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Oligonucleotide therapeutics in sports? An antidoping perspective

Maria K. Parr[1] | Annekathrin M. Keiler[2,3]

[1]Institute of Pharmacy, Pharmaceutical and Medicinal Chemistry, Freie Universität Berlin, Berlin, Germany

[2]Institute of Doping Analysis & Sports Biochemistry, Kreischa, Germany

[3]Environmental Monitoring & Endocrinology, Faculty of Biology, Technische Universität Dresden, Dresden, Germany

Correspondence
Maria K. Parr, Institute of Pharmacy, Pharmaceutical and Medicinal Chemistry, Freie Universität Berlin, Königin-Luise-Straße 2+4, 14195 Berlin, Germany.
Email: maria.parr@fu-berlin.de

Annekathrin M. Keiler, Institute of Doping Analysis & Sports Biochemistry, Dresdner Str. 12, 01731 Kreischa, Germany.
Email: a.keiler@idas-kreischa.de

Abstract

Within the last two decades, the European Medicines Agency and the US Food and Drug Administration have approved several gene therapies. One category is oligonucleotide therapeutics, which allow for the regulation of the expression of target genes. Besides already approved therapeutics, there are several preclinical and clinical trials ongoing. The World Anti-Doping Agency prohibits the use of "nucleic acids or nucleic acid analogs that may alter genome sequences and/or alter gene expression by any mechanism" as a nonspecified method at all times. Hence, the administration of nucleic acids or analogs by athletes would cause an Anti-Doping Rule Violation. Herein, we discuss types of oligonucleotide therapeutics, their potential to be misused in sports, and considerations to sample preparation and mass spectrometric approaches with regard to antidoping analysis.

KEYWORDS
doping, gene therapy, mass spectrometry

1 | INTRODUCTION

Gene therapy generally refers to the introduction of nucleic acids in humans with the aim of either introducing healthy genes to be expressed, modulating the activity of the endogenous genes, or editing endogenous genes.

From a regulatory point of view, the European Medicines Agency (EMA) has categorized gene therapy medicinal products as a subgroup of "Advanced Therapy Medicinal Products" (ATMPs). These products are further defined as "biological products with an active substance which contains a recombinant nucleic acid with a view to regulate, repair, replace, add, or delete a genetic sequence and that will have a therapeutic, prophylactic, or diagnostic effect on the patient."[1] In contrast, vaccines are not included in this category due to their preventive effect on infectious diseases. The scope of gene therapy is the treatment of cancer or rare genetic diseases.

The first gene therapies approved by the EMA and the US Food and Drug Administration (FDA) in 2012 and 2017, respectively, were both based on the delivery of genes by adeno-associated viruses (AAV).[2,3] This vector-based introduction of foreign deoxyribonucleic acid (DNA) causes the expression of a functional protein in vivo. In contrast to this principle, research has focused on other approaches aiming at the modulation of endogenous gene expression or gene editing. The latter is based on the CRISPR/CAS9-mediated editing of the genome.

Besides, the development of so-called oligonucleotide-based therapeutics allows for a multitude of gene expression regulations. Oligonucleotides are small synthetic nucleic acids, either single- or double-stranded, and act on their targets by Watson–Crick base pairing with the complementary ribonucleic acid (RNA) or DNA sequences. Oligonucleotide therapeutics include antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), microRNA (miRNAs) mimics, small hairpin RNAs

(shRNAs), messenger RNAs (mRNAs), and small activating RNAs (saRNAs).

ASOs are short single-stranded molecules (13–30 nucleotides), which bind to a complementary RNA target. Based on their mode of action, they can be categorized into two groups, that is, gene expression inhibitors and splicing modulators.[4–6] Inhibition of the targeted genes is commonly achieved by RNase H which degrades the target RNA of the ASO-RNA hetero-duplex.[7] Pre-mRNA splicing can be altered by ASOs that either mediate exon skipping or exon inclusion.[7,8] Besides, ASOs can act indirectly on the expression of a target gene by binding to miRNAs thereby preventing miRNA binding to the target mRNA. These ASOs are called antagomiRs or miRNA inhibitors.[9]

To reduce fast digestion by endogenous nucleases, ASOs have been modified extensively. These modifications comprise backbone modifications (phosphorothioates, PS; phosphorodiamidate morpholino oligomers, PMOs), sugar modifications introducing 2-*O*′-methyl (2′-OMe), 2-*O*′-methoxy-ethyl (2′-MOE), open ring sugar derivatives, or 2′-fluoro (2′-F) moieties, nucleobase modifications introducing 5-methylcytosine (5mC) or N-acetylgalactosamine (GalNAc) conjugation enabling liver-specific delivery.[6,10–12] Furthermore, these modifications are advantageous as the immunomodulatory effects are decreased and no extra carrier is necessary for delivery.

Besides ASOs, siRNAs are the second most common type of oligonucleotide therapeutics. A siRNA is a double-stranded RNA, which is highly specific for one mRNA. Gene silencing is hereby achieved by binding one siRNA strand (guide strand) to the Argonaute 2 (Ago2) protein of the RNA-induced silencing complex (RISC). The activated RISC degrades the target mRNA, which is sequence-specifically recognized and bound by the siRNA guide strand. Ribose (2′OMe, 2′-F) and backbone modifications are also introduced for chemical stabilization as described for ASOs. Additionally, most of the siRNA therapeutics are GalNAc-conjugates targeting the liver due to a facilitated cellular uptake into hepatocytes via the asialoglycoprotein receptor.

