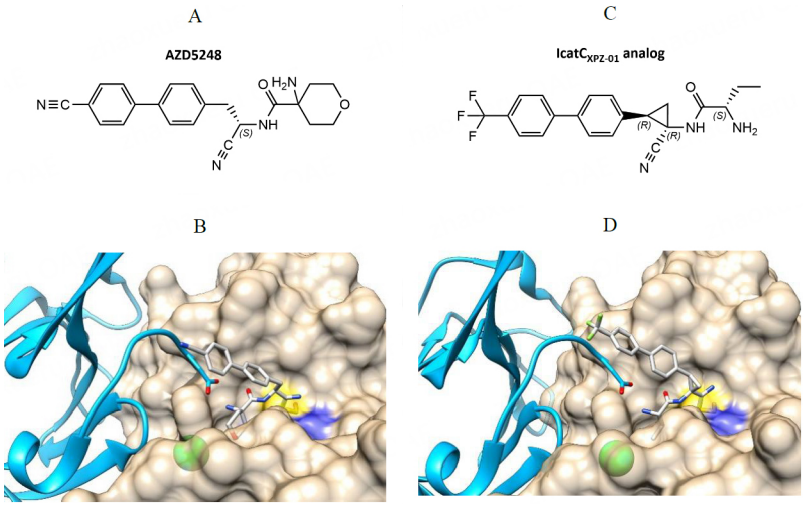


Figure 2. Reversible nitrile warhead-containing inhibitors of cathepsin C. (A) Chemical structure of compounds AZD5258 developed by AstraZeneca; (B) Crystal structure of AZDB5248 bound into the active site of cathepsin C. (PDB accession code 4CDE); (C) Chemical structure of the IcatC$_{XPZ-01}$ analog used for crystallization; (D) Crystal structure of IcatC$_{XPZ-01}$ analog bound into the active site of cathepsin C (PDB accession code 6IC6). Chemical structures were drawn with ChemDraw software. The crystal structures in panels B and D were drawn with UCSF Chimera software[113].

AZD7986 was the first nitrile-based cathepsin C inhibitor to reach clinical trials[104]. In 2016, Insmed, Inc (USA) announced a license agreement with AstraZeneca for exclusive worldwide rights to AZD7986. The compound was renamed INS1007 (trade name brensocatib) and was used in a trial study for the treatment of non-cystic fibrosis bronchiectasis, a persistent dilatation of the airways associated with neutrophil-mediated inflammation[76]. In the 24-week phase 2 study, which ended in 2020, patients treated with brensocatib showed reduced NSP activity, which was associated with the clinical effect of bronchiectasis[105]. These results confirmed the efficacy of brensocatib in the treatment of chronic neutrophil-related inflammatory diseases in the lung. A phase 3 clinical trial is currently underway[7]. Neutrophils and their peptidases are also associated with the severity of COVID-19[106,107]. Therefore, brensocatib has also been evaluated for its beneficial effects in hospitalized patients with COVID-19. Unfortunately, brensocatib did not improve the clinical condition of COVID-19 patients[108].

Another promising nitrile-based inhibitor of cathepsin C is the compound IcatC$_{XPZ-01}$ ((S)-2-amino-N-((1R,2R)-1-cyano-2-(4'-(4-methylpiperazin-1-ylsulfonyl)biphenyl-4-yl)cyclopropyl)butanamide) [Table 1]. IcatC$_{XPZ-01}$ is a reversible, potent, and cell-permeable inhibitor with an IC_{50} value of 15 ± 1 nM and excellent selectivity for cathepsin C[78]. The crystal structure of one of its derivatives bound to the active site of cathepsin C revealed a binding pose analogous to that of AZD5248 [Figure 2][109]. The compound successfully inhibited the activation of NSPs in bone marrow in both cell-based assays and primate experiments[78]. In mice, sufficient concentrations of IcatC$_{XPZ-01}$ were accumulated in the bone marrow to inhibit cathepsin C, and subcutaneous administration of the inhibitor showed significant anti-arthritic activity in an anti-collagen-induced rheumatoid arthritis mouse model[109]. Moreover, preoperative treatment of mice prior to lung transplantation improved early graft function and decreased active NSP levels in the graft, indicating a novel potential use of cathepsin C inhibitors[110]. Despite its promising potential, there is currently no information on its further exploration as a candidate for human clinical trials.

In addition to AZD7986, two other cathepsin C inhibitors have entered clinical trials. Compound GSK2793660 [Table 1] was developed by GlaxoSmithKline (UK) and is a dipeptide-based irreversible inhibitor with an α,β-unsaturated amide-reactive group. Unfortunately, clinical trials were discontinued after several volunteers in a phase 1 study showed symptoms of epidermal desquamation of the palms and soles after repeated administration of the inhibitor, which bore some resemblance to PLS patients. In addition, no significant reduction in NSP activity was observed[111]. The third compound, BI1291583 (undisclosed structure), developed by Boehringer Ingelheim (Germany), has just started a phase 2 trial for the treatment of bronchiectasis (NCT05238675) after successfully completing phase 1[112] and is expected to be completed in Q1 of 2024.

Based on the successes and failures of these inhibitors, novel non-peptidic, non-covalent inhibitors of cathepsin C are emerging in recent years[114]. Chen *et al.* synthesized and characterized a series of inhibitors based on a pyridine scaffold. The best compound had a reported IC_{50} value of 57.4 ± 0.7 nM and was selective for cathepsin C *in vitro*[115] [Table 1]. Its administration also reduced NSP activation in rat bone marrow and showed anti-inflammatory activity in a rat model of COPD[115]. The compound was recently further optimized to improve its pharmacokinetic properties[116]. Further research will shed light on the efficacy and safety of these compounds.

In addition, a substantial number of cathepsin C inhibitors have been reported that have provided only *in vitro* data and no *in vivo* follow-up studies to date. Using a structure-based approach, Radzey *et al.* used E-64c hydrazide as a lead structure for the development of irreversible cathepsin C inhibitors[117]. The best resulting inhibitor, (2S,3S)-3-(2-butylhydrazine carbonyl)-N-((S)-1-(isopentylamino)-4-methyl-1-oxopentan-2-yl)oxirane-2-carboxamide [Table 1], exhibits significantly improved potency (k_{inact} = (5.6 ± 0.17) × 10^4 M^{-1} s^{-1}) compared to E-64c-hydrazide (k_{inact} = 140 ± 5 M^{-1} s^{-1}). It also reacts more rapidly with cathepsin C than with cathepsin L, which is the opposite of E-64c and its hydrazide, which have a strong preference for cathepsin L[117]. The authors therefore proposed this compound as a starting point for the development of optimized inhibitors that bind to the S1'-S2' sites of cathepsin C[117].

Azapeptides, i.e., peptide analogs in which one or more amino acids have been replaced by a semicarbazide group, have been reported as inhibitors of hepatitis C virus NS3 peptidase[118], human rhinovirus 3C peptidase[119], and several papain-like cysteine peptidases[120]. Azapeptides with weak leaving groups cannot acylate the enzyme and therefore show competitive inhibition, whereas azapeptides with reactive leaving groups form carbamoyl-enzyme complexes that are more stable than normal acyl enzymes. For cathepsin C, one of the best inhibitors from this class was 1-(2S-2-aminobutanoyl)-4-{2S-N-[2S-3-(m-fluoro-phenyl)propan-2-yl-amide]-4-phenylbutan-2-yl-amide}semi- carbazide [Table 1] with an IC_{50} value of 31 ± 3 nM and a K_i value of 45 ± 2 nM. The compound acted as a reversible, competitive inhibitor and was selective for cathepsin C. It was not cytotoxic to HepG2 cells and showed about 50% inhibition of intracellular cathepsin C activity in this cell line[121].

Drag *et al.* described a series of phosphonate dipeptide analogs as non-covalent inhibitors of cathepsin C [122]. The most potent compounds, diethyl 2-(L-phenylalanyl) amino-1-hydroxymethane phosphonate and monomethyl 2-(L-phenylalanyl) amino-1-hydroxyethane phosphonate [Table 1], inhibited cathepsin C with K_i values in the nanomolar range (23 ± 12 nM and 51 ± 16 nM, respectively). Unfortunately, these compounds exhibited low selectivity for cathepsin C, as they have also been shown to be potent inhibitors of other cysteine peptidases such as papain, cathepsin B, and cathepsin K[122]. Nevertheless, the phosphonate dipeptide analogs identified in this study could serve as lead compounds for the development of more specific inhibitors of cathepsin C and/or other cysteine cathepsins. Similarly, a group of tripeptide

aminophosphonates were recently identified as weak or moderate inhibitors of cathepsin C[123], further highlighting the potential of the phosphonate group as transition state analogs.

Moreover, two known epidermal growth factor receptor (EGFR) inhibitors containing Cys-reactive acrylamide warheads were identified as cathepsin C inhibitors by activity-based protein profiling, highlighting the potential of drug repurposing for the targeting of cathepsin C. In 2016, Canertinib, a former drug candidate developed by Pfizer (USA) for the treatment of cancer, was identified as a potent cathepsin C inhibitor with an IC_{50} value of 0.12 μM [Table 1]. Based on the structure of Canertinib, novel molecular probes with additional tags and/or click chemistry-compatible reactive groups were synthesized, providing the potential basis for further development of selective cathepsin C inhibitors[124]. Later, the same group reported that the EGFR inhibitor WZ4002 inhibits cathepsin C with an IC_{50} value in the micromolar range[125]. A new series of inhibitors developed on this scaffold led to the identification of a highly potent and selective inhibitor, N-(5-((6-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)- 3-(piperidin-1-yl) pyridin-2-yl)oxy)-2-methylphenyl)acrylamide (compound 22), [Table 1]. *In vitro*, the compound showed concentration-dependent inhibition of cathepsin C activity in both THP1 and U937 cell-based assays, with IC_{50} values of 2 and 3 nM, respectively. It also showed good metabolic stability and oral bioavailability and resulted in efficient inhibition of downstream neutrophil serine peptidases in both bone marrow and blood of mice[125].

Naturally occurring compounds are a rich source of pharmacologically active molecules and inhibition of cathepsin C by such compounds has also been investigated to some extent. A recent study has shown that polyglucoside from the vine *Triperygium wilfordii* inhibited cathepsin C activity in the serum, synovial fluid, and tissues in a rat model of collagen-induced arthritis, highlighting the possibility that the suppression of rheumatoid arthritis traditionally associated with *Tripergyium wilfordii* polyglucoside may be related to the inhibition of cathepsin C and downstream serine peptidases[126]. In addition, we found that caffeic acid and its derivatives inhibit cysteine cathepsins, including cathepsin C[31,127], *in vitro*. Since polyphenolic compounds such as caffeic acid have a broad spectrum of pharmacological activity and biological targets, their use for specific targeting of individual protein targets is not feasible. Nevertheless, caffeic acid is considered a promising pharmacophore for the development of specific inhibitors[128]. Alternative methods for targeting cathepsin C are also emerging. We have identified 3'-nitrophthalanilic acid as a partial inhibitor of cathepsins K and C that presumably acts by binding outside of the active site[31,129]. Such allosteric drugs are a popular and promising approach for the targeting of membrane receptors[130] and strategies of targeting sites away from the active site have also been explored in several PLPs, including human cathepsin K[131,132] and plasmodial cathepsin C homologs[133]. However, in the case of human cathepsin C, the development of specific partial inhibitors suitable for use in cell culture and *in vivo* and the evaluation of the efficacy of such an approach are still in their infancy.

CONCLUSION

Although cathepsin C was among the first peptidases to be identified, interest in this peptidase as a drug target has only emerged in the last two decades, primarily leading to facilitated investigation of its physiological and pathological roles. With two inhibitors currently in clinical trials for the treatment of non-cystic fibrosis bronchiectasis, cathepsin C is currently the most important and promising human cysteine cathepsin from a clinical perspective. Considering the recent failures in the field of peptidase targeting, e.g., the cathepsin K inhibitor odanacatib (Merck & Co., USA) for the treatment of osteoporosis which was discontinued after the completion of phase 3 clinical trials[134], the outcomes of these trials may be crucial to (re)establish not only cathepsin C, but cysteine cathepsins and peptidases in general as valid drug targets in human diseases. From the perspective of basic protein science, cathepsin C is proving to be an interesting

model protein for studying the molecular mechanisms and evolution of protein oligomerization[4]. Further research in this direction could, in turn, provide novel strategies for the targeting of this important peptidase.

DECLARATIONS

Authors' contributions

Wrote and revised the manuscript: Stojkovska Docevska M, Novinec M

Availability of data and materials

Not applicable.

Financial support and sponsorship

The authors acknowledge the financial support of the Slovenian Research Agency (project grant No. N1-0211 to MN and research core funding No. P1-0179).

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Tallan HH, Jones ME, Fruton JS. On the proteolytic enzymes of animal tissues. X. beef spleen cathepsin C. *J Biol Chem* 1952;194:793-805. PubMed
2. Rawlings ND, Barrett AJ, Thomas PD, Huang X, Bateman A, Finn RD. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. *Nucleic Acids Res* 2018;46:D624-32. DOI PubMed PMC
3. Novinec M, Lenarčič B. Papain-like peptidases: structure, function, and evolution. *Biomol Concepts* 2013;4:287-308. DOI PubMed
4. Varda N, Novinec M. Evolutionary analysis of dipeptidyl peptidase I. *Int J Mol Sci* 2022;23:1852. DOI PubMed PMC
5. Wu C, Jin X, Tsueng G, Afrasiabi C, Su AI. BioGPS: building your own mash-up of gene annotations and expression profiles. *Nucleic Acids Res* 2016;44:D313-6. DOI PubMed PMC
6. Korkmaz B, Caughey GH, Chapple I, et al. Therapeutic targeting of cathepsin C: from pathophysiology to treatment. *Pharmacol Ther* 2018;190:202-36. DOI
7. Chalmers JD, Usansky H, Rubino CM, et al. Pharmacokinetic/pharmacodynamic evaluation of the dipeptidyl peptidase 1 inhibitor brensocatib for non-cystic fibrosis bronchiectasis. *Clin Pharmacokinet* 2022;61:1457-69. DOI PubMed PMC
8. Nakano A, Nomura K, Nakano H, et al. Papillon-Lefèvre syndrome: mutations and polymorphisms in the cathepsin C gene. *J Invest Dermatol* 2001;116:339-43. DOI PubMed
9. Hart TC, Hart PS, Bowden DW, et al. Mutations of the cathepsin C gene are responsible for Papillon-Lefèvre syndrome. *J Med Genet* 1999;36:881-7. DOI
10. Hart TC, Hart PS, Michalec MD, et al. Haim-munk syndrome and papillon-Lefèvre syndrome are allelic mutations in cathepsin C. *J Med Genet* 2000;37:88-94. DOI PubMed PMC
11. Sreeramulu B, Shyam ND, Ajay P, Suman P. Papillon-Lefèvre syndrome: clinical presentation and management options. *Clin Cosmet Investig Dent* 2015;7:75-81. DOI PubMed PMC
12. Turk V, Stoka V, Vasiljeva O, et al. Cysteine cathepsins: from structure, function and regulation to new frontiers. *Biochim Biophys Acta* 2012;1824:68-88. DOI PubMed PMC
13. Turk D, Janjic V, Stern I, et al. Structure of human dipeptidyl peptidase I (cathepsin C): exclusion domain added to an endopeptidase framework creates the machine for activation of granular serine proteases. *EMBO J* 2001;20:6570-82. DOI PubMed PMC