In contrast to the siRNAs, a miRNA mimic can modulate many targets and may be used for multiple-target therapy. Together, the mechanism mediated by siRNAs and miRNA mimics is referred to as RNA interference. So far, no miRNA mimic therapeutic has been approved until now.

In addition, another possible mode of action is RNA activation (RNAa) by saRNAs. Endogenously, these double-stranded RNAs (21 nucleotides) increase gene expression reversibly by binding to promoter regions or even sustainably by epigenetic changes.[13,14] Similar to siRNA, saRNAs are bound to Ago2 forming the RNA-induced transcriptional activation complex (RITA) together with RNA helicase A and an RNA polymerase-associated protein.[15] Even if not approved yet, there is also interest in using this mechanism for therapeutic approaches with candidate therapeutics in the preclinical development stage.[16–19] Moreover, there is one saRNA drug for liver cancer treatment that is currently being investigated in a phase II clinical trial.[20]

mRNAs are single-stranded RNA molecules consisting of an open reading frame (ORF) that is flanked by a 5′ and a 3′ untranslated region (UTR). At the 5′-end, there is a modified guanine located (so-called 5′ cap) and the 3′-end is characterized by a sequence of multiple adenosines (so-called poly(A) tail). mRNA therapeutics have been described for vaccination against infectious diseases, for protein replacement, or for tumor immunotherapy.[21,22]

Additionally, only recently antibody-oligonucleotide conjugates (AOCs) came into focus. Similarly to antibody–drug conjugates, AOCs combine the selectivity of antibodies for specific target cells with the effectivity of the attached payload.[23] In the case of currently reported AOCs, this payload either consists of siRNA or ASOs. Specific aspects of AOCs were recently reviewed by Dugal-Tessier et al.[23] Due to its highly promising effects in a current clinical trial, the US FDA has granted breakthrough therapy designation for the first AOC, delpacibart etedesiran (del-desiran), in 2023. It is expected that further AOCs will be generated in the near future.

2 | ANTIDOPING REGULATIONS

Within its policies and rules, the World Anti-Doping Agency (WADA) annually publishes a list of substances and methods that are prohibited in athletes ("Prohibited List of Substances and Methods," Figure 1). Included substances are prohibited in competition (all listed subgroups) or even at all times (substances out of subgroups S0-S5 and all methods), with beta-blockers only prohibited in specified sports.[24] Since its first publication in 2004, "gene and cell doping" is classified as "non-specified methods" and prohibited at all times. These include "normal or genetically modified cells" and "nucleic acids or analogs" with the potential to alter gene expression or genome sequences. Hence, the administration of any kind of oligonucleotide therapeutic by an athlete would cause an Anti-Doping Rule Violation.

3 | APPROVED DRUGS

As of May 2024, 16 oligonucleotide-based therapeutics have been approved by the FDA and EMA; among them, 10 ASOs, five siRNAs, and one RNA-based aptamer (Table 1).[6,7] In addition, several others are currently examined in phase III trials,[25] and thus, have to be considered in the pipeline for approval.

Among the therapeutical drugs, the majority is targeting the liver (mipomersen, patisiran, inotersen, givosiran, volanesorsen, lumasiran, inclisiran, and vuttrisiran). The muscle is the target tissue of four out of the 16 approved oligonucleotide-based therapeutics, two therapeutics target the eye (formivirsen and pegaptanib), and two the central nervous system (nusinersen and tofersen).

4 | POTENTIALLY DOPING-RELEVANT THERAPEUTICAL TARGETS

Regarding a potential misuse by athletes, targets related to performance enhancement or accelerated recovery might be of potential interest. The annually published antidoping testing figures show that

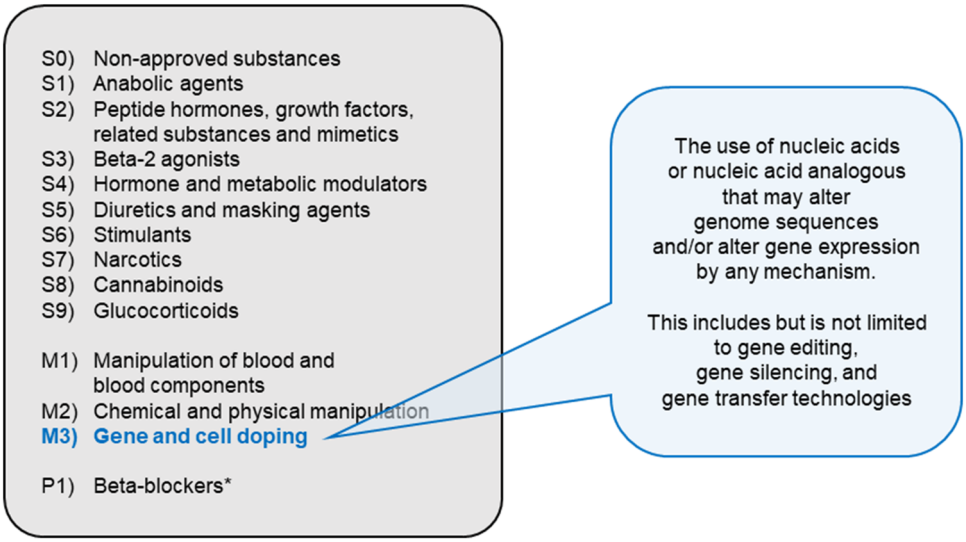

FIGURE 1 Classes of prohibited substances and methods in sports as per the 2024 list of the World Anti-Doping Agency (WADA),[24] *beta-blockers are prohibited in specified sports only.

not only approved pharmaceuticals are misused for performance enhancement.[26] Hence, we herein include approved oligonucleotide therapeutics as well as nonapproved ones which have been only tested in preclinical models so far with effects that might be reasonable targets for sports doping.

First, targeting the musculoskeletal system by inhibiting muscle protein degradation or enhancing myoblast proliferation is conceivable. Muscular dystrophy (MD) comprises genetic diseases causing weakness and a progressive loss of muscle mass. Among all known muscular dystrophies, Duchenne muscular dystrophy (DMD) has the highest, and Becker muscular dystrophy (BMD) has the second highest prevalence worldwide.[27] The responsible mutations are in the X chromosome-linked dystrophin gene (DMD) encoding a protein that is crucial for muscular strength and stability of muscle fibers.[28] Besides pharmaco-therapeutic approaches such as anti-inflammatory or antioxidative treatments or approaches to normalize intracellular Ca^{2+} levels,[29] antisense and gene therapeutic approaches have become of interest for research and development.[30–34] The first study investigating the potential of an antisense-based approach to restore functional dystrophin expression in an MD mouse model was published in 2001.[35] By now, there are five oligonucleotide therapeutics approved for the treatment of DMD (Exondys 51, Vyondys 53, Waylivra, Viltepso, Amondys) and one for spinal muscular dystrophy (Spinraza), all six being ASOs (Table 1). Regarding a potential misuse for sports performance enhancement, an administration of the currently approved oligonucleotide therapeutics for rare diseases seems unlikely due to the fact that they are only effective in persons bearing the mutations on the dystrophin gene.

However, there are other musculoskeletal targets that might be of interest. The most studied one among them is myostatin, a myokine that negatively regulates muscle growth. By inhibiting myostatin gene expression by siRNA, an increase in muscle fiber size and mass has been shown in mice and fish.[36–38] Besides, the usage of an octa-guanidine morpholino oligomer also induced increased skeletal muscle mass in normal and cachexic mice.[39] Only recently, an ASO targeting a splicing enhancer in exon 1 of the MSTN gene has been described as a newly identified myostatin inhibitor.[40] Although only preclinical studies in rodents or fish have been performed, there is potential for oligonucleotide-based approaches to increase muscle mass by targeting myostatin. Moreover, myostatin expression can be inhibited by targeting regulating miRNA, as it was shown that an intramuscular injection of a miR-27a-expressing plasmid in mice significantly increased the myofiber cross-sectional area.[41]

In general, elements of the myostatin signaling pathway are also possible targets for an oligonucleotide-based therapeutic approach with the objective of increasing muscle mass. Among those mediators, the transcription factor FoxO-1 is crucial for the regulation of skeletal muscle differentiation, and its inhibition by an ASO in mice has been linked to increased muscle mass.[42] Moreover, the myostatin-binding protein follistatin (Fst) is of interest as it blocks the myostatin signaling pathway, thereby increasing skeletal muscle mass as well. Based on this, myostatin inhibitors including Fst are prohibited in sports under category S4 (Figure 1). However, besides the administration of recombinant Fst, Fst gene therapy to increase the in vivo expression of the protein is possible. While an increase in skeletal muscle mass in response to AAV-mediated delivery of the Fst gene was shown in preclinical studies[43–47] and clinical trials on muscular dystrophy patients,[33,34] there is no drug approved for Fst gene therapy until now.

The protein α-klotho (klotho) in its transmembrane form is a co-receptor for different fibroblast growth factor receptors (FGFRs), and its cleaved soluble form a proteohormone with various functions.[48,49] Since its first description, it has been named the 'longevity gene' due to the extended life span of mice with over-expressed klotho genes.[50] Besides, a function of klotho regarding muscles has been shown as well, as klotho loss has been linked to

TABLE 1 Overview of oligonucleotide-based therapeutics approved by the European Medicines Agency and the Food and Drug Administration.