14. Cigic B, Pain RH. Location of the binding site for chloride ion activation of cathepsin C. *Eur J Biochem* 1999;264:944-51. DOI PubMed
15. Lindley H. The specificity of dipeptidyl aminopeptidase I (cathepsin C) and its use in peptide sequence studies. *Biochem J* 1972;126:683-5. DOI PubMed PMC
16. Wang F, Krai P, Deu E, et al. Biochemical characterization of plasmodium falciparum dipeptidyl aminopeptidase 1. *Mol Biochem Parasitol* 2011;175:10-20. DOI PubMed PMC
17. de Vries LE, Sanchez MI, Groborz K, et al. Characterization of P. falciparum dipeptidyl aminopeptidase 3 specificity identifies differences in amino acid preferences between peptide-based substrates and covalent inhibitors. *FEBS J* 2019;286:3998-4023. DOI PubMed PMC
18. Poreba M, Mihelic M, Krai P, et al. Unnatural amino acids increase activity and specificity of synthetic substrates for human and malarial cathepsin C. *Amino Acids* 2014;46:931-43. DOI PubMed PMC
19. Łęgowska M, Hamon Y, Wojtysiak A, et al. Development of the first internally-quenched fluorescent substrates of human cathepsin C: the application in the enzyme detection in biological samples. *Arch Biochem Biophys* 2016;612:91-102. DOI
20. Metrione RM, MacGeorge NL. The mechanism of action of dipeptidyl aminopeptidase. Inhibition by amino acid derivatives and amines; activation by aromatic compounds. *Biochemistry* 1975;14:5249-52. DOI PubMed
21. Mcdonald JK, Callahan PX, Zeitman BB, Ellis S. Inactivation and degradation of glucagon by dipeptidyl aminopeptidase I (Cathepsin C) of rat liver. *J Biol Chem* 1969;244:6199-208. DOI PubMed
22. Dolenc I, Turk B, Pungercic G, Ritonja A, Turk V. Oligomeric structure and substrate induced inhibition of human cathepsin C. *J Biol Chem* 1995;270:21626-31. DOI PubMed
23. Gorter J, Gruber M. Cathepsin C: an allosteric enzyme. *Biochim Biophys Acta* 1970;198:546-55. DOI PubMed
24. Nilsson KK, Fruton JS. Polymerization reactions catalyzed by intracellular proteinases. iv. factors influencing the polymerization of dipeptide amides by cathepsin C. *Biochemistry* 1964;3:1220-4. DOI PubMed
25. Gittel C, Schmidtchen FP. Directed N-terminal elongation of unprotected peptides catalyzed by cathepsin c in water. *Bioconjug Chem* 1995;6:70-6. DOI PubMed
26. Tavano OL. Protein hydrolysis using proteases: an important tool for food biotechnology. *J Mol Catal B-Enzym* 2013;90:1-11. DOI
27. Cigić B, Dahl SW, Pain RH. The residual pro-part of cathepsin C fulfills the criteria required for an intramolecular chaperone in folding and stabilizing the human proenzyme. *Biochemistry* 2000;39:12382-90. DOI PubMed
28. Dahl SW, Halkier T, Lauritzen C, et al. Human recombinant pro-dipeptidyl peptidase I (cathepsin C) can be activated by cathepsins L and S but not by autocatalytic processing. *Biochemistry* 2001;40:1671-8. DOI
29. Lamort AS, Hamon Y, Czaplewski C, et al. Processing and maturation of cathepsin C zymogen: a biochemical and molecular modeling analysis. *Int J Mol Sci* 2019;20:4747. DOI PubMed PMC
30. Santilman V, Jadot M, Mainferme F. Importance of the propeptide in the biosynthetic maturation of rat cathepsin C. *Eur J Cell Biol* 2002;81:654-63. DOI PubMed
31. Rebernik M, Snoj T, Klemenčič M, Novinec M. Interplay between tetrameric structure, enzymatic activity and allosteric regulation of human dipeptidyl-peptidase I. *Arch Biochem Biophys* 2019;675:108121. DOI PubMed
32. Lauritzen C, Pedersen J, Madsen MT, Justesen J, Martensen PM, Dahl SW. Active recombinant rat dipeptidyl aminopeptidase I (cathepsin C) produced using the baculovirus expression system. *Protein Expr Purif* 1998;14:434-42. DOI
33. Burge V, Mainferme F, Wattiaux R. Transient membrane association of the precursors of cathepsin C during their transfer into lysosomes. *Biochem J* 1991;275:797-800. DOI PubMed PMC
34. Muno D, Ishidoh K, Ueno T, Kominami E. Processing and transport of the precursor of cathepsin C during its transfer into lysosomes. *Arch Biochem Biophys* 1993;306:103-10. DOI PubMed
35. Clair J, Shi GP, Sutherland RE, Chapman HA, Caughey GH, Wolters PJ. Cathepsins L and S are not required for activation of dipeptidyl peptidase I (cathepsin C) in mice. *Biol Chem* 2006;387:1143-6. DOI PubMed PMC
36. Hamon Y, Legowska M, Hervé V, et al. Neutrophilic cathepsin C is maturated by a multistep proteolytic process and secreted by activated cells during inflammatory lung diseases. *J Biol Chem* 2016;291:8486-99. DOI PubMed PMC
37. Olsen JG, Kadziola A, Lauritzen C, Pedersen J, Larsen S, Dahl SW. Tetrameric dipeptidyl peptidase I directs substrate specificity by use of the residual pro-part domain. *FEBS Lett* 2001;506:201-6. DOI PubMed
38. Cigić B, Pain RH. Competitive inhibition of cathepsin C by guanidinium ions and reexamination of substrate inhibition. *Biochem Biophys Res Commun* 1999;258:6-10. DOI PubMed
39. Rebernik M, Lenarčič B, Novinec M. The catalytic domain of cathepsin C (dipeptidyl-peptidase I) alone is a fully functional endoprotease. *Protein Expr Purif* 2019;157:21-7. DOI PubMed
40. Wolters PJ, Laig-Webster M, Caughey GH. Dipeptidyl peptidase I cleaves matrix-associated proteins and is expressed mainly by mast cells in normal dog airways. *Am J Respir Cell Mol Biol* 2000;22:183-90. DOI PubMed
41. Kuribayashi M, Yamada H, Ohmori T, Yanai M, Imoto T. Endopeptidase activity of cathepsin C, dipeptidyl aminopeptidase I, from bovine spleen. *J Biochem* 1993;113:441-9. DOI PubMed
42. Pham CT. Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol* 2006;6:541-50. DOI
43. Perera NC, Schilling O, Kittel H, Back W, Kremmer E, Jenne DE. NSP4, an elastase-related protease in human neutrophils with arginine specificity. *Proc Natl Acad Sci U S A* 2012;109:6229-34. DOI PubMed PMC
44. Salvesen G, Enghild JJ. An unusual specificity in the activation of neutrophil serine proteinase zymogens. *Biochemistry*

1990;29:5304-8. DOI PubMed

45. Wolters PJ, Pham CT, Muilenburg DJ, Ley TJ, Caughey GH. Dipeptidyl peptidase I is essential for activation of mast cell chymases, but not tryptases, in mice. *J Biol Chem* 2001;276:18551-6. DOI
46. Pham CT, Ley TJ. Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B *in vivo*. *Proc Natl Acad Sci U S A* 1999;96:8627-32. DOI PubMed PMC
47. Freer ST, Kraut J, Robertus JD, Wright HT, Xuong NH. Chymotrypsinogen: 2.5-angstrom crystal structure, comparison with alpha-chymotrypsin, and implications for zymogen activation. *Biochemistry* 1970;9:1997-2009. DOI PubMed
48. Reiling KK, Krucinski J, Miercke LJ, Raymond WW, Caughey GH, Stroud RM. Structure of human pro-chymase: a model for the activating transition of granule-associated proteases. *Biochemistry* 2003;42:2616-24. DOI PubMed
49. Gullberg U, Andersson E, Garwicz D, Lindmark A, Olsson I. Biosynthesis, processing and sorting of neutrophil proteins: insight into neutrophil granule development. *Eur J Haematol* 1997;58:137-53. DOI PubMed
50. Garwicz D, Lindmark A, Persson A, Gullberg U. On the role of the proform-conformation for processing and intracellular sorting of human cathepsin G. *Blood* 1998;92:1415-22. DOI PubMed
51. Garwicz D, Lennartsson A, Jacobsen SE, Gullberg U, Lindmark A. Biosynthetic profiles of neutrophil serine proteases in a human bone marrow-derived cellular myeloid differentiation model. *Haematologica* 2005;90:38-44. PubMed
52. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science* 2004;303:1532-5. DOI
53. Korkmaz B, Horwitz MS, Jenne DE, Gauthier F. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. *Pharmacol Rev* 2010;62:726-59. DOI PubMed PMC
54. Ruffell B, Affara NI, Cottone L, et al. Cathepsin C is a tissue-specific regulator of squamous carcinogenesis. *Genes Dev* 2013;27:2086-98. DOI PubMed PMC
55. Wolters PJ, Raymond WW, Blount JL, Caughey GH. Regulated expression, processing, and secretion of dog mast cell dipeptidyl peptidase I. *J Biol Chem* 1998;273:15514-20. DOI PubMed
56. Dikov MM, Springman EB, Yeola S, Serafin WE. Processing of procarboxypeptidase A and other zymogens in murine mast cells. *J Biol Chem* 1994;269:25897-904. DOI PubMed
57. Gullberg U, Lindmark A, Nilsson E, Persson A, Olsson I. Processing of human cathepsin G after transfection to the rat basophilic/mast cell tumor line RBL. *J Biol Chem* 1994;269:25219-25. DOI
58. McEuen AR, Ashworth DM, Walls AF. The conversion of recombinant human mast cell prochymase to enzymatically active chymase by dipeptidyl peptidase I is inhibited by heparin and histamine. *Eur J Biochem* 1998;253:300-8. DOI PubMed
59. Le QT, Min HK, Xia HZ, Fukuoka Y, Katunuma N, Schwartz LB. Promiscuous processing of human alphabeta-protryptases by cathepsins L, B, and C. *J Immunol* 2011;186:7136-43. DOI PubMed PMC
60. Le QT, Gomez G, Zhao W, et al. Processing of human protryptase in mast cells involves cathepsins L, B, and C. *J Immunol* 2011;187:1912-8. DOI PubMed PMC
61. Clair J, Pham CT, Villalta SA, Caughey GH, Wolters PJ. Mast cell dipeptidyl peptidase I mediates survival from sepsis. *J Clin Invest* 2004;113:628-34. DOI PubMed PMC
62. Jenne D, Rey C, Haefliger JA, Qiao BY, Groscurth P, Tschopp J. Identification and sequencing of cDNA clones encoding the granule-associated serine proteases granzymes D, E, and F of cytolytic T lymphocytes. *Proc Natl Acad Sci U S A* 1988;85:4814-8. DOI PubMed PMC
63. Caputo A, Garner R, Winkler U, Hudig D, Bleackley R. Activation of recombinant murine cytotoxic cell proteinase-1 requires deletion of an amino-terminal dipeptide. *J Biol Chem* 1993;268:17672-5. DOI PubMed
64. Brown GR, McGuire MJ, Thiele DL. Dipeptidyl peptidase I is enriched in granules of *in vitro*- and *in vivo*-activated cytotoxic T lymphocytes. *J Immunol* 1993;150:4733-42. DOI PubMed
65. Mcguire M, Lipsky P, Thiele D. Generation of active myeloid and lymphoid granule serine proteases requires processing by the granule thiol protease dipeptidyl peptidase I. *J Biol Chem* 1993;268:2458-67. DOI PubMed
66. Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP, Ley TJ. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* 2004;104:2840-8. DOI
67. Pham CT, Thomas DA, Mercer JD, Ley TJ. Production of fully active recombinant murine granzyme B in yeast. *J Biol Chem* 1998;273:1629-33. DOI
68. Alam S, Liu Q, Liu S, et al. Up-regulated cathepsin C induces macrophage M1 polarization through FAK-triggered p38 MAPK/NF-κB pathway. *Exp Cell Res* 2019;382:111472. DOI
69. Dai J, Liu J, Zhang Q, et al. Cathepsin C is involved in macrophage M1 polarization via p38/MAPK pathway in sudden cardiac death. *Cardiovasc Ther* 2021;2021:6139732. DOI PubMed PMC
70. Liu Q, Zhang Y, Liu S, et al. Cathepsin C promotes microglia M1 polarization and aggravates neuroinflammation via activation of Ca^{2+}-dependent PKC/p38MAPK/NF-κB pathway. *J Neuroinflamm* 2019;16:1-18. DOI PubMed
71. Adkison AM, Kelley DG, Pham CTN, et al. Dipeptidyl peptidase I activates neutrophil-derived serine proteases and regulates the development of acute experimental arthritis find the latest version: dipeptidyl peptidase I activates neutrophil-derived serine proteases and regulates the development. *J Clin Invest* 2002;109:363-71. DOI
72. Chu Y, Guo Y, Walls AF, Zhou X. The regulatory role of dipeptidyl peptidase I on the activation of immune granulocytes. *Cell Biol Int* 2017;41:1093-102. DOI
73. Pagano MB, Bartoli MA, Ennis TL, et al. Critical role of dipeptidyl peptidase I in neutrophil recruitment during the development of

experimental abdominal aortic aneurysms. *Proc Natl Acad Sci U S A* 2007;104:2855-60. DOI PubMed PMC