Compound	Drug name	Indication	Target tissue	Sequence	Approval year
	Antisense oligonucleotides (ASOs)				
Fomivirsen	Vitravene	Cytomegalovirus retinitis	Eye	5′-**G**ʃ**C**ʃ**G**ʃ **T**ʃ**T**ʃ**T**ʃ **G**ʃ**C**ʃ**T**ʃ **C**ʃ**T**ʃ**T**ʃ **C**ʃ**T**ʃ**T**ʃ **C**ʃ**T**ʃ**T**ʃ **G**ʃ**C**ʃ**G**-3′	1998
Mipomersen	Kynamro	homozygous familial hypercholesterolemia	Liver	5′-**G**ʃ**C***ʃ**C***ʃ **T**ʃ**C***ʃdAʃ dGʃdTʃdC*ʃ dTʃdGʃdC*ʃ dTʃdTʃdC*ʃ **G**ʃ**C***ʃ**A**ʃ **C***ʃ**C***-3′	2013
Eteplirsen	Exondys 51	Duchenne Muscle Dystrophy	Muscle	PMO—5′-CTC CAA CAT CAA GGA AGA TGG CAT TTC TAG-3′	2016
Nusinersen	Spinraza	Spinal Muscular Atrophy	CNS	5′-**T***ʃ**C***ʃ**A** ʃ**C***ʃ**T**ʃ**T** ʃ**T**ʃ**C***ʃ**A** ʃ**T**ʃ**A**ʃ**A** ʃ**T**ʃ**G**ʃ**C*** ʃ**T**ʃ**G**ʃ**G**-3′	2016
Inotersen	Tegsedi	Hereditary Transthyretin Amyloidosis	Liver	5′-**TC*T** TGdG dTdTdA dC*dAdT dGdAdA **ATC*** **C*C***-3′	2018
Golodirsen	Vyondys 53	Duchenne Muscle Dystrophy	Muscle	PMO—5′-GTT GCC TCC GGT TCT GAA GGT GTT C-3′	2019
Volanesorsen	Waylivra	Duchenne Muscle Dystrophy	Liver	5′-**T**ʃ**A**ʃ**T**ʃ ʃ**T**ʃ**T**ʃdC*ʃ dGʃdAʃdC*ʃd C*ʃdTʃdGʃ dTʃdTʃdC*ʃ **T**ʃ**T**ʃ**C***ʃ **G**ʃ**A**-3′	2019
Viltolarsen	Viltepso	Duchenne Muscle Dystrophy	Muscle	PMO—5′-CCT CCG GTT CTG AAG GTG TTC-3′	2020
Casimersen	Amondys	Duchenne Muscle Dystrophy	Muscle	PMO—5′-CAA TGC CAT CCT GGA GTT CCT G-3′	2021
Tofersen	Qalsody	Amyotrophic Lateral Sclerosis	CNS	5′-**C***ʃ**AG**ʃ **GA**ʃdT ʃdAʃdC*ʃdA ʃdTʃdTʃdT ʃdC*ʃdTʃdA ʃ**C*A**ʃ**G C***ʃ**U***-3′	2023
	Small interfering RNAs (siRNAs)				
Patisiran	Onpattro	Hereditary Transthyretin Amyloidosis	Liver	Passenger 5′-GmUA AmCmC AAG AGmU AmUmU mCmCA mUdTdT-3′ Guide 5′-AUG GAA mUAC UCU UGG UmUA CdTdT-3′	2018
Givosiran	Givlaari	Acute Hepatic Porphyria	Liver	Passenger 5′-mCʃmAʃmG mAmAmA fGmAfG mUfGmU fCmUmC mAmUmC mUʃmUʃmA-3′ triantennary GalNAc Guide 5′-mUʃfAʃfA fGmAfU mGfAmG fAmCfA mCfUmC fUmUfU mCfUmG mGmU-3′	2019
Lumasiran	Oxlumo	Primary Hyperoxaluria Type I	Liver	Passenger 5′-mGʃmAʃmC mUmUmU fCmAfU fCfCmU mGmGmA mAmAmU mAmUmA-3′ triantennary GalnAc Guide 5′-mUʃfAʃmU mAmUfU mUfCfC mAmGmG mAfUmG fAmAmA mGmUmC ʃmCʃmA-3′	2020
Inclisiran	Leqvio	Heterozygous Familial Hypercholesterolemia/Clinical Atherosclerotic Vascular Disease	Liver	Passenger 5′-mCʃmUʃmA mGmAmC fCmUfG mUdTmU mUmGmC mUmUmU mUmGGmU-3′ triantennary GalnAc Guide	2020

TABLE 1 (Continued)

Compound	Drug name	Indication	Target tissue	Sequence	Approval year
Vutrisiran	Amvuttra	Hereditary Transthyretin Amyloidosis	Liver	5′-mA⌈fC⌈mA fAfAfA mGfCmA fAmCfA mGfGmU fCmUmA mG⌈mA⌈mA-3′ Passenger 5′-mU⌈mG⌈mG mGmAmU fUmUfC fAfUmG mUmAmA mCmCmA mAmGmA-3′ triantennary GalnAc Guide 5′-mU⌈fC⌈mU mUmGfG mUmUfA mCmAmU mGfAmA fAmUmC mCmCmA⌈ mU⌈mC-3′	2022
	RNA-based aptamer				
Pegaptanib	Macugen	Neovascular Age-Related Macular Degeneration	Eye	PEG—5′-fCmGmG AAfU fCmAmG fUmGmA mAfUmG fCfUmA fUmAfC mAUC CmG-3′-3′-dT	2004

Abbreviations: *, 5′-methyl-base; ⌈, phosphorothioate modification; bold bases, 2′-O-(2-methoxyethyl)-modified bases; d, deoxyribonucleotide; f, 2′-fluor; m, 2′-O-methyl modification; PEG, polyethylene glycol; PMO, phosphorodiamidate morpholino oligomer backbone.

sarcopenia in mice and humans.[51,52] In contrast, the overexpression of klotho, either in a transgenic mouse strain or by AAV-delivery, increased the performance in mice and increased muscle strength,[53,54] which is based on klotho's impact on muscle regeneration and muscle stem cell function.[55,56] Moreover, higher klotho levels in middle-aged and senior humans have been positively correlated with muscle strength and negatively with frailty.[57] Based on these observations, klotho is an interesting therapeutic target,[58] although no approaches have been described in humans so far.