74. John DS, Aschenbach J, Krüger B, et al. Deficiency of cathepsin C ameliorates severity of acute pancreatitis by reduction of neutrophil elastase activation and cleavage of E-cadherin. *J Biol Chem* 2019;294:697-707. DOI PubMed PMC
75. Fan K, Wu X, Fan B, et al. Up-regulation of microglial cathepsin C expression and activity in lipopolysaccharide-induced neuroinflammation. *J Neuroinflamm* 2012;9:1-13. DOI PubMed
76. Keir HR, Shoemark A, Dicker AJ, et al. Neutrophil extracellular traps, disease severity, and antibiotic response in bronchiectasis: an international, observational, multicohort study. *Lancet Respir Med* 2021;9:873-84. DOI
77. Kim S, Nadel JA. Role of neutrophils in mucus hypersecretion in COPD and implications for therapy. *Treat Respir Med* 2004;3:147-59. DOI PubMed
78. Guarino C, Hamon Y, Croix C, et al. Prolonged pharmacological inhibition of cathepsin C results in elimination of neutrophil serine proteases. *Biochem Pharmacol* 2017;131:52-67. DOI
79. Sanchez Klose FP, Björnsdottir H, Dahlstrand Rudin A, et al. A rare CTSC mutation in Papillon-Lefèvre syndrome results in abolished serine protease activity and reduced NET formation but otherwise normal neutrophil function. *PLoS One* 2021;16:e0261724. DOI PubMed PMC
80. Bullón P, Castejón-Vega B, Román-Malo L, et al. Autophagic dysfunction in patients with Papillon-Lefèvre syndrome is restored by recombinant cathepsin C treatment. *J Allergy Clin Immunol* 2018;142:1131-1143.e7. DOI
81. Suban D, Zajc T, Renko M, Turk B, Turk V, Dolenc I. Cathepsin C and plasma glutamate carboxypeptidase secreted from Fischer rat thyroid cells liberate thyroxin from the N-terminus of thyroglobulin. *Biochimie* 2012;94:719-26. DOI PubMed
82. Bernardini F, Warburton MJ. Lysosomal degradation of cholecystokinin-(29-33)-amide in mouse brain is dependent on tripeptidyl peptidase-I: implications for the degradation and storage of peptides in classical late-infantile neuronal ceroid lipofuscinosis. *Biochem J* 2002;366:521-9. DOI PubMed PMC
83. Sleat DE, Donnelly RJ, Lackland H, et al. Association of mutations in a lysosomal protein with classical late-infantile neuronal ceroid lipofuscinosis. *Science* 1997;277:1802-5. DOI
84. Kim KH, Pham CT, Sleat DE, Lobel P. Dipeptidyl-peptidase I does not functionally compensate for the loss of tripeptidyl-peptidase I in the neurodegenerative disease late-infantile neuronal ceroid lipofuscinosis. *Biochem J* 2008;415:225-32. DOI PubMed PMC
85. Zhang GP, Yue X, Li SQ. Cathepsin C interacts with TNF-α/p38 MAPK signaling pathway to promote proliferation and metastasis in hepatocellular carcinoma. *Cancer Res Treat* 2020;52:10-23. DOI PubMed PMC
86. Kim S, Lee S-I, Kim N, et al. Decursin inhibits cell growth and autophagic flux in gastric cancer via suppression of cathepsin C. *Am J Cancer Res* 2021;11:1304. PubMed PMC
87. Khaket TP, Singh MP, Khan I, Bhardwaj M, Kang SC. Targeting of cathepsin C induces autophagic dysregulation that directs ER stress mediated cellular cytotoxicity in colorectal cancer cells. *Cell Signal* 2018;46:92-102. DOI PubMed
88. Xiao Y, Cong M, Li J, et al. Cathepsin C promotes breast cancer lung metastasis by modulating neutrophil infiltration and neutrophil extracellular trap formation. *Cancer Cell* 2021;39:423-437.e7. DOI
89. Korkmaz B, Lamort AS, Domain R, et al. Cathepsin C inhibition as a potential treatment strategy in cancer. *Biochem Pharmacol* 2021;194:114803. DOI
90. Kavčič N, Butinar M, Sobotič B, et al. Intracellular cathepsin C levels determine sensitivity of cells to leucyl-leucine methyl ester-triggered apoptosis. *FEBS J* 2020;287:5148-66. DOI PubMed
91. Nicklin MJ, Barrett AJ. Inhibition of cysteine proteinases and dipeptidyl peptidase I by egg-white cystatin. *Biochem J* 1984;223:245-53. DOI PubMed PMC
92. Tušar L, Usenik A, Turk B, Turk D. Mechanisms applied by protein inhibitors to inhibit cysteine proteases. *Int J Mol Sci* 2021;22:997. DOI PubMed PMC
93. Hamilton G, Colbert JD, Schuettelkopf AW, Watts C. Cystatin F is a cathepsin C-directed protease inhibitor regulated by proteolysis. *EMBO J* 2008;27:499-508. DOI PubMed PMC
94. Halfon S, Ford J, Foster J, et al. Leukocystatin, a new Class II cystatin expressed selectively by hematopoietic cells. *J Biol Chem* 1998;273:16400-8. DOI
95. Božič J, Dolenc I. Feedback regulation of cathepsin C by the propeptide dipeptides of granzymes A and B. *ACSi* 2019;66:501-9. DOI
96. Méthot N, Rubin J, Guay D, et al. Inhibition of the activation of multiple serine proteases with a cathepsin C inhibitor requires sustained exposure to prevent pro-enzyme processing. *J Biol Chem* 2007;282:20836-46. DOI
97. Mølgaard A, Arnau J, Lauritzen C, Larsen S, Petersen G, Pedersen J. The crystal structure of human dipeptidyl peptidase I (cathepsin C) in complex with the inhibitor Gly-Phe-CHN2. *Biochem J* 2007;401:645-50. DOI PubMed PMC
98. Green GD, Shaw E. Peptidyl diazomethyl ketones are specific inactivators of thiol proteinases. *J Biol Chem* 1981;256:1923-8. DOI PubMed
99. Laine DI, Busch-Petersen J. Inhibitors of cathepsin C (dipeptidyl peptidase I). *Expert Opin Ther Pat* 2010;20:497-506. DOI PubMed
100. Furber M, Tiden AK, Gardiner P, et al. Cathepsin C inhibitors: property optimization and identification of a clinical candidate. *J Med Chem* 2014;57:2357-67. DOI
101. Gardiner P, Wikell C, Clifton S, Shearer J, Benjamin A, Peters SA. Neutrophil maturation rate determines the effects of dipeptidyl peptidase 1 inhibition on neutrophil serine protease activity. *Br J Pharmacol* 2016;173:2390-401. DOI PubMed PMC
102. Bragg RA, Brocklehurst S, Gustafsson F, et al. Aortic binding of AZD5248: mechanistic insight and reactivity assays to support lead optimzation. *Chem Res Toxicol* 2015;28:1991-9. DOI

103. Doyle K, Lönn H, Käck H, et al. Discovery of second generation reversible covalent DPP1 inhibitors leading to an oxazepane amidoacetonitrile based clinical candidate (AZD7986). *J Med Chem* 2016;59:9457-72. DOI
104. Palmér R, Mäenpää J, Jauhiainen A, et al. Dipeptidyl peptidase 1 inhibitor AZD7986 induces a sustained, exposure-dependent reduction in neutrophil elastase activity in healthy subjects. *Clin Pharmacol Ther* 2018;104:1155-64. DOI PubMed PMC
105. Chalmers JD, Haworth CS, Metersky ML, et al; WILLOW Investigators. Phase 2 trial of the DPP-1 inhibitor brensocatib in bronchiectasis. *N Engl J Med* 2020;383:2127-37. DOI
106. Sinha S, Rosin NL, Arora R, et al. Dexamethasone modulates immature neutrophils and interferon programming in severe COVID-19. *Nat Med* 2022;28:201-11. DOI PubMed PMC
107. Seren S, Derian L, Keleş I, et al. Proteinase release from activated neutrophils in mechanically ventilated patients with non-COVID-19 and COVID-19 pneumonia. *Eur Respir J* 2021;57:2003755. DOI PubMed PMC
108. Keir HR, Long MB, Abo-Leyah H, et al; STOP-COVID19 Investigators. Dipeptidyl peptidase-1 inhibition in patients hospitalised with COVID-19: a multicentre, double-blind, randomised, parallel-group, placebo-controlled trial. *Lancet Respir Med* 2022;10:1119-28. DOI PubMed PMC
109. Korkmaz B, Lesner A, Wysocka M, et al. Structure-based design and *in vivo* anti-arthritic activity evaluation of a potent dipeptidyl cyclopropyl nitrile inhibitor of cathepsin C. *Biochem Pharmacol* 2019;164:349-67. DOI
110. Rehm SRT, Smirnova NF, Morrone C, et al. Premedication with a cathepsin C inhibitor alleviates early primary graft dysfunction in mouse recipients after lung transplantation. *Sci Rep* 2019;9:9925. DOI PubMed PMC
111. Miller BE, Mayer RJ, Goyal N, et al. Epithelial desquamation observed in a phase I study of an oral cathepsin C inhibitor (GSK2793660). *Br J Clin Pharmacol* 2017;83:2813-20. DOI PubMed PMC
112. Badorrek P, Diefenbach C, Kögler H, et al. Phase I characterization of the novel cathepsin C inhibitor BI 1291583. *Am J Resp Crit Care* 2022;205:A4777. DOI
113. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera-a visualization system for exploratory research and analysis. *J Comput Chem* 2004;25:1605-12. DOI PubMed
114. Shen XB, Chen X, Zhang ZY, Wu FF, Liu XH. Cathepsin C inhibitors as anti-inflammatory drug discovery: challenges and opportunities. *Eur J Med Chem* 2021;225:113818. DOI
115. Chen X, Yan Y, Zhang Z, et al. Discovery and *in vivo* anti-inflammatory activity evaluation of a novel non-peptidyl non-covalent cathepsin C inhibitor. *J Med Chem* 2021;64:11857-85. DOI
116. Chen X, Yan Y, Du J, et al. Non-peptidyl non-covalent cathepsin C inhibitoEEr bearing a unique thiophene-substituted pyridine: design, structure-activity relationship and anti-inflammatory activity *in vivo*. *Eur J Med Chem* 2022;236:114368. DOI PubMed
117. Radzey H, Rethmeier M, Klimpel D, Grundhuber M, Sommerhoff CP, Schaschke N. E-64c-hydrazide: a lead structure for the development of irreversible cathepsin C inhibitors. *ChemMedChem* 2013;8:1314-21. DOI PubMed
118. Zhang R, Durkin JP, Windsor WT. Azapeptides as inhibitors of the hepatitis C virus NS3 serine protease. *Bioorg Med Chem Lett* 2002;12:1005-8. DOI PubMed
119. Venkatraman S, Kong J, Nimkar S, Wang QM, Aubé J, Hanzlik RP. Design, synthesis, and evaluation of azapeptides as substrates and inhibitors for human rhinovirus 3C protease. *Bioorg Med Chem Lett* 1999;9:577-80. DOI PubMed
120. Verhelst SH, Witte MD, Arastu-Kapur S, Fonovic M, Bogyo M. Novel aza peptide inhibitors and active-site probes of papain-family cysteine proteases. *Chembiochem* 2006;7:943-50. DOI PubMed
121. Bondebjerg J, Fuglsang H, Valeur KR, et al. Novel semicarbazide-derived inhibitors of human dipeptidyl peptidase I (hDPPI). *Bioorg Med Chem* 2005;13:4408-24. DOI
122. Drąg M, Wieczerzak E, Pawełczak M, Berlicki Ł, Grzonka Z, Kafarski P. Toward very potent, non-covalent organophosphonate inhibitors of cathepsin C and related enzymes by 2-amino-1-hydroxy-alkanephosphonates dipeptides. *Biochimie* 2013;95:1640-9. DOI PubMed
123. Jewgiński MP, Makowski M, Pawełczak M, et al. Synthesis of hybrid tripeptide peptidomimetics containing dehydroamino acid and aminophosphonic acid in the chain and evaluation of their activity toward cathepsin C. *Chem Biodivers* 2022;19:e202101019. DOI
124. Sun H, Ren Y, Hou W, et al. Focusing on probe-modified peptides: a quick and effective method for target identification. *Chem Commun* 2016;52:10225-8. DOI
125. Hou W, Sun H, Ma Y, Liu C, Zhang Z. Identification and optimization of novel cathepsin C inhibitors derived from EGFR inhibitors. *J Med Chem* 2019;62:5901-19. DOI PubMed
126. Wang J, Chu Y, Zhou X. Inhibitory effect of triperygium wilfordii polyglucoside on dipeptidyl peptidase I *in vivo* and *in vitro*. *Biomed Pharmacother* 2017;96:466-70. DOI PubMed
127. Ulčakar L, Novinec M. Inhibition of human cathepsins B and L by caffeic acid and its derivatives. *Biomolecules* 2020;11:31. DOI PubMed PMC
128. Touaibia M, Jean-François J, Doiron J. Caffeic acid, a versatile pharmacophore: an overview. *Mini Rev Med Chem* 2011;11:695-713. DOI PubMed
129. Novinec M, Lenarčič B, Baici A. Probing the activity modification space of the cysteine peptidase cathepsin K with novel allosteric modifiers. *PLoS One* 2014;9:e106642. DOI PubMed PMC
130. Changeux JP, Christopoulos A. Allosteric modulation as a unifying mechanism for receptor function and regulation. *Cell* 2016;166:1084-102. DOI PubMed

131. Novinec M, Korenč M, Caflisch A, Ranganathan R, Lenarčič B, Baici A. A novel allosteric mechanism in the cysteine peptidase cathepsin K discovered by computational methods. *Nat Commun* 2014;5:3287. DOI PubMed
132. Panwar P, Søe K, Guido RV, Bueno RV, Delaisse JM, Brömme D. A novel approach to inhibit bone resorption: exosite inhibitors against cathepsin K. *Br J Pharmacol* 2016;173:396-410. DOI PubMed PMC
133. Sanchez MI, de Vries LE, Lehmann C, et al. Identification of plasmodium dipeptidyl aminopeptidase allosteric inhibitors by high throughput screening. *PLoS One* 2019;14:e0226270. DOI PubMed PMC
134. McClung MR, O'Donoghue ML, Papapoulos SE, et al; LOFT Investigators. Odanacatib for the treatment of postmenopausal osteoporosis: results of the LOFT multicentre, randomised, double-blind, placebo-controlled trial and LOFT extension study. *Lancet Diabetes Endo* 2019;7:899-911. DOI PubMed

Chu *et al. Rare Dis Orphan Drugs J* 2023;2:15
DOI: 10.20517/rdodj.2023.28

Rare Disease and Orphan Drugs Journal

Correction

Open Access

Correction: A report and review of the recurrent c.811C > T variant and mutation spectrum of Kindler syndrome in East Asians: a diagnostic odyssey of 2 weeks versus 49 years

Annie Tsz Wai Chu[1,#], Joshua Chun Ki Chan[2,#], Jasmine Lee Fong Fung[1], Wenshu Tang[1], Mianne Lee[3], Sze Man Wong[4], Man Ho Chung[4], Geoffrey Yu[2], Vivien Li[2], Calvin Tik Hei Ng[3], Hong Kong Genome Project, Brian Hon Yin Chung[1,3]

[1]Hong Kong Genome Institute, Hong Kong Special Administrative Region, Hong Kong, China.
[2]Department of Paediatrics and Adolescent Medicine, Queen Mary Hospital, Hong Kong Special Administrative Region, Hong Kong, China.
[3]Department of Paediatrics and Adolescent Medicine, School of Clinical Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region, Hong Kong, China.
[4]Division of Dermatology, Department of Medicine, Clinical School of Medicine, The University of Hong Kong, Hong Kong, China.
[#]Authors contributed equally and considered joint first authors.

Correspondence to: Dr. Brian Hon Yin Chung, Hong Kong Genome Institute, 2/F, Building 20E, Hong Kong Science Park, Hong Kong, China. E-mail: bhychung@genomics.org.hk

How to cite this article: Chu ATW, Chan JCK, Fung JLF, Tang W, Lee M, Wong SM, Chung MH, Yu G, Li V, Ng CTH, Project HKG, Chung BHY. Correction: A report and review of the recurrent c.811C > T variant and mutation spectrum of Kindler syndrome in East Asians: a diagnostic odyssey of 2 weeks versus 49 years. *Rare Dis Orphan Drugs J* 2023;2:15. https://dx.doi.org/10.20517/rdodj.2023.28

Received: 14 Aug 2023 **Accepted:** 18 Aug 2023 **Published:** 21 Aug 2023

Academic Editor: Daniel Scherman **Copy Editor:** Dan Zhang **Production Editor:** Dan Zhang

The Case Report was published on 3 Mar 2023.

The authors wish to add Dr. Sze Man Wong as a co-author of the paper and add the Authors' contributions in the Declaration part of the paper. The two cases reported in the paper were under Dr Wong's care. The dermatological symptoms and signs are under vigorous dermatological verification by her, together with initiating a referral for a genetic test, as in these two reported cases. She provided details of the dermatological description, reflecting the expert input after that by dermatologists.

The authors apologize for any inconvenience caused and state that the scientific conclusions are unaffected.