Further therapeutic targets with potential relevance for sports doping are growth hormone (GH) and insulin-like growth factor 1 (IGF-1). The option to increase GH levels was shown in mice using a nonviral, nonintegrating liposome-based vector system, for which low toxicity as a result of the injection and long-term therapeutic GH serum levels have been described after one to two single injections.[59] There are no data on potential human trials available by now. Aside from delivering the *Igf-1* gene, IGF-1 signaling can also be enhanced by targeting IGF-binding proteins (IGFBP). Only recently, Yavas et al. showed the possibility of enhancing IGF-1 signaling by ASO-mediated downregulation of IGFBP-1 and IGFBP-3 in C2C12 cells.[60] In contrast to their in vitro results, no exon skipping by the ASOs tested in the C2C12 cells could be proven in a mouse model by Yavas and colleagues. The authors explain this discrepancy by the poor delivery of the ASOs to the target tissues.[60] In addition, the possible use of IGF-1 mRNA locally injected into the skeletal muscle might also be an option for performance enhancement. A recent study showed the regenerative potential of an IGF-1 mRNA carrying optimized signal peptides in a mouse model for muscle injury and in a rabbit model for spinal disc herniation.[61]

Targets relevant for performance enhancement also include those involved in an increase in stamina, hemoglobin mass, or maximal oxygen transfer. One therapeutic drug based on AAV-mediated delivery of the erythropoietin gene is Repoxygen® which had been only tested in preclinical animal models and whose further development had been discontinued in 2003.[62] Anyway, it has already been reported in connection to a court case related to doping in sports in 2006. Moreover, there is an active patent on different ASOs for the treatment of EPO-related diseases.[63] However, no clinical trial has been started until now. Besides, the administration of EPO mRNA is also conceivable based on studies performed in mice,[64–66] rats,[67] pigs,[65] and monkeys,[67] causing an increase in the EPO protein and the according blood cell counts. In addition, the overexpression of an exogenously administered EPO gene may result in relevant performance enhancement.

The recent detection of transgenic EPO DNA in two black market products labeled as EPO gene and IGF-1 gene-containing materials for potential gene doping in sports points to the availability of nonapproved gene therapeutic material.[68] Hence, the approaches described above might be available and potentially misused in sports already.

In general, the application of nonapproved products represents a tremendous health risk for users. As access to oligonucleotide-therapeutics might be limited due to high costs or for lack of approval

as a therapeutic, the black market might be a potential source. However, preparations available on the black market might be labeled incorrectly, contaminated, or impure as it has been described for illicit products used for performance enhancement containing, for instance, anabolic androgenic steroids or growth hormone-releasing peptides.[69–74]

5 | ANALYTICAL PERSPECTIVES

5.1 | General analytical considerations

Oligonucleotide drugs represent a class of large molecule drugs that are generally characterized by large structural variability and their related complexity. The limited stability of oligonucleotide drugs does not only raise challenges for the shelf-life stability of the drug itself but also particularly with respect to limited stability during body passage. Thus, the majority of oligonucleotide therapeutics contain modifications, such as noncanonical nucleosides, seconucleosides (i.e., derivatives of nucleosides with open ring structure, often cleaved between C2′ and C3′), locked nucleic acids, a phosphothioate backbone, 2′-modifications, GalNac conjugation, or end-capping with alkylic chains or 5′-methylenephosphonates, and so forth.[75–81] However, it has to be noted that a large number of inserted modifications may also affect the efficacy and safety of the drugs.[82,83]

While small molecule drugs are generally applied as chemically strictly defined molecules, the structures of large molecule drugs are generally much more diverse. Thus, the analysis of oligonucleotide drugs results in challenges similar to those observed with other large molecule drugs such as peptides and proteins. From the perspective of an anti-doping-related applicability, methods using mass spectrometry in combination with separation methods are considered as most relevant techniques for their detection in athletes' samples. High-resolution mass spectrometry (HRMS) combined with (ultra) high-performance liquid chromatography (UHPLC) is currently seen as the most promising analytical approach for oligonucleotides. Similar to protein mass spectrometry, especially electrospray ionization (ESI), results in the generation of multiply charged ions for oligonucleotides. The observed charge envelope is exemplified for a sgRNA in Figure 2. Due to their properties, a negative ionization mode is generally applied. It was reported that analysis gets more and more challenging with the size of the oligonucleotides.[84] As sgRNAs used in CRISPR-Cas9 gene editing may easily comprise 100 nucleotides, their analysis requires even more sophisticated approaches.[84] Thus, for the identification of oligonucleotides, approaches similar to protein analysis are applied. Different strategies such as intact oligonucleotide analysis (top-down strategy), oligomer analysis after restricted hydrolysis (middle-down strategy), and oligo mapping analysis (bottom-up strategy) have been reported. Furthermore, full cleavage and subsequent analysis of monomeric nucleotides or nucleobases may complement the analytical portfolio. The different approaches are visualized in Figure 3. Especially relevant for small oligomers, tandem mass spectrometry (MS/MS) may be used for further increase in analytical selectivity or sequence identification based on fragmentation analysis. The most commonly observed fragmentations occur at the bonds of the phosphate-sugar backbone. Similar to fragmentations of peptides, they are named as “a” to “d” ions from the 5′ fragmentation products, and “w” to “z” for the 3′ fragmentation products as illustrated in Figure 4.