DECLARATIONS

Authors' contributions

Conception and design: Chung BHY, Chu ATW
Drafting the article: Chu ATW, Chan JCK, Fung JLF
Data analysis and interpretation: Fung JLF, Tang W, Lee M
Critical revision: Chung BHY, Chu ATW
Final approval of the version to be published: Chung BHY, Chu ATW, Chan JCK, Fung JLF, Tang W, Lee M, Wong SM, Chung MH, Yu G, Li V, Ng CTH
Patient recruitment and data collection: Hong Kong Genome Project

Magnifico *et al. Rare Dis Orphan Drugs J* 2023;2:16
DOI: 10.20517/rdodj.2023.17

Rare Disease and Orphan Drugs Journal

Systematic Review

Open Access

A systematic review of real-world applications of genome sequencing for newborn screening

Giuditta Magnifico, Irene Artuso, Stefano Benvenuti

Fondazione Telethon ETS, Milan IT 20129, Italy.

Correspondence to: Dr. Stefano Benvenuti, Fondazione Telethon ETS, via Carlo Poerio 14, Milan IT20129, Italy. E-mail: sbenvenuti@telethon.it

How to cite this article: Magnifico G, Artuso I, Benvenuti S. A systematic review of real-world applications of genome sequencing for newborn screening. *Rare Dis Orphan Drugs J* 2023;2:16. https://dx.doi.org/10.20517/rdodj.2023.17

Received: 21 Jun 2023 **Revised:** 9 Aug 2023 **Accepted:** 22 Aug 2023 **Published:** 29 Aug 2023

Academic Editors: Daniel Scherman, Virginie Bros-Facer **Copy Editor:** Yanbing Bai **Production Editor:** Yanbing Bai

Abstract

Aim: With the costs of genomic sequencing falling quickly and an ever-increasing number of clinical laboratories equipped with new-generation sequencing machines, healthcare systems around the world are getting ready to enter the era of genomic newborn screening (NBS). However, the adoption of Genomic Sequencing (GS), encompassing whole-exome sequencing (WES) and whole-genome sequencing (WGS), in NBS programs raises a number of clinical, ethical, and legal questions as well as organizational and economic challenges. This systematic review is part of a feasibility study to assess the introduction of WGS for NBS in Lombardy region with the specific aim of gathering evidence from existing pilots in the field whose results have been published.

Methods: Three different sources were identified for the selection of articles in order to obtain a various and unbiased set of publications. 33 articles were retained for analysis to answer the following questions:

1. Clinical: Does genomic sequencing demonstrate clinical utility in the context of NBS? What are the limitations of these kind of programs?

2. Societal: What are the social, ethical and psychological implications of using GS for NBS?

3. Governance: What are the legal, economic, and organizational challenges for GS-based NBS programs?

Results: There is a general consensus in the literature on the key principles that should guide the adoption of GS in NBS, such as the inclusion of actionable genes only, the need for informed consent from the parents, the right of the newborn to an open future, which means the exclusion of late-onset diseases even when those are considered treatable. However, there are still several differences in how these principles are detailed and applied.

Conclusion: Real-world evidence from a handful of pilot projects (namely BabySeq and NC-Nexus, both carried out

in the USA) have been published recently; however, this evidence is not yet sufficient to put an end to the broad and animated debate on the use of GS for NBS. Ethical, legal, and social issues still constitute great challenges and major barriers to wide and uniform adoption of GS in NBS. On the clinical side, a number of issues remain unaddressed, such as the benefits and limitations of the different approaches (targeted sequencing, GS only versus GS+standard NBS), the genes/diseases to include and the frequency of incidental findings, identification of carrier status, and variants of uncertain significance (VUS). Further pilots and consultations with involved stakeholders will be necessary before GS-based NBS can be accepted and systematically implemented in national healthcare programs.

Keywords: Newborn screening, genome sequencing, whole exome sequencing, whole genome sequencing

INTRODUCTION

Newborn screening (NBS) programs have been running successfully for more than 50 years since its introduction in the 1960s. In many countries, the first disorder included in screening programs was Phenylketonuria (PKU). With the advent of Tandem Mass Spectrometry (MS/MS), the number of conditions screened increased to around 50, although with great disparities among countries[1]. The introduction of MS/MS was therefore a key driver for the expansion of the number of conditions screened, with an increase in the order of 10 folds. Now, with the costs of genomic sequencing falling quickly and an ever-increasing capacity of laboratories as more and more are getting equipped with new generation sequencing instruments, a further scale-up of NBS programs is technically possible, also in the order of 10 folds (from 50 to 500 conditions)[2-6]. However, it is important to consider that one disease can be linked to one or more genes, and for each gene, there could be several variants, pathogenic or not. A major limitation of the GS approach is that several variants cannot be classified either as non-pathogenic or pathogenic and are actually classified as variants of uncertain/unknown significance (VUS). The specific criteria for selecting the genes and the conditions to be screened are not yet unanimously accepted, even if there is a general agreement that only pathogenic or likely-pathogenic variants should be reported and the principles set by Wilson and Jungner are still basically valid[7]. Moreover, the adoption of Genomic Sequencing (GS), meaning whole-exome sequencing (WES) or whole-genome sequencing (WGS), poses a number of clinical, ethical, and legal questions[8-12] together with organizational and economic challenges[3,13-14].

This systematic review is part of a feasibility study assessing the introduction of GS for NBS in Lombardy region (Italy) and is co-funded by the regional government (Regione Lombardia) and Fondazione Telethon. The study is conducted according to the Responsible Research and Innovation (RRI) principles[15-18] and is inspired by the EUNetHTA Core Model®[19,20]. RRI principles include, among others, engagement of all societal actors, gender balance both within the research teams and in the group of consulted stakeholders, ethics, and governance, with the intent to enable a positive impact of the research on society.

Considering the nine domains of EUNetHTA Core Model®, the purpose of this review is to inform the activities of the feasibility study in the following domains while addressing relevant and associated issues:

(1) Health Problem and Current Use of the Technology with a special focus on pilot projects that tested GS for NBS;

(2) Description and technical characteristics of the technology with a focus on the discussion within the scientific community on the list of genes that should (or should not) be included in the analysis;

(3) Safety with a focus on incidental findings, false negatives, and false positives;

(4) Clinical Effectiveness trying to answer the question: What is the number of newborns per year we could expect to identify as positive?

(5) Costs and economic evaluation to investigate which methods and models were used to estimate the costs of GS-based NBS by ongoing initiatives;

(6) Ethical analysis considering in particular that in the case of NBS the patient cannot make any decision by himself/herself, as all decisions are taken by the parents;

(7) Organizational aspects - again looking at recent pilots, trying to identify the major obstacle(s) to the full deployment as part of the standard of care of a GS-NBS program;

(8) Patients and Social aspects with a focus on the acceptability of GS-based screening programs by citizens and the methodology adopted by other pilot programs to consult and engage citizens;

(9) Legal aspects to first answer the question of whether a genomic screening program could be made mandatory (as it is now for the traditional Italian NBS program) or should be voluntary.

Trying to cover all the above-mentioned issues, we selected a wide search algorithm without limiting our review to a specific domain but limiting it to newborn/neonatal screening AND WGS (that includes, as a MeSH term, WES). For results and conclusions, we grouped the above-listed domains into three main areas: Clinical (covering issues 1 to 4), Societal (covering issues 7 and 8), and Governance (covering issues 5, 6, and 8).

METHODS

Search strategy

Three different sources were identified for the selection of the papers in order to obtain a various and unbiased set of articles. The sources included (1) the PubMed online database via a query performed on September 28th, 2022; (2) the Mendeley library shared within the clinicians working group; and (3) the final selection of articles that were selected for Downie *et al.*'s 2021 systematic review "Principles of Genomic Newborn Screening Programs: a systematic review[21]".

The search algorithm used in PubMed was defined according to the objective of the review, i.e., to provide the practical and theoretical background for the application of WGS or WES techniques to population-wide NBS programs. The search was performed for all study types published in English, with the full texts available using MeSH terms (whole-genome sequencing) AND (neonatal screening). These MeSH terms were selected because they include all the possible synonyms, and in the case of WGS, it includes WES as well. The query on the PubMed online database with that algorithm gave 147 articles as a result.

The Mendeley Library has been populated by the multidisciplinary team working on the feasibility study mentioned in the introduction. 79 articles were identified and used to guide the conception, design, and start-up phases of the study.

Downie *et al.*'s systematic review "Principles of Genomic Newborn Screening Programs: a systematic review" published in 2021 was considered the benchmark and the 36 final articles were included in our initial database[21].

The three sources all together yielded 262 articles, some of which were duplicated in two or all the sources, as shown in Figure 1. The final articles to be screened were 219.

Documents selection

The selection of articles to be included in the review followed two steps, both of which were performed independently by two people:

(1) Titles screening

The first screening was made considering the title of the articles. Articles focusing on one disease only, carrier screening, case reports, protocols only, and infective outbreaks in the Neonatal Intensive Care Unit (NICU) were excluded. After this first step, 151 articles were excluded for relevance reasons.

(2) Abstracts screening

The second step consisted of reading the abstracts and assigning each article to one of 14 pre-identified categories grouped in three areas: clinical, social, and governance [see Figure 2]. Reading the abstracts allowed a stricter selection of articles with a clear focus on the application of Genome Sequencing (GS) to population-wide NBS, while excluding the publications that used GS as a diagnostic tool. Finally, we reduced the redundancy based on the article's topic and publication date (e.g., for articles on the same topic, the most recent was preferred). After this step, 36 articles were excluded.

The final number of articles retained for the review was 33.

A flowchart of the selection of the articles can be found in Figure 3.

RESULTS

The mixed methods search brought to the identification of 33 articles distributed as in Figure 4.

For the Clinical subject, 14 publications were identified. Five papers were focused on wide GS discussion in the last 15 years[22-24,7] Methods to manage the genomic data produced in the GS analysis and a definition of the clinical actionable conditions have been explored in three publications[2,6,5]. Results and/or discussions about the impact, feasibility, benefits, and costs of the GS in the clinical care of newborns have been reported in five publications[14,26-29].

For the Social subject, 12 publications were identified. Three records dealt with the BabySeq Project, surveying parents and clinicians involved in the trial, parents who denied participation, and a third one analyzing the changed protocol and the concept of family benefit[8,12,30]. The study NC-NEXUS was also taken into consideration, with a publication regarding a Decision Aid tool to support parents in the decision-making process. If GS is to be implemented in NBS, communication and education are key elements that must be considered and promoted[31]. Opinions from genetics professionals were also considered through a paper that presented a survey to the American College of Medical Genetics and

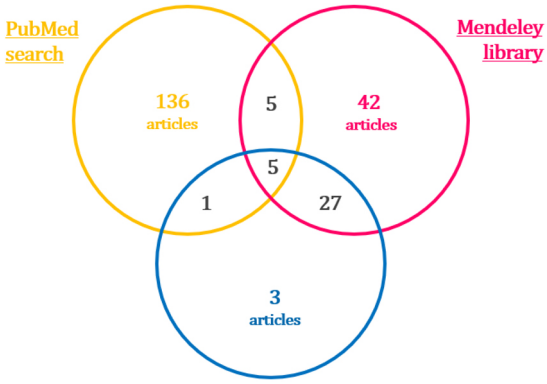


Figure 1. Venn diagram that shows the different sources of the articles.

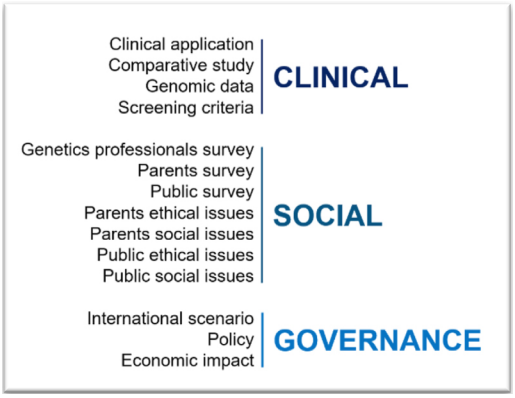


Figure 2. Grouping of the papers' categories.

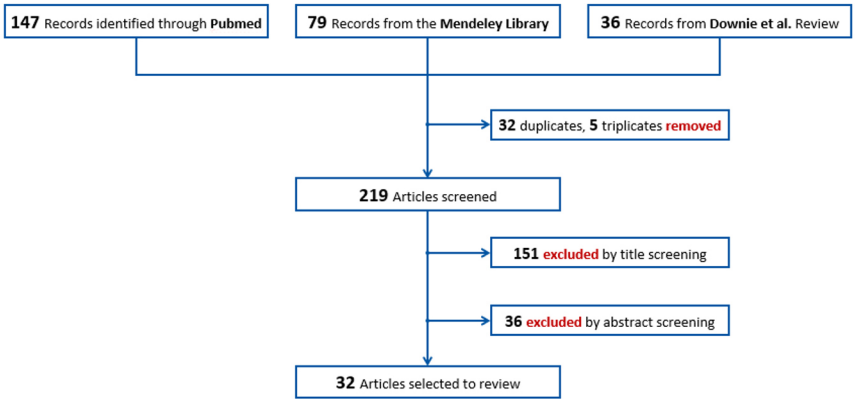


Figure 3. Study flowchart.

Genomics (ACMG) members[32]. Lastly, public views on the incorporation of GS in NBS were also included[33]. Recommendations by the NSIGH Ethics and Policy Advisory Boards were also a result of the search, sharing their opinion regarding the use of GS applied to diagnostic and universal NBS[34]. Finally, two independent opinions and a parent survey were considered[35-37].

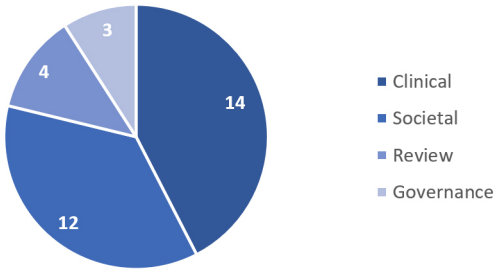


Figure 4. Distribution of the selected articles in the studied areas.

For the Governance subject, three publications were identified[9,38,39]. Two[9,38] have a legal focus, analyzing the constitutional framework for the adoption of GS-based NBS programs in the US. The third paper[39] has a policy perspective and lists eight recommendations for the introduction of GS in NBS. These recommendations were elaborated by the Pediatric Task Team of the Global Alliance for Genomics and Health.

Clinical

Wilson and Jungner originally defined the screening criteria to guide the selection of conditions that would have been suitable for screening. Among these criteria, early-stage detectability and treatment availability are still solidly respected. However, the advent of the genomic era with advanced medical technologies and the increased interest in genome screening requested a revision of Wilson and Jungner screening criteria[7]. Certainly, the screening criteria should be further and constantly discussed to reflect people's evolving interests and needs.