Hydrolysis is mainly carried out by RNA digesting enzymes, whereas RNases with varying specificities are considered depending on the analytical target profile. A selection of different RNases and their selectivities are presented in Table 2.

RNase T1 cleaves single-stranded RNA backbones after the 3′-phosphate of G residues, which is reported to frequently yield relatively short oligomers. This digestion is especially interesting for the analysis of polyA-tails.[86] An enzymatic digestion using a combination of RNase T1, RNase A, and snake venom phosphodiesterase I digested double- as well as single-stranded RNA and DNA to nucleosides.[92] Similarly, chemical digestion with hydrofluoric acid (HF) leads to full digestion of RNA, resulting in pyrimidine nucleosides and purine nucleobases.[93] In this approach, peptides remain unaffected and can be easily separated before analysis.

Aiming to achieve high sequence coverage, RNase 4 was reported to result in an optimum number of digestion products with 4–40 nucleotides as well as unique monoisotopic masses and fragmentation patterns that allow for proper assignment within an oligonucleotide. This was found to be especially important for uridine-depleted or uridine-modified oligonucleotides, as this procedure reportedly also cleaves at nucleobase-modified uridine sites while modifications at the ribose moiety inhibited the cleavage.[90] Among others, the authors used this digestion for the identification of

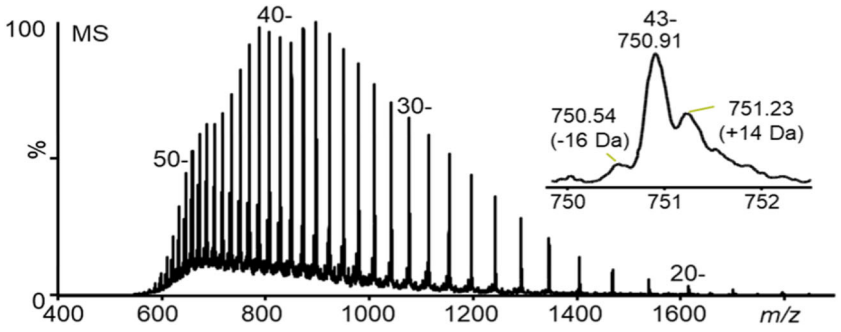

FIGURE 2 Mass spectrum of sgRNA showing the charge envelope resulting from the various obtained ionization states; the insert illustrates the zoom-in spectrum of charge state 43- showing modified species, copied from Crittenden et al.[84] with permission from ACS Publications.

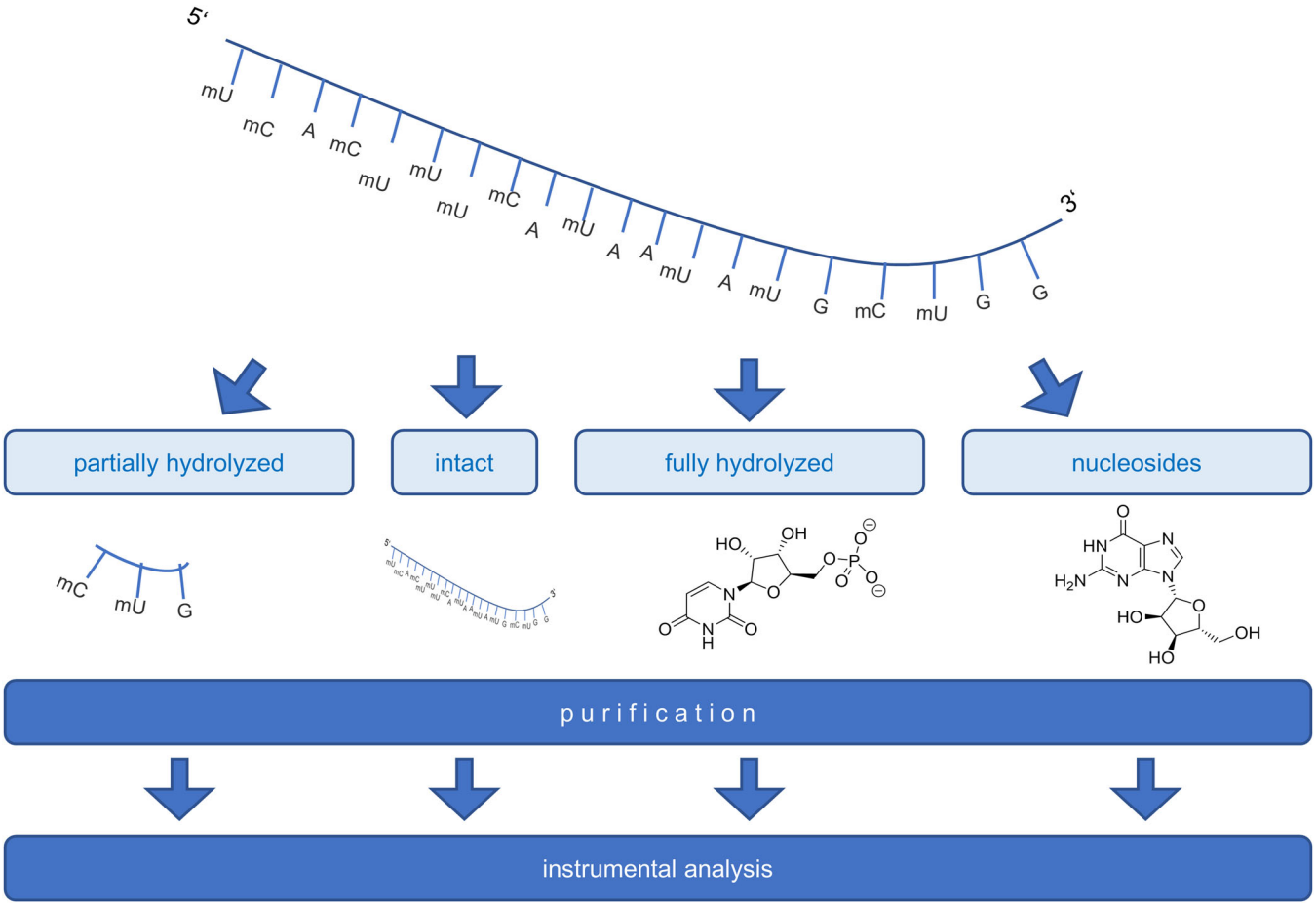

FIGURE 3 Different strategies for oligonucleotide analysis involving mass spectrometric detection, exemplified using the oligonucleotide sequence of nusinersen.

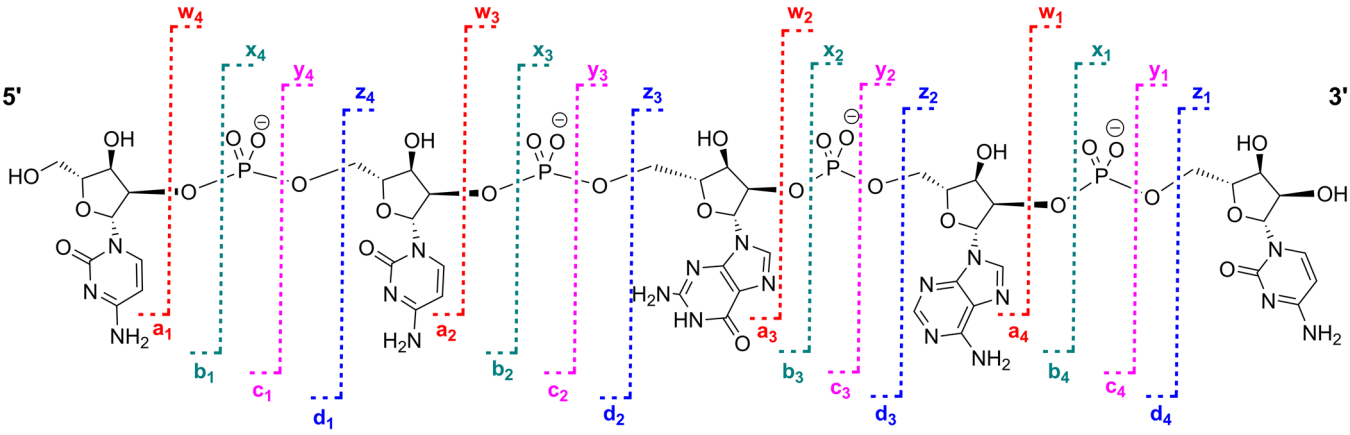

FIGURE 4 Tandem-MS fragmentation of oligonucleotides illustrating the conventionally used letter code for MS/MS fragment assignments.

capped and uncapped erythropoietin (EPO) mRNA. Contrary to RNase 4, the authors found MC1 to be capable of cleaving at Um and m5Um sites, while the presence of 1-methylpseudouridine resulted in noncleavage in this case. For complex samples such as blood, urine, or tissue, further enzymes may contribute to the prepurification of the sample, for example, proteases or DNases to eliminate non-RNA components from the matrix.

When analyzing (oligo)nucleotide drugs, their high polarity and potential to chelate metal ions need to be considered as both may result in significant challenges especially when opting for liquid

TABLE 2 Enzymes used in RNA digestion.

Enzyme	Cleavage site	References
RNase A	3′ end of Up ↓ N or Cp ↓ N	[85]
RNase T1	3′-end of Gp ↓ N	[86]
RNase MC1	5′ end of Up ↓ N, however not in –GU-	[87]
Cusativin	3′-end of Cp ↓ N (except for Cp-C), additionally cleaves Up ↓ A and Ap ↓ U bonds (lower efficiency).	[87]
RNase I	all dinucleotide pairs (no base preference)	
mRNA interferase MazF	5′-end of ACA sequence resulting in 2′,3′-cyclic phosphate at one side and a free 5′-OH group at the other. 2′-OH group of the residue preceding the recognized sequence is thereby absolutely essential	[88]
RNase U2	3′ end of Ap ↓ N or Gp ↓ N	[89]
hRNase 4	3′ end of Up ↓ N	[90]
Colicin E5	GU-specific	[91]