The clinical utility of genetic testing and the efforts to guarantee transparency and quality of the results have been widely discussed in Europe and the USA. The Public and Professional Policy Committee (PPPC) and the Quality Committee of the European Society of Human Genetics (ESHG) addressed these challenges in the past years, and the final recommendations were approved and published in December 2012[24]. Whole-genome analysis might be applied in several circumstances, such as diagnosis in symptomatic patients, research, pharmacogenomics, investigation in pre-symptomatic patients, and population screening programs. In order to develop best practices in implementing WGS/WES into health care:

(1) Stakeholders from different fields should participate in the discussions about WGS/WES implementations, sharing their experience and contributing to the development of national and international guidelines;

(2) A targeted approach should be adopted to avoid unsolicited findings, e.g., known genetic variants with limited or no clinical utility;

(3) WGS/WES analysis should be applied when necessary, ensuring a balance of benefits and limitations for the patient. Genetic experts should explain the benefits and limitations of genetic testing for screening, informing prospective parents and raising public awareness;

(4) A protocol is essential to guide the communication of secondary findings and report how the data will be shared and stored;

(5) Guidelines for informed consent on genomic testing, sample uses (e.g., research studies) and storage need to be developed and widely shared within the appropriate workforce;

The European initiative EuroGentest was established by the European Commission to promote accurate and high-quality genetic diagnostics across Europe, and it was integrated as a working group with the European Society of Human Genetics (ESHG), with whom in 2016 they published the guidelines for diagnostic applications of Next Generation Sequencing (NGS) for rare genetic diseases, consisting of 38 statements with a particular focus on WES and sequencing on selected genes identifying small germline variants (Single Nucleotide Variants (SNVs) and insertions/deletions). In 2021, an update of EuroGentest guidelines for NGS has been published, including five additional statements (a total of 44 statements) by the Solve-RD, a Horizon2020-funded project, born with the aim of finding a diagnosis for a large number of rare diseases (www.solve-rd.eu)[22].

GS-based NBS pilot projects

The implementation of GS in newborns triggered great interest in the setting of explorative pilot projects to assess medical, economic, ethical, and social impact in the healthcare system and among the general population.

The BabySeq project (ClinicalTrials.gov Identifier: NCT02422511) is a randomized trial on newborns with the aim to assess the impact of genomic sequencing in the newborn period to screen healthy infants for current and future health risks and provides data about the feasibility, risks, benefits, and costs of the integration of exome sequencing in the clinical care of newborns. The BabySeq2 Project (ClinicalTrials.gov Identifier: NCT05161169) is currently in the recruitment phase and aims to expand and improve the results obtained in the first study. Results reported for the BabySeq project were obtained by the clinical trial on 159 children from the well-baby nursery at Brigham and Women's Hospital (127 healthy newborns) and from the neonatal and pediatric intensive care units at Boston Children's Hospital in Massachusetts General Hospital (32 ill newborns)[28]. 1,514 genes [Supplementary List 1] were curated and classified into three categories (A, B, or C). Category A includes genes with definitive or strong evidence to cause a highly penetrant childhood-onset disorder; Category B includes genes based on actionability during childhood; Category C includes genes that did not meet criteria to be returned in the newborn genome sequencing report[6]. A table including an example of genes from category A from Ceyhan-Birsoy *et al.* (2017) has been appended[28] [Supplementary Table 1].

After testing, a newborn genomic sequencing report is generated, including information on pathogenic and likely pathogenic variants, monogenic disease variants, recessive carrier variants for childhood-onset or actionable conditions, and pharmacogenomic variants. The analysis also contains information on variants of uncertain/unknown significance (VUS) indications. However, only a randomized group of families received newborn GS reports and the results obtained from the study were disclosed to the newborn's parents during an in-person consultation by a genetic counselor and physician. The reports are available in both hospitals and online through a GeneInsight Clinic instance[14].

In the BabySeq project, WES analysis uncovered the risk of childhood-onset diseases in 15/159 (9,4%) of newborns, and none of these was expected based on the clinical histories of babies and their parents. Only parents of 85/159 newborns accepted to receive information on adult-onset actionable conditions, and in 3/85 cases a risk was identified. 88% of newborns were carriers of recessive disease and 5% were carriers of pharmacogenomics variants. Among the newborns with carrier-status variants, 8 of 140 (6%) also had VUS in one of the reported carrier genes. The number of carrier-status variants ranged from one to seven

variants in a single newborn[28].

Regarding the yield of the GS approach compared to standard NBS methods, the BabySeq project's results were discordant compared with conventional NBS and NBS plus WES[29]: 84% of newborns were NBS and WES negative; 1/159 infants were positive for the same disorder by both approaches; 9/159 infants were NBS positive and WES negative. Among the latter, 7 were reported as false positives after subsequent analysis. 15/159 infants were WES positive and NBS negative, indicating the risk of genetic conditions not detectable through the conventional NBS approach[29]. However, the BabySeq project results demonstrated the efficacy of newborn GS in detecting risk and carrier status for a wide range of disorders that cannot be detected by current NBS assays[28].

The North Carolina Newborn Exome Sequencing for Universal Screening (NC NEXUS) project (ClinicalTrials.gov Identifier: NCT02826694) was concluded in 2020 and examined the use of WES for NBS versus the conventional NBS approach. 106 infants were enrolled, including two cohorts: 61 healthy infants whose parents were approached for participation in the study prenatally and 45 ill infants affected by inborn errors of metabolism (17) and hearing loss (28), already detected by conventional NBS methods. Trio analysis was not performed. However, a follow-up parental sequencing has been performed in cases for which compound heterozygosity was suspected.

In the NC NEXUS project, WES correctly identified 88% of the cases with already diagnosed metabolic disorders and only 18% with already diagnosed hearing loss. Moreover, actionable findings that would not have been revealed by conventional NBS were revealed in four newborns. Some parents were selected to receive additional information about childhood-onset conditions with low or no clinical actionability, clinically actionable adult-onset conditions, and carrier status for autosomal-recessive conditions[27]. Carrier findings in newborns whose parents requested this information were detected with an average of 1.8 per infant (with a maximum of 7 variants).

Clinical actionability was detected using the age-based semiquantitative metric[5].

(1) Conditions were categorized into four categories:

(2) Pediatric conditions with high medical actionability;

(3) pediatric conditions with low or no medical actionability;

(4) adult conditions with high medical actionability;

(5) adult conditions with low or no medical actionability.

According to these criteria, 755 gene-disease pairs were categorized (the list of 755 genes from Milko *et al.* (2019) has been included [Supplementary List 2][5]. An abnormal or positive screen GS-NBS result related to high medical actionability conditions was reported by observing likely pathogenic and/or pathogenic variants in genes associated with pediatric conditions. A normal or negative GS-NBS result was defined by the absence of likely pathogenic or pathogenic variants. Positive results were associated with the presence of likely pathogenic or pathogenic variants found in gene(s) reported in the metabolic or hearing loss diagnostic list. Inconclusive results included, for example, a single heterozygous variant found in a gene associated with an autosomal-recessive condition and/or variants of uncertain significance (VUS) in genes

on the diagnostic list. Negative results indicated no detection of any pathogenic or likely pathogenic variants or any VUS on the diagnostic gene lists. 15/17 (88,2%) of patients affected by metabolic conditions resulted as GS-NBS positive. In the hearing loss cohort, "inconclusive" findings, not providing definitive results, were reported (some participants were heterozygous or homozygous for different VUSs in genes associated with hearing loss). Two false negative results were detected: one patient had a single heterozygous pathogenic variant in a gene associated with maple syrup urine disease and a patient with Malonyl-CoA decarboxylase deficiency had a homozygous missense VUS. However, since the authors did not have sufficient information to better identify the genetic etiology of the patient's disease, both were reported as "inconclusive findings". One patient was a carrier for another condition. 5/28 (17,9%) patients affected by hearing loss tested GS-NBS positive and two of them had positive screen results unrelated to their condition[27].

After the conclusion of the NC NEXUS project, it has been stated that using a GS approach could not widely substitute current screening tests. However, genomic information could be useful to perform a "secondary" or "indication-based" analysis, improving the sensitivity and specificity of NBS for inborn errors of metabolism[27].

In the Netherlands, the NBS (NGSf4NBS) project is a technical feasibility study also aiming at assessing the ethical, legal, social, and financial aspects to explore the adoption of NGS approaches as a first-tier method in NBS[26]. The study will proceed in three steps. In Step 1, inherited metabolic disorders eligible for NGS as a first-tier test will be identified based on treatability. In Step 2, the feasibility, limitations, and comparability of different technical NGS approaches and analysis workflows for NBS will be tested. In Step 3, the results will be incorporated into the current Dutch NBS program, including guidelines for the referral of a child after a positive NGS test result[26].

Methods to evaluate the criteria for inclusion of genes in GS studies

NBS through WGS and WES should be based on a clear path of clinical utility and/or actionability[23]. The magnitude of the genomic information generated, and its management are key challenges of introducing GS in the clinical setting. Other issues that must be taken into account are the definition of a subset of clinically actionable findings, the use of standardized protocols, and the introduction of appropriate and shared informed consensus for the families involved. In 2016, Berg *et al.* defined a semiquantitative metric for evaluating clinical actionability by assessing five criteria: the severity and likelihood of manifesting a particular condition, the efficacy and acceptability of the intervention, and the overall knowledge base of the gene-disease association[2]. The metric did not take into account the individual's age and sex, the timing of the onset of the disease, and the availability and cost of any preventive strategy.

The North Carolina Newborn Exome Sequencing for Universal Screening (NC NEXUS) project implemented the semiquantitative metric and assessed an age-based framework for evaluating genome-scale sequencing results in NBS. The age-based, semiquantitative metric categorized gene-disease pairs into groups based on age of onset or timing of interventions, improving the past method and facilitating the definition of inclusion criteria in the GS studies[5].

Additionally, a list of genes with putative pediatric relevance based on the framework released by the Clinical Genome Resource (ClinGen) working group has been assessed to manage the return of results in the BabySeq project. The generation of the gene-disease pair association was curated for the following criteria: validity of gene-disease association, age of onset, penetrance, and inheritance pattern. Based on the selected criteria, three categories of classification of gene-disease pairs were defined: category A: genes

included in the newborn genomic sequencing report with definitive or strong evidence to cause a highly penetrant childhood-onset disorder; category B: genes included in the newborn genomic sequencing report based on actionability during childhood; category C: genes that did not meet criteria to be returned in the newborn genomic sequencing report[6].

A comparison between the NC NEXUS age-based framework and the BabySeq categorization approach revealed differences in the methods used to define each category. The NC NEXUS age-based semiquantitative metric includes several components to achieve actionability score criteria, whereas the BabySeq criteria differ between each of the three categories. BabySeq category A is focused on clear evidence of gene-disease relationship without actionability considerations. Category B includes potential actionability. Category C includes low penetrance, insufficient evidence or late-onset conditions, and non-invasive intervention in childhood. A solution proposed was to report actionable genomic information at the corresponding age-appropriate stage (e.g., infancy, childhood, adult) to overcome any potential social, ethical, or psychological issue related to non-actionability conditions[5].

Societal

Incorporating WGS/WES into population-wide NBS programs triggers significant ethical and policy concerns, as it implies the generation of incidental health information of known and unknown clinical significance for millions of infants annually[36]. When implementing a new technology in a state-run program, it is particularly important to reach clarity in the evaluation of benefits and limitations. This is notably valid when the technology is GS, as test results present a heterogeneous, complex, and unsure nature[33].

Conventional (biochemical-based) NBS is considered a standard of care and is often a mandatory, state-supported activity, e.g., in Canada and the US, where parental consent is typically implied[40,41]. Introducing NGS technologies could dramatically change the context, shifting the balance between clinical benefits and risks and raising new questions that could threaten the universality and moral authority of NBS. GS technology has raised fundamental challenges to the traditional ways genomic information is communicated. If GS was to be incorporated into standard NBS practice, clinicians, public health officials, and other stakeholders would need to agree on the type of information that they should seek and communicate to parents[31].

Ulm *et al.* in 2015 surveyed members of the American College of Medical Genetics and Genomics (ACMG) to gather genetic professionals' opinions regarding the use of WGS in NBS[32]. Starting from the premise that 86% of the respondents believe WGS should not be included in NBS yet, many critical challenges were identified, such as the introduction of pre- and post-counseling, the interpretation of results, and follow-up access. Informed consent should be required from parents to enable them to decide which information to receive but with the confidence of knowing that laws and policies are being implemented to protect against discrimination and privacy[32]. It is interesting to notice that at the time the participants filled out the survey (November-December 2012), 28% believed WGS would have been implemented in 5 years (by 2017) and 23% in 6-10 years (by 2018-2022).

Informed consent and return of results

Given the nature of NBS, for which the primary beneficiary is the newborn, parents have a substantial role in the process. Joseph *et al.* conducted four focus groups with socioeconomically and ethnically diverse pregnant women to examine their views and perspectives regarding the potential application of WGS to NBS. For many women, knowledge and information are fundamental tools to have a sense of control over

labor and childbirth - and consultations and education regarding NBS are key topics of conversation that should happen before the test, in order to understand the process and have the opportunity to ask questions[37]. Formal permission or written consent was, however, a secondary priority for parents, while it was felt more urgent in case NBS was performed with WGS, given the increased complexity of genetic information. The need for formal parental permission implies the possibility that parents opt out, thus altering the universality principle that always characterizes NBS[34].

Genetti *et al.* in 2018 evaluated parental interest in a randomized trial of GS-NBS, in particular analyzing causes for declining participation, before and after an enrolment meeting with a genetic counselor. Risk communication was found to be a key element during the education process for informed consent, given the sensitivity of genetic information and the apprehension that this information would be recorded in their infants' medical documents[30].

Psychological distress

Families and professionals involved in newborn genetic screening are challenged with complex and onerous questions that can lead to an increased amount of new knowledge which can be difficult to deal with. Parents have the authority, both legal and moral, of making decisions for their newborns, including medical decisions that are, supposedly, in their child's best interest. When using GS, a large number of gene variants are possibly detected, including genes encoding for adult-onset disorders. Such timing of testing, being in the neonatal period, makes it impossible for the primary beneficiaries, i.e., newborns, to make their own decisions depriving them of future adult autonomy and confidentiality[11,12,35,37].

While the use of GS as a diagnostic tool is accepted, the uncertainty and ambiguity of some results of GS as a screening tool could transform healthy newborns into pre-sick or "patient-in-waiting"[42], risking premature medicalization of infants and causing significant distress and worry in parents[34].

Many other potential drawbacks for the screened family are the damage to the child's self-esteem, stigmatization, and the sense of guilt of transmitting a pathogenic variant to your child; this information could also be the cause of discrimination, lack of privacy in different circumstances, with issues accessing medical insurance being the first difficulties on a potentially long list[35].

Genetic professionals and laboratorians are also suffering from potential moral and ethical dilemmas: Ross *et al.* in 2019 reported a case in the BabySeq project where the discovery of an actionable adult-onset disease in a newborn led to a dilemma of the personnel that could not return a result that was widely considered actionable[12]. On the basis of this case, the BabySeq protocol was then modified, invoking the principle of family benefit, for which the best interest of the child includes his parents' well-being. Following these modifications, parents could decide whether they wanted to receive information on adult-onset variants, even though it is still widely accepted[43] that children should not be tested for adult-onset conditions. For Ross & Clayton, one solution could have been to modify the BabySeq analytical process in order not to discover those variants, designing the study to limit the search to relevant genes and reduce the risk of finding stress-inducing information[12].

A survey conducted by Pereira *et al.* published in 2019 demonstrated that parents and clinicians would prefer NBS without GS, even though parents showed more trust than clinicians towards GS. This shows that what is considered a clinical benefit to the clinicians is different from the perception of the parents (i.e., parental/personal utility), which might have a broader range of expectations, showing once again how relevant and crucial the education process is in these circumstances[8].

Considering how fast today's society evolves and how complex and sensitive this field is - more frequent societal consultations are key to understanding whether there is a community consensus.