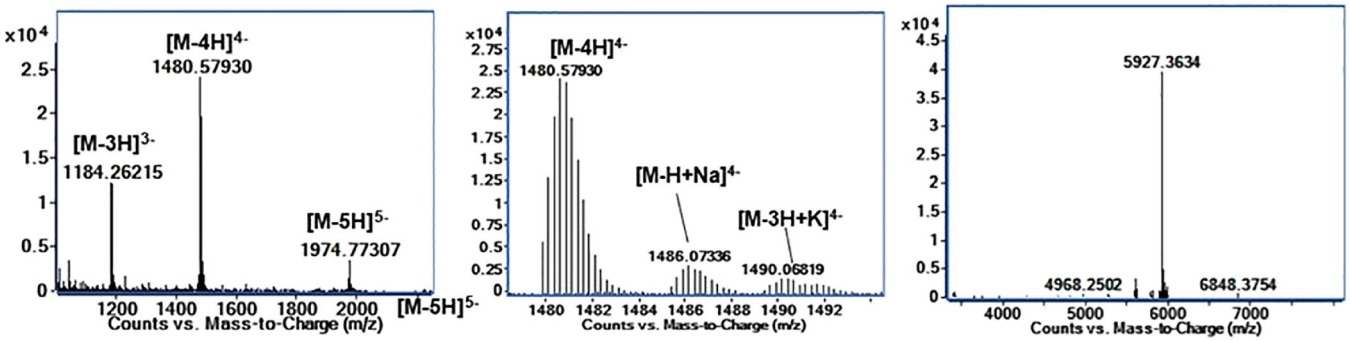

FIGURE 5 Mass spectrum (HRMS, full scan) of 18-meric RNA (all PS, 5′ U⌈C⌈A⌈C⌈U⌈U⌈U⌈C⌈A⌈U⌈A⌈A⌈U⌈G⌈C⌈U⌈G⌈G 3′; ⌈: phosphorothioate modification) showing various ionization states (left), adduct formation (center), and the resulting deconvoluted mass (right), copied from Vosahlova et al.[97] with permission from Elsevier.

chromatography–mass spectrometry (LC-MS) analysis.[94–96] Various metal ion adducts may be observed in the spectra as exemplified in Figure 5, due to the chelating effects of oligonucleotides. Thus, special emphasis should be given to proper sample preparation.[97] Furthermore, interactions of oligonucleotides with metal ions may result in chromatographic challenges.

Possibilities to overcome the bad peak shape of chelating agents, such as nucleotides, are either metal-free instruments including metal-free columns,[98,99] or deactivation of the instrumentation.[96] The latter can be achieved by utilization of neutral or high pH mobile phases, the addition of metal complexing agents such as citrate, acetylacetone, or medronic acid to the mobile phase, ideally in combination with a pre-analytical passivation of the system by overnight reaction using phosphoric acid.[96,98,100,101] As suggested by Simeone et al., a system suitability test using adenosine 5′-(α,β-methylene)diphosphate as test substrate may be used to probe for system inertness.[95]

In 2022, Fekete et al.[102] reviewed literature discussing a variety of chromatographic techniques for mRNA pharmaceuticals. They presented details of utilizing anion exchange chromatography (AEX), reversed phase chromatography using an ion-pairing reagent (IP-RP-LC), or hydrophilic interaction chromatography (HILIC). Electrophoresis and other techniques were only briefly mentioned by the authors,[102] as the application focus of their review was pharmaceutical analysis mainly for quality assurance. From the perspective of doping control analysis, it has to be noted that MS detection is challenging for AEX and even ion-pairing reagents in the system will pose challenges for direct coupling with MS detection.[103,104] Thus, Li et al. successfully applied ion-pair free RP-LC-MS/MS for the separation of patisiran and its potential truncated impurities utilizing a terephthalate-bonded silica-based stationary phase.[105]

Besides LC-based methods, capillary electrophoresis has also been reported for the separation of oligonucleotide therapeutics. A recent review by Wei et al. summarizes aspects related to capillary gel electrophoresis and capillary zone electrophoresis including their coupling to mass spectrometric detection for intact and hydrolyzed analytes. Oligonucleotides of up to 5000 nucleotides (such as mRNA-based

COVID-19 vaccines) were successfully determined thereby.[106] For the analysis of monomers (nucleobases, nucleosides, and nucleotides) as well as for small oligomers (i.e., tetramers), chromatography using supercritical carbon dioxide (supercritical fluid chromatography, SFC) was also reported.[107–109] As stationary phases in SFC, amide, silica, (ethyl-)pyridine, picolylamine, or diol-based stationary phases were used, mainly in combination with methanol or ethanol as a modifier, if used in combination with MS detection. Water and ammonium acetate or ammonium formate have been reported as potential additives. Another interesting approach for the determination of nucleosides involves chemical derivatization for increased sensitivity in LC-MS analysis. This technique is especially used in the detection of nucleoside modifications[110,111] using Girard T or P reagent, 4-(dimethylamino) benzoic anhydride, dansylhydrazine, or 8-(diazomethyl)quinoline. Cis-diols are converted to their respective ketals using acetone or other ketones. After *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatization, 5-methylcytosine was even amenable to gas chromatography–mass spectrometry analysis.[112] Finally, it has to be noted that Tozaki et al.[113] used MALDI-TOF mass spectrometry for the identification of a phosphothioated model oligonucleotide in equine blood. Software-assisted identification of the potential target gene of the oligonucleotide was performed demonstrating the suitability of this approach also for nontargeted analysis.

5.2 | Bioanalytical methods

For bioanalytical investigations, Vosahlova[97] successfully established an ion-pair-free HILIC-MS method using an Aquity Premier BEH Amide column and water/acetonitrile with ammonium acetate to support ionization thereby allowing for lower thresholds of detection. Accepting the drawbacks of ion-pairing reagents, Studzinska et al.[114] achieved the separation of nusinersen and several metabolites on a C18 column using N,N-dimethylbutylamine