Governance

The psychological distress and worry around using GS in NBS bring governance and policy consequences that must be taken into consideration. For example, parental worry could cause follow-up visits, tests, and services that may not be medically indicated[36].

Moreover, when clinicians or other healthcare professionals have the role of returning results to patients, time management is a concern, since counseling parents and educating them on procedures and next steps will be time- and energy-consuming and, therefore, costly. It has to be taken into consideration that all positive screen results will need follow-up care, confirmatory testing, and monitoring, ensuing even more time and costs to the healthcare system[34].

Genetics professionals surveyed by Ulm *et al.* think that the complexity implied in the use of GS in NBS should lead to a new counseling paradigm, forcing a non-mandatory program that envisages consent and the option to opt out in a setting where genetic discrimination is prevented[32]. These changes and challenges should thus require a new setting and an infrastructure boosting education and training of the workforce involved[35].

On the same line, two papers[9,38] analyzed the US legal framework with respect to the introduction of GS in NBS programs. Both concluded that the current "constitutional boundaries" do not allow the introduction of mandatory neonatal screening programs using GS. The first argument is that mandatory screening is based on two fundamental legal bases:

(1) *Police power* that allows the state to intervene in order to protect the health and safety of citizens AND

(2) *Parens patrie* that allows public authority to make decisions in the best interest of the children despite the opinion of the parents.

Both principles do not seem to be applicable to genomic screening unless it is limited to a strict number of genes (and variants on those genes) that cause severe but treatable conditions with an almost certain pediatric onset[9,38,39].

From a health policy perspective, there is a consensus regarding the introduction of GS-based NBS programs which should not substitute the current conventional NBS programs, meaning that the costs for implementing the new program are on top of the existing one with limited overlap[39]. Another important aspect considered by all the three papers[9,38,39] is equity: despite being subject to consent from the parents, once introduced, GS-based NBS should be equally accessible to all newborns. An interesting concept linked with equity concerns is the possibility for the families to have raw data from GS analyzed and interpreted independently; if families can get access to raw data, some of them, the wealthier and more educated, could look for deeper analysis and interpretation even for a portion of the genome not included in the NBS program. Is that ethical? Is that fair, considering that other families will not have that possibility?[9]

LIMITATIONS

The rapid evolution of the field and the increasing number of pilot programs using GS for NBS make it difficult to give a snapshot without the risk of missing the most recently published evidence. To make an

example, while preparing this manuscript, a rapid evidence review on the implementation of large-scale genomic screening was published by Alarcón Garavito *et al.*[44].

Moreover, the decision to focus exclusively on NBS programs using WES or WGS forces to neglect some works on disease-specific genetic screening that could provide some additional evidence, especially on topics such as acceptability by the parents and management of incidental findings and VUS.

Finally, for this work, only peer-reviewed articles were taken into consideration. This could have limited the identification of relevant information, especially on governance and legal aspects that could have been included in grey literature, such as project public deliverables, reports, and policy guidelines.

CONCLUSIONS

Although there is a broad and animated debate on the use of GS for NBS, there is still little real-world evidence available from a few pilot projects (namely BabySeq and NC-Nexus, both carried out in the USA). Other pilot projects have been recently launched in Europe and the UK and more evidence will become available in the coming years. Despite a consensus in the literature on the key principles that should guide the use of GS in NBS, many important issues are still to be adequately addressed and solved.

All authors agree that NBS should include only actionable genes, but the definition of actionable is still a matter of debate, as well as the criteria and ideal frequency of updates of the list of genes-diseases to be screened for. Currently, informed consent from the parents seems to be the preferred approach, but there is still an open discussion on how to manage incidental findings or information on the status of the carrier.

Ethical, legal, social, and budgetary issues still constitute great challenges and major barriers to the wide, equitable, and uniform adoption of GS in NBS. When looking at these aspects, it is important to also consider the other side of the coin, i.e., the burden that inherently accompanies a family who did not get the chance of an early diagnosis or the management of critically ill patients in NICUs. Early diagnosis could also generate cost savings for the healthcare systems as it allows them to prevent severe symptoms that may require frequent hospitalizations. These savings could at least partially balance the additional costs generated by GS-NBS, which, according to the majority of authors, should not substitute the current NBS programs but run in parallel as additional screening. Unfortunately, it was not possible to find any published studies with information on cost-effectiveness and the estimation of potential savings of healthcare resources by using GS in NBS.

The management of genomic data of newborns for secondary use (e.g., for research purposes) should be balanced with the right of children to an "open future" and to autonomously make decisions on the use of their own genomic profile. As shown by this literature review, no easy or straightforward solutions have emerged so far. Moreover, a one-size-fits-all approach will probably never work, as GS-based NBS should take into consideration the specific value and ethical frame of the community where it is deployed. Ten years ago, 50% of the surveyed experts of the ACMG expected GS to be implemented in the NBS everyday practice. Evidently, we are not there yet. Further pilots and consultations with the stakeholders will be necessary before GS-based NBS programs can be widely implemented.

DECLARATIONS

Acknowledgments

The authors would like to thank Virginie Bros-Facer, David Pearce, and Daniel Scherman for their precious support. Special thanks also to Maria Iascone for the valuable suggestions.

Authors' contributions
Design of the methodology and the search algorithm: Magnifico G, Benvenuti S
Blind selection of the articles: Magnifico G, Artuso I
Writing of the manuscript: Magnifico G, Artuso I, Benvenuti S

Availability of data and materials
Not applicable.

Financial support and sponsorship
This work was supported in part by Regione Lombardia, ITALY [FT5132], and by Fondazione Telethon [GSP21003].

Conflicts of interest
All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

REFERENCES
1. Loeber JG, Platis D, Zetterström RH, et al. Neonatal screening in europe revisited: An ISNS perspective on the current state and developments since 2010. *Int J Neonatal Screen* 2021;7:15. DOI PubMed PMC
2. Berg JS, Foreman AK, O'Daniel JM, et al. A semiquantitative metric for evaluating clinical actionability of incidental or secondary findings from genome-scale sequencing. *Genet Med* 2016;18:467-75. DOI PubMed PMC
3. Berg JS, Agrawal PB, Bailey DB Jr, et al. Newborn sequencing in genomic medicine and public health. *Pediatrics* 2017:139. DOI PubMed PMC
4. Burlina A, Jones SA, Chakrapani A, et al. A new approach to objectively evaluate inherited metabolic diseases for inclusion on newborn screening programmes. *Int J Neonatal Screen* 2022;8:25. DOI PubMed PMC
5. Milko LV, O'Daniel JM, DeCristo DM, et al. An age-based framework for evaluating genome-scale sequencing results in newborn screening. *J Pediatr* 2019;209:68-76. DOI PubMed PMC
6. Ceyhan-Birsoy O, Machini K, Lebo MS, et al. A curated gene list for reporting results of newborn genomic sequencing. *Genet Med* 2017;19:809-18. DOI PubMed PMC
7. Andermann A, Blancquaert I, Beauchamp S, Déry V. Revisiting Wilson and Jungner in the genomic age: a review of screening criteria over the past 40 years. *Bull World Health Organ* 2008;86:317-9. DOI PubMed PMC
8. Pereira S, Robinson JO, Gutierrez AM, et al; BabySeq Project Group. Perceived benefits, risks, and utility of newborn genomic sequencing in the babyseq project. *Pediatrics* 2019;143:S6-S13. DOI PubMed PMC
9. Zacharias RL, Smith ME, King JS. The legal dimensions of genomic sequencing in newborn screening. *Hastings Cent Rep* 2018;48 Suppl 2:S39-41. DOI PubMed
10. Tarini BA, Goldenberg AJ. Ethical issues with newborn screening in the genomics era. *Annu Rev Genomics Hum Genet* 2012;13:381-93. DOI PubMed PMC
11. Lantos JD. Ethical and psychosocial issues in whole genome sequencing (WGS) for newborns. *Pediatrics* 2019;143:S1-5. DOI PubMed
12. Ross LF, Clayton EW. Ethical issues in newborn sequencing research: the case study of babyseq. *Pediatrics* 2019:144. DOI PubMed PMC
13. Pichini A, Ahmed A, Patch C, et al. Developing a national newborn genomes program: an approach driven by ethics, engagement and co-design. *Front Genet* 2022;13:866168. DOI PubMed PMC
14. Holm IA, Agrawal PB, Ceyhan-Birsoy O, et al; BabySeq project team. the babyseq project: implementing genomic sequencing in newborns. *BMC Pediatr* 2018;18:225. DOI PubMed PMC
15. Schuijff M, Dijkstra AM. Practices of responsible research and innovation: a review. *Sci Eng Ethics* 2020;26:533-74. DOI PubMed

16. Loeber A, Bernstein MJ, Nieminen M. Implementing responsible research and innovation: from new public management to new public governance. In: Blok V, editor. Putting Responsible Research and Innovation into Practice. Cham: Springer International Publishing; 2023. pp. 211-28. DOI
17. Responsible research and innovation: Europe's ability to respond to societal challenges Available from: https://data.europa.eu/doi/10.2777/11739 [Last accessed on 24 Aug 2023].
18. European commission. Open innovation, open science, open to the world. Available from: http://europa.eu/rapid/press-release_SPEECH-15-5243_en.htm [Last accessed on 24 Aug 2023].
19. HTA core model[R] version 3.0. Available from: https://www.eunethta.eu/hta-core-model/ [Last accessed on 29 Aug 2023].
20. HTA core model version 3.0. Available from: https://www.eunethta.eu/wp-content/uploads/2018/03/HTACoreModel3.0-1.pdf [Last accessed on 29 Aug 2023].
21. Downie L, Halliday J, Lewis S, Amor DJ. Principles of genomic newborn screening programs: a systematic review. *JAMA Netw Open* 2021;4:e2114336. DOI PubMed PMC
22. Souche E, Beltran S, Brosens E, et al. Recommendations for whole genome sequencing in diagnostics for rare diseases. *Eur J Hum Genet* 2022;30:1017-21. DOI PubMed PMC
23. Hendricks-Sturrup RM, Lu CY. When should genomic and exome sequencing be implemented in newborns? *Genet Med* 2020;22:809-10. DOI
24. van El CG, Cornel MC, Borry P, et al; ESHG Public and Professional Policy Committee. Whole-genome sequencing in health care: recommendations of the European society of human genetics. *Eur J Hum Genet* 2013;21:580-4. DOI PubMed PMC
25. Morava E, Baumgartner M, Patterson M, Peters V, Rahman S. Newborn screening: to WES or not to WES, that is the question. *J Inherit Metab Dis* 2020;43:904-5. DOI PubMed
26. Veldman A, Kiewiet MBG, Heiner-Fokkema MR, et al. Towards next-generation sequencing (NGS)-based newborn screening: a technical study to prepare for the challenges ahead. *Int J Neonatal Screen* 2022;8:17. DOI PubMed PMC
27. Roman TS, Crowley SB, Roche MI, et al. Genomic sequencing for newborn screening: results of the NC NEXUS project. *Am J Hum Genet* 2020;107:596-611. DOI PubMed PMC
28. Ceyhan-Birsoy O, Murry JB, Machini K, et al; BabySeq Project Team. Interpretation of genomic sequencing results in healthy and ill newborns: results from the BabySeq project. *Am J Hum Genet* 2019;104:76-93. DOI PubMed PMC
29. Wojcik MH, Zhang T, Ceyhan-Birsoy O, et al; BabySeq Project Team. Discordant results between conventional newborn screening and genomic sequencing in the BabySeq Project. *Genet Med* 2021;23:1372-5. DOI PubMed PMC
30. Genetti CA, Schwartz TS, Robinson JO, et al; BabySeq Project Team. Parental interest in genomic sequencing of newborns: enrollment experience from the BabySeq project. *Genet Med* 2019;21:622-30. DOI PubMed PMC
31. Lewis MA, Paquin RS, Roche MI, et al. Supporting parental decisions about genomic sequencing for newborn screening: the NC NEXUS decision Aid. *Pediatrics* 2016;137 Suppl 1:S16-23. DOI PubMed PMC
32. Ulm E, Feero WG, Dineen R, Charrow J, Wicklund C. Genetics professionals' opinions of whole-genome sequencing in the newborn period. *J Genet Couns* 2015;24:452-63. DOI PubMed
33. Bombard Y, Miller FA, Hayeems RZ, et al. Public views on participating in newborn screening using genome sequencing. *Eur J Hum Genet* 2014;22:1248-54. DOI PubMed PMC
34. Johnston J, Lantos JD, Goldenberg A, Chen F, Parens E, Koenig BA; members of the NSIGHT Ethics and Policy Advisory Board. Sequencing newborns: a call for nuanced use of genomic technologies. *Hastings Cent Rep* 2018;48 Suppl 2:S2-6. DOI PubMed PMC
35. Reinstein E. Challenges of using next generation sequencing in newborn screening. *Genet Res* 2015;97:e21. DOI PubMed PMC
36. Grob R, Roberts S, Timmermans S. Families' experiences with newborn screening: a critical source of evidence. *Hastings Cent Rep* 2018;48 Suppl 2:S29-31. DOI PubMed
37. Joseph G, Chen F, Harris-Wai J, Puck JM, Young C, Koenig BA. Parental views on expanded newborn screening using whole-genome sequencing. *Pediatrics* 2016;137 Suppl 1:S36-46. DOI PubMed PMC
38. King JS, Smith ME. Whole-genome screening of newborns? *Pediatrics* 2016;137 Suppl 1:S8-15. DOI PubMed PMC
39. Friedman JM, Cornel MC, Goldenberg AJ, Lister KJ, Sénécal K, Vears DF; Global Alliance for Genomics and Health Regulatory and Ethics Working Group Paediatric Task Team. Genomic newborn screening: public health policy considerations and recommendations. *BMC Med Genomics* 2017;10:9. DOI PubMed PMC
40. Morrison A, Dowler J. Newborn screening for disorders and abnormalities in Canada. Available from: https://www.cadth.ca/sites/default/files/pdf/Newborn_Screening_es-26_e.pdf [Last accessed on 24 Aug 2023].
41. Goldenberg AJ, Sharp RR. The ethical hazards and programmatic challenges of genomic newborn screening. *JAMA* 2012;307:461-2. DOI PubMed PMC
42. Timmermans S, Buchbinder M. Patients-in-waiting: living between sickness and health in the genomics era. *J Health Soc Behav* 2010;51:408-23. DOI PubMed
43. COMMITTEE ON BIOETHICS, COMMITTEE ON GENETICS, AND, AMERICAN COLLEGE OF MEDICAL GENETICS AND, GENOMICS SOCIAL, ETHICAL, LEGAL ISSUES COMMITTEE. Ethical and policy issues in genetic testing and screening of children. *Pediatrics* 2013;131:620-2. DOI PubMed
44. Alarcón Garavito GA, Moniz T, Déom N, Redin F, Pichini A, Vindrola-Padros C. The implementation of large-scale genomic screening or diagnostic programmes: a rapid evidence review. *Eur J Hum Genet* 2023;31:282-95. DOI PubMed PMC

Tsipouras *et al. Rare Dis Orphan Drugs J* 2023;2:17
DOI: 10.20517/rdodj.2023.15

Rare Disease and Orphan Drugs Journal

Opinion

Open Access

Could federated data analysis be the catalyst accelerating the introduction of newborn genome screening for the detection of genetic disease?

Petros Tsipouras[1], Maria Chatzou Dunford[2], Hadley Sheppard[2], Hannah Gaimster[2], Theoklis Zaoutis[3,4]

[1]FirstSteps Greece, Newborn genome screening Initiative, Athens 106 80, Greece.
[2]Lifebit Biotech Ltd., London EC2A 2AP, United Kingdom.
[3]National Public Health Organization (EODY), Athens 151 23, Greece.
[4]The 2nd Department of Pediatrics, National and Kapodistrian University of Athens, 'P. & A. Kyriakou' Children's Hospital, Athens 106 80, Greece.

Correspondence to: Dr. Petros Tsipouras, FirstSteps Greece, Newborn genome screening Initiative, Skoufa 64, Athens 106 80, Greece. E-mail: petros.tsipouras@beginnings.gr

How to cite this article: Tsipouras P, Dunford MC, Sheppard H, Gaimster H, Zaoutis T. Could federated data analysis be the catalyst accelerating the introduction of newborn genome screening for the detection of genetic disease? *Rare Dis Orphan Drugs J* 2023;2:17. https://dx.doi.org/10.20517/rdodj.2023.15

Received: 14 Jun 2023 **First Decision:** 7 Sep 2023 **Revised:** 14 Sep 2023 **Accepted:** 22 Sep 2023 **Published:** 27 Sep 2023

Academic Editor: Virginie Bros-Facer **Copy Editor:** Dan Zhang **Production Editor:** Dan Zhang

Abstract

Data federation intermediated through trusted research environments can help accelerate the adoption and utilization of newborn genome screening worldwide. Data federation will protect individual datasets from unauthorized security breaches, allow analysis *in situ*, and bypass the need for cumbersome data sharing agreements between parties. Finally, data federation could accelerate the adoption of new therapies for rare genetic diseases with the use of synthetic clinical trials.

Keywords: Newborn genome screening, data federation, trusted research environment

INTRODUCTION

Worldwide, millions of children are born with a rare genetic disease[1,2]. Newborn screening (NBS) has been effective in identifying babies who are at risk of developing a genetic disease and initiating a therapeutic intervention. The first genetic disease for which NBS was introduced is phenylketonuria (PKU), where early

dietary intervention prevents serious mental deficiency[3]. In the past fifty years, mandated NBS has expanded to include other, mostly Mendelian, diseases where early therapeutic intervention has been effective in preventing and/or ameliorating irreversible tissue damage. In many countries, states, and regions of the world, public health programs are in place to collect blood specimens from babies soon after birth[4]. Analytes extracted from dried blood spots collected on filter paper are assayed using gas chromatography/mass spectrometry (GC/MS) or tandem MS.

A second layer of screening based on Next-Generation Sequencing (NGS) technology could expand the scope of the existing NBS programs[5,6]. This additional layer of screening will not replace what is currently used, but it will increase the current offering substantially to include a broader spectrum of disorders not detectable by tandem MS.

Newborn genome sequencing could evolve to become the new paradigm for healthcare delivery, where early detection could result in better clinical outcomes. Rapid Whole Genome Sequencing (rWGS) has been shown to be an effective diagnostic test linked to decreased infant mortality and improved outcomes in babies admitted to Neonatal Intensive Care Units (NICU)[7,8].

Extending the use of genome sequencing as a screening test to all newborns is only a matter of time. However, before newborn genome screening is widely adopted, several factors will need to be carefully considered, including:

1. Accurate definition of pathogenic genomic variants in diverse populations.

2. Defined care paths for the follow-up of a screen-positive finding.

3. Evidence that early intervention leads to improved clinical outcomes.

4. Detailed cost analysis.

Persuasive answers to the above will be required by the key stakeholders whose support is essential, i.e., parents, health care providers, public health policymakers, and the pharmaceutical industry.

Several newborn genome screening (including whole genome sequencing and whole exome sequencing) initiatives have been launched, or they will be launched soon[9-11].

We anticipate that no one project will have the necessary solutions to satisfactorily address all or some of the above-mentioned problems. Thus, aggregation of information collected from different sources could provide part of the solution for critical mass and momentum.

Data aggregation of such magnitude presents significant legal, ethical, and technical challenges related to (i) the security and privacy of sensitive information; (ii) the size and varied nature of stored genomic data; and (iii) legal requirements for data sharing. A viable near- and mid-term solution that can help address these issues will be using trusted research environments (TREs) and data federation for secure storage, access, and analysis of genomic data[12]. A comparison of risks and benefits between existing and federated databases for genomic data is shown in Table 1.

Table 1. The data aggregation challenge. Comparison of risks and benefits between existing and federated databases

	Databases	Federated databases
Security and compliance	Movement and copying of sensitive information increases the risk of data breach	In a TRE and federation environment, data are not moved or copied, reducing security risk
Data size and interoperability	Lack of standardized formats and pipelines limits interoperability, and negatively impacts scalability, cost, and efficiency	Fully standardized data, securely accessible by cloud-based platforms through federation, can be combined with global cohorts and disparate datasets
Collaboration	Data cannot leave jurisdictional borders. Data sharing agreements are frequently difficult to negotiate and implement, hindering collaboration	Federated approaches will eliminate a major barrier across individual datasets, vastly improving the statistical power of research

TRE: trusted research environment.

Federated data analysis platforms, which facilitate secure data access from multiple sources without the need for data movement- where data could be vulnerable to interception, have emerged as a promising part of a solution for safely sharing anonymized genomic data. Here, genomic data remains secure in the TRE, which can then be linked virtually using a set of Application Programming Interfaces (APIs).

Traditional data access methods involve researchers downloading data to an institutional computing cluster. With federated analysis, the analysis is brought to where the distributed data lies, thereby eliminating the risky movement of data and removing many existing barriers to accessibility[13]. Such technology means that data can be made securely accessible but that data controllers (e.g., biobanks and healthcare providers) retain jurisdictional autonomy over data, a key concern in international data sharing.

International initiatives such as the Global Alliance for Genomics and Health (GA4GH)[14] set standards to promote the international sharing of genomic and health-related data, in part by setting interoperability standards and providing open-source APIs.

Common Data Models (CDMs) are crucial to ensuring data is interoperable, with several growing in popularity in the life sciences sector recently, including OMOP (Observational Medical Outcomes Partnership) CDM from the OHDSI (Observational Health Data Sciences and Informatics)-specifically for clinical-genomic data. Examples of health organizations utilizing OMOP as their CDM include the UK Biobank and All of Us from the US National Institutes for Health (NIH)[15,16].

Additionally, extraction, transformation, and loading (ETL) pipelines that can automate this work to process and convert raw data to analysis-ready data help further simplify this process for researchers. Normalizing all data to internationally recognized standards allows researchers to perform joint analyses across distributed datasets, which is key to ensuring diversity and representation of as many populations as possible in studies.

These standardized and interoperable datasets could be combined seamlessly for analysis via federation, enabling researchers to analyze this data collaboratively in conjunction with other complementary datasets. Standardization of data formats and analytical approaches within and even between health systems can bring substantial benefits in terms of comparability of data and contribute to continually improving processes.

Illustrative examples with potential multiplier effects could include:

Sharing pathogenic variants: Defining the frequency and prevalence of a pathogenic variant in diverse populations is essential. Access to the pathogenic variant libraries of the various initiatives will impact the predictive value of a screen positive, and it might help in the reclassification of Variants of Uncertain Significance (VUS).

Sharing care paths: Newborn genome screening is a risk stratification test that places a person in a high- or low-risk group for a particular genetic disease. The accuracy and validity of establishing the presence of disease have an enormous impact on the well-being of the person and the family, the timing of therapeutic intervention, possibly the modality of intervention, and ultimately healthcare cost.

Sharing clinical outcomes: Managing individuals with latent or early-stage disease can potentially increase the burden on health care providers and the health care system. Therapeutic interventions, to the extent possible, will need to be evidence-based. Individual genetic illnesses are often uncommon, and randomized clinical studies are difficult to conduct. Sharing clinical outcomes, on the other hand, may provide an incentive for synthetic clinical studies.

Sharing the analytic modality used to generate a variant: The validity of a variant is frequently linked to the analytical platform used to generate the information, i.e., panel, short-read NGS, and long-read NGS. Providing a barcode record could be helpful in assessing a variant and its possible value as a biomarker.

Recently, a pioneering example of a multi-party federation between Genomics England and Cambridge Biomedical Research Centre (BRC) was demonstrated. This allowed secure data analysis across TREs in the UK's first known demonstration of genomic data federation. This highlights that the technology now facilitates secure data access via federation for authorized researchers to perform joint secure data analysis on global cohorts. Data sharing via federated databases also decreases the danger of unauthorized access and encourages the adoption of advanced privacy-preserving encryption methods when analyzing data[17]. It is conceivable to imagine that this technology could be used in an undiagnosed disease program targeting newborns to help the integration of clinical, genomic, therapeutic, and outcome inputs residing in different datasets. A summary of how this could work is provided in Figure 1.

The Federated European Genome-Phenome Archive (EGA) is another program that uses federation to provide global discovery and access to human data for research while still adhering to jurisdictional data protection rules. The Federated EGA promotes data reuse, facilitates reproducibility, and accelerates biomedical research by providing a solution to increasing issues in the safe and efficient handling of human omics and related data[18].

While there are significant advantages to moving towards a genomic approach to newborn screening, these programs also have challenges. These include considerations of the ethical, legal, and social implications (ELSI) of newborn genomic screening - these can include concerns surrounding sensitive data sharing, patient autonomy, and consent. A detailed discussion of these issues is out of the scope of this piece, but we refer the reader to other references that have discussed these issues more completely[19,20].

As federation is an emerging technology, careful consideration must be given to scaling up federation across different TREs, particularly surrounding governance and assurances in particular across different jurisdictions. Additionally, genomic data federation could potentially have risks, which may include improper use of data, hacking, and identification of incidental findings such as detection of variants associated with pathologies not immediately treatable or relevant to the newborn[21]. It is important that

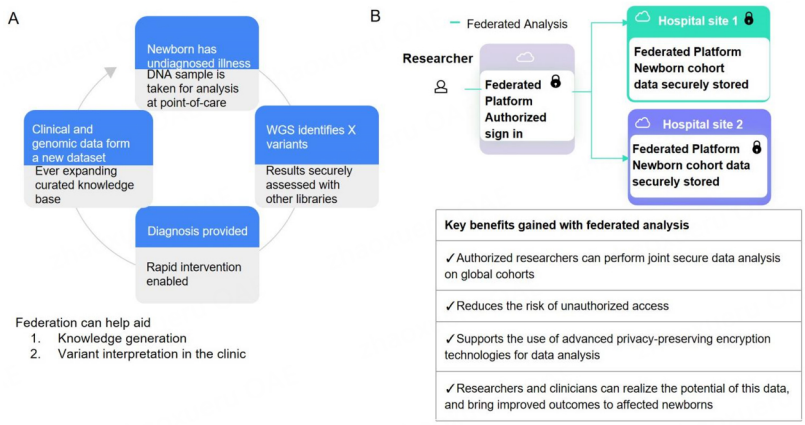


Figure 1. An overview of how federated data analysis can be incorporated into an undiagnosed disease program targeting newborns to help enable secure data access across research laboratories and clinics worldwide. (A) The steps involved in diagnosing a rare disease in an affected newborn; (B) A summary of how federated data analysis is performed and the benefits that can be gained.

these all be considered and addressed as federated approaches continue to be developed.

CONCLUSION

Newborn genome screening is a promising approach to early disease detection with considerable advantages compared to traditional approaches, but the integration into clinical care comes with complex technical challenges, which must be meaningfully explored to ensure effective and equitable impact. Standardized data federation could provide part of a crucial solution as a collaboration framework for the various newborn genome screening initiatives underway worldwide. Such efforts to facilitate secure joint data access and analysis to information among relevant stakeholders will accelerate the existing momentum of collaboration between global newborn sequencing initiatives, ultimately improving outcomes for patients.

DECLARATIONS

Authors' contributions

Wrote the paper: Tsipouras P, Sheppard H, Gaimster H
Reviewed the paper: Chatzou Dunford M, Zaoutis T

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Hageman IC, van Rooij IALM, de Blaauw I, Trajanovska M, King SK. A systematic overview of rare disease patient registries: challenges in design, quality management, and maintenance. *Orphanet J Rare Dis* 2023;18:106. DOI PubMed PMC
2. Pogue RE, Cavalcanti DP, Shanker S, et al. Rare genetic diseases: update on diagnosis, treatment and online resources. *Drug Discov Today* 2018;23:187-95. DOI
3. Guthrie R. Blood screening for phenylketonuria. *JAMA* 1961;178:863. DOI
4. Fidan Ç, Örün H, Alper AB, et al. Expanded newborn bloodspot screening: developed country examples and what can be done in Turkey. *Intractable Rare Dis Res* 2022;11:63-9. DOI PubMed PMC
5. Bick D, Ahmed A, Deen D, et al. Newborn screening by genomic sequencing: opportunities and challenges. *Int J Neonatal Screen* 2022;8:40. DOI PubMed PMC
6. Ceyhan-Birsoy O, Machini K, Lebo MS, et al. A curated gene list for reporting results of newborn genomic sequencing. *Genet Med* 2017;19:809-18. DOI PubMed PMC
7. Kingsmore SF, Smith LD, Kunard CM, et al. A genome sequencing system for universal newborn screening, diagnosis, and precision medicine for severe genetic diseases. *Am J Hum Genet* 2022;109:1605-19. DOI
8. Kingsmore SF; BeginNGS Consortium. Dispatches from biotech beginning BeginNGS: rapid newborn genome sequencing to end the diagnostic and therapeutic odyssey. *Am J Med Genet C Semin Med Genet* 2022;190:243-56. DOI PubMed
9. Gaff CL, M Winship I, M Forrest S, et al. Preparing for genomic medicine: a real world demonstration of health system change. *NPJ Genom Med* 2017;2:16. DOI PubMed PMC
10. Holm IA, Agrawal PB, Ceyhan-Birsoy O, et al; BabySeq Project Team. The BabySeq project: implementing genomic sequencing in newborns. *BMC Pediatr* 2018;18:225. DOI PubMed PMC
11. Pichini A, Ahmed A, Patch C, et al. Developing a national newborn genomes program: an approach driven by ethics, engagement and co-design. *Front Genet* 2022;13:866168. DOI PubMed PMC
12. Alvarellos M, Sheppard HE, Knarston I, et al. Democratizing clinical-genomic data: how federated platforms can promote benefits sharing in genomics. *Front Genet* 2022;13:1045450. DOI PubMed PMC
13. Chaterji S, Koo J, Li N, Meyer F, Grama A, Bagchi S. Federation in genomics pipelines: techniques and challenges. *Brief Bioinform* 2019;20:235-44. DOI PubMed PMC
14. Rehm HL, Page AJH, Smith L, et al. GA4GH: international policies and standards for data sharing across genomic research and healthcare. *Cell Genom* 2021;1:100029. DOI PubMed PMC
15. Papez V, Moinat M, Voss EA, et al. Transforming and evaluating the UK Biobank to the OMOP Common Data Model for COVID-19 research and beyond. *J Am Med Inform Assoc* 2022;30:103-11. DOI PubMed PMC
16. Mayo KR, Basford MA, Carroll RJ, et al. The all of Us data and research center: creating a secure, scalable, and sustainable ecosystem for biomedical research. *Annu Rev Biomed Data Sci* 2023;6:443-64. DOI
17. Nik-Zainal S, Seeger T, Fennessy R, et al. Multi-party trusted research environment federation: Establishing infrastructure for secure analysis across different clinical-genomic datasets. *Zenodo* ;2022:online ahead of print. DOI
18. Rueda M, Ariosa R, Moldes M, Rambla J. Beacon v2 reference implementation: a toolkit to enable federated sharing of genomic and phenotypic data. *Bioinformatics* 2022;38:4656-7. DOI PubMed
19. Hunter A, Lewis C, Hill M, et al. Public and patient involvement in research to support genome services development in the UK. *J Transl Genet Genom* 2023;7:17-26. DOI
20. Goldenberg AJ, Lloyd-Puryear M, Brosco JP, et al; Bioethics and Legal Workgroup of the Newborn Screening Translational Research Network. Including ELSI research questions in newborn screening pilot studies. *Genet Med* 2019;21:525-33. DOI
21. Richards S, Aziz N, Bale S, et al; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015;17:405-24. DOI PubMed PMC

AUTHOR INSTRUCTIONS

1. Submission Overview

Before you decide to publish with *Rare Disease and Orphan Drugs Journal* (*RDODJ*), please read the following items carefully and make sure that you are well aware of Editorial Policies and the following requirements.

1.1 Topic Suitability

The topic of the manuscript must fit the scope of the journal. Please refer to Aims and Scope for more information.

1.2 Open Access and Copyright

The journal adopts Gold Open Access publishing model and distributes content under the Creative Commons Attribution 4.0 International License. Copyright is retained by authors. Please make sure that you are well aware of these policies.

1.3 Publication Fees

The publication fee for each submission is $299. There are no additional charges based on color, length, figures, or other elements. OAE provides expense deduction for authors as appropriate. For more details, please refer to OAE Publication Fees.

1.4 Language Editing

All submissions are required to be presented clearly and cohesively in good English. Authors whose first language is not English are advised to have their manuscripts checked or edited by a native English speaker before submission to ensure the high quality of expression. A well-organized manuscript in good English would make the peer review even the whole editorial handling more smoothly and efficiently.
If needed, authors are recommended to consider the language editing services provided by Charlesworth to ensure that the manuscript is written in correct scientific English before submission. Authors who publish with OAE journals enjoy a special discount for the services of Charlesworth via the following two ways.
Submit your manuscripts directly at http://www.charlesworthauthorservices.com/~OAE;
Open the link http://www.charlesworthauthorservices.com/, and enter Promotion Code "OAE" when you submit.

1.5 Work Funded by the National Institutes of Health

If an accepted manuscript was funded by National Institutes of Health (NIH), the authors may inform Editors of the NIH funding number. The Editors are able to deposit the paper to the NIH Manuscript Submission System on behalf of the authors.

2. Submission Preparation

2.1 Cover Letter

A cover letter is required to be submitted accompanying each manuscript. It should be concise and explain why the study is significant, why it fits the scope of the journal, and why it would be attractive to readers, *etc.*
Here is a guideline of a cover letter for authors' consideration:
In the first paragraph: include the title and type (e.g., Original Article, Review, Case Report, *etc.*) of the manuscript, a brief on the background of the study, the question the author sought out to answer and why;
In the second paragraph: concisely explain what was done, the main findings and why they are significant;
In the third paragraph: indicate why the manuscript fits the Aims and Scope of the journal, and why it would be attractive to readers;
In the fourth paragraph: confirm that the manuscript has not been published elsewhere and not under consideration of any other journal. All authors have approved the manuscript and agreed on its submission to the journal. Journal's specific requirements have been met if any.
If the manuscript is contributed to a special issue, please also mention it in the cover letter.
If the manuscript was presented partly or entirely in a conference, the author should clearly state the background information of the event, including the conference name, time and place in the cover letter.

2.2 Types of Manuscripts

There is no restriction on the length of manuscripts, number of figures, tables and references, provided that the manuscript is concise and comprehensive. The journal publishes Original Article, Review, Meta-Analysis, Case Report, Commentary, *etc.* For more details about paper type, please refer to the following table.

Manuscript Type	Definition	Abstract	Keywords	Main Text Structure
Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author includes an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
Case Report	A Case Report details symptoms, signs, diagnosis, treatment, and follows up an individual patient. The goal of a Case Report is to make other researchers aware of the possibility that a specific phenomenon might occur.	Unstructured abstract. No more than 150 words.	3-8 keywords	The main text consists of three sections with fixed section titles: Introduction, Case Report, and Discussion.
Meta-Analysis	A Meta-Analysis is a statistical analysis combining the results of multiple scientific studies. It is often an overview of clinical trials.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Systematic Review	A Systematic Review collects and critically analyzes multiple research studies, using methods selected before one or more research questions are formulated, and then finding and analyzing related studies and answering those questions in a structured methodology.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Technical Note	A Technical Note is a short article giving a brief description of a specific development, technique or procedure, or it may describe a modification of an existing technique, procedure or device applied in research.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Commentary	A Commentary is to provide comments on a newly published article or an alternative viewpoint on a certain topic.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Editorial	An Editorial is a short article describing news about the journal or opinions of senior editors or the publisher.	None required	None required	/
Letter to Editor	A Letter to Editor is usually an open post-publication review of a paper from its readers, often critical of some aspect of a published paper. Controversial papers often attract numerous Letters to Editor	Unstructured abstract (optional). No more than 250 words.	3-8 keywords (optional)	/
Opinion	An Opinion usually presents personal thoughts, beliefs, or feelings on a topic.	Unstructured abstract (optional). No more than 250 words.	3-8 keywords	/
Perspective	A Perspective provides personal points of view on the state-of-the-art of a specific area of knowledge and its future prospects. Links to areas of intense current research focus can also be made. The emphasis should be on a personal assessment rather than a comprehensive, critical review. However, comments should be put into the context of existing literature. Perspectives are usually invited by the Editors.	Unstructured abstract. No more than 150 words.	3-8 keywords	/

2.3 Manuscript Structure

2.3.1 Front Matter

2.3.1.1 Title

The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

2.3.1.2 Authors and Affiliations

Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

2.3.1.3 Abstract

The abstract should be a single paragraph with word limitation and specific structure requirements (for more details please refer to Types of Manuscripts). It usually describes the main objective(s) of the study, explains how the study was done, including any model organisms used, without methodological detail, and summarizes the most important results and their significance. The abstract must be an objective representation of the study: it is not allowed to contain results which are not presented and substantiated in the manuscript or exaggerate the main conclusions. Citations should not be included in the abstract.

2.3.1.4 Keywords

Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

2.3.2 Main Text

Manuscripts of different types are structured with different sections of content. Please refer to Types of Manuscripts to make sure which sections should be included in the manuscripts.

2.3.2.1 Introduction

The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether the aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

2.3.2.2 Methods

Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

2.3.2.3 Results

This section contains the findings of the study. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

2.3.2.4 Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

2.3.2.5 Conclusion

It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

2.3.3 Back Matter

2.3.3.1 Acknowledgments

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

2.3.3.2 Authors' Contributions

Each author is expected to have made substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data, or the creation of new software used in the work, or have drafted the work or substantively revised it.

Please use Surname and Initial of Forename to refer to an author's contribution. For example: made substantial contributions to conception and design of the study and performed data analysis and interpretation: Salas H, Castaneda WV; performed data acquisition, as well as provided administrative, technical, and material support: Castillo N, Young V.

If an article is single-authored, please include "The author contributed solely to the article." in this section.

2.3.3.3 Availability of Data and Materials

In order to maintain the integrity, transparency and reproducibility of research records, authors should include this section in their manuscripts, detailing where the data supporting their findings can be found. Data can be deposited into data repositories or published as supplementary information in the journal. Authors who cannot share their data should state that the data will not be shared and explain it. If a manuscript does not involve such issue, please state "Not applicable." in this section.

2.3.3.4 Financial Support and Sponsorship

All sources of funding for the study reported should be declared. The role of the funding body in the experiment design, collection, analysis and interpretation of data, and writing of the manuscript should be declared. Any relevant grant numbers and the link of funder's website should be provided if any. If the study is not involved with this issue, state "None." in this section.

2.3.3.5 Conflicts of Interest

Authors must declare any potential conflicts of interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there are no conflicts of interest, please state "All authors declared that there are no conflicts of interest." in this section. Some authors may be bound by confidentiality agreements. In such cases, in place of itemized disclosures, we will require authors to state "All authors declare that they are bound by confidentiality agreements that prevent them from disclosing their conflicts of interest in this work.". If authors are unsure whether conflicts of interest exist, please refer to the "Conflicts of Interest" of *RDODJ* Editorial Policies for a full explanation.

2.3.3.6 Ethical Approval and Consent to Participate

Research involving human subjects, human material or human data must be performed in accordance with the Declaration of Helsinki and approved by an appropriate ethics committee. An informed consent to participate in the study should also be obtained from participants, or their parents or legal guardians for children under 16. A statement detailing the name of the ethics committee (including the reference number where appropriate) and the informed consent obtained must appear in the manuscripts reporting such research.

Studies involving animals and cell lines must include a statement on ethical approval. More information is available at Editorial Policies.

If the manuscript does not involve such issue, please state "Not applicable." in this section.

2.3.3.7 Consent for Publication

Manuscripts containing individual details, images or videos, must obtain consent for publication from that person, or in the case of children, their parents or legal guardians. If the person has died, consent for publication must be obtained from the next of kin of the participant. Manuscripts must include a statement that a written informed consent for publication was obtained. Authors do not have to submit such content accompanying the manuscript. However, these documents must be available if requested. If the manuscript does not involve this issue, state "Not applicable." in this section.

2.3.3.8 Copyright

Authors retain copyright of their works through a Creative Commons Attribution 4.0 International License that clearly states how readers can copy, distribute, and use their attributed research, free of charge. A declaration "© The Author(s) 2021." will be added to each article. Authors are required to sign License to Publish before formal publication.

2.3.3.9 References

Preferably original research articles that directly support the statements should be cited. Review articles could be cited when they specifically address the statement made in the manuscript. An abstract should not be used as a reference. Non-specific citations should be avoided.

References should be numbered in order of appearance at the end of manuscripts. In the text, reference numbers should be placed in square brackets and the corresponding references are cited thereafter. If the number of authors is less than or equal to six, we require to list all authors' names. If the number of authors is more than six, only the first three authors' names are required to be listed in the references, other authors' names should be omitted and replaced with "et al.". Abbreviations of the journals should be provided on the basis of Index Medicus. Information from manuscripts accepted but not published

should be cited in the text as "Unpublished material" with written permission from the source.

Types	Examples
Journal articles by individual authors	Weaver DL, Ashikaga T, Krag DN, et al. Effect of occult metastases on survival in node-negative breast cancer. *N Engl J Med* 2011;364:412-21. [PMID: 21247310 DOI: 10.1056/NEJMoa1008108]
Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002;40:679-86. [PMID: 12411462]
Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. *J Urol* 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. *J Appl Clin Pediatr* 2012;27:1903-7. (in Chinese)
Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. BJOG 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A*. Forthcoming 2002.

For other types of references, please refer to U.S. National Library of Medicine.
The journal also recommends that authors prepare references with a bibliography software package, such as EndNote to avoid typing mistakes and duplicated references.

2.3.3.10 Supplementary Materials

Additional data and information can be uploaded as Supplementary Materials to accompany the manuscripts. The supplementary materials will also be available to the referees as part of the peer-review process. Any file format is acceptable, such as data sheet (word, excel, csv, cdx, fasta, pdf or zip files), presentation (powerpoint, pdf or zip files), image (cdx, eps, jpeg, pdf, png or tiff), table (word, excel, csv or pdf), audio (mp3, wav or wma) or video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv). All information should be clearly presented. Supplementary materials should be cited in the main text in numeric order (e.g., Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2, *etc.*). The style of supplementary figures or tables complies with the same requirements on figures or tables in main text. Videos and audios should be prepared in English and limited to a size of 500 MB.

2.4 Manuscript Format

2.4.1 File Format

Manuscript files can be in DOC and DOCX formats and should not be locked or protected.

2.4.2 Length

There are no restrictions on paper length, number of figures, or amount of supporting documents. Authors are encouraged to present and discuss their findings concisely.

2.4.3 Language

Manuscripts must be written in English.

2.4.4 Multimedia Files

The journal supports manuscripts with multimedia files. The requirements are listed as follows:
Videos or audio files are only acceptable in English. The presentation and introduction should be easy to understand. The frames should be clear, and the speech speed should be moderate.
A brief overview of the video or audio files should be given in the manuscript text.
The video or audio files should be limited to a size of up to 500 MB.
Please use professional software to produce high-quality video files, to facilitate acceptance and publication along with the

submitted article. Upload the videos in mp4, wmv, or rm format (preferably mp4) and audio files in mp3 or wav format.

2.4.5 Figures

Figures should be cited in numeric order (e.g., Figure 1, Figure 2) and placed after the paragraph where it is first cited;
Figures can be submitted in format of tiff, psd, AI or jpeg, with resolution of 300-600 dpi;
Figure caption is placed under the Figure;
Diagrams with describing words (including, flow chart, coordinate diagram, bar chart, line chart, and scatter diagram, *etc.*) should be editable in word, excel or powerpoint format. Non-English information should be avoided;
Labels, numbers, letters, arrows, and symbols in figure should be clear, of uniform size, and contrast with the background;
Symbols, arrows, numbers, or letters used to identify parts of the illustrations must be identified and explained in the legend;
Internal scale (magnification) should be explained and the staining method in photomicrographs should be identified;
All non-standard abbreviations should be explained in the legend;
Permission for use of copyrighted materials from other sources, including re-published, adapted, modified, or partial figures and images from the internet, must be obtained. It is authors' responsibility to acquire the licenses, to follow any citation instruction requested by third-party rights holders, and cover any supplementary charges.

2.4.6 Tables

Tables should be cited in numeric order and placed after the paragraph where it is first cited;
The table caption should be placed above the table and labeled sequentially (e.g., Table 1, Table 2);
Tables should be provided in editable form like DOC or DOCX format (picture is not allowed);
Abbreviations and symbols used in table should be explained in footnote;
Explanatory matter should also be placed in footnotes;
Permission for use of copyrighted materials from other sources, including re-published, adapted, modified, or partial tables from the internet, must be obtained. It is authors' responsibility to acquire the licenses, to follow any citation instruction requested by third-party rights holders, and cover any supplementary charges.

2.4.7 Abbreviations

Abbreviations should be defined upon first appearance in the abstract, main text, and in figure or table captions and used consistently thereafter. Non-standard abbreviations are not allowed unless they appear at least three times in the text. Commonly-used abbreviations, such as DNA, RNA, ATP, *etc*., can be used directly without definition. Abbreviations in titles and keywords should be avoided, except for the ones which are widely used.

2.4.8 Italics

General italic words like *vs*., *et al., etc*., *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

2.4.9 Units

SI Units should be used. Imperial, US customary and other units should be converted to SI units whenever possible. There is a space between the number and the unit (i.e., 23 mL). Hour, minute, second should be written as h, min, s.

2.4.10 Numbers

Numbers appearing at the beginning of sentences should be expressed in English. When there are two or more numbers in a paragraph, they should be expressed as Arabic numerals; when there is only one number in a paragraph, number < 10 should be expressed in English and number > 10 should be expressed as Arabic numerals. 12345678 should be written as 12,345,678.

2.4.11 Equations

Equations should be editable and not appear in a picture format. Authors are advised to use either the Microsoft Equation Editor or the MathType for display and inline equations.

2.5 Submission Link

Submit an article via https://oaemesas.com/login?JournalId=rdodj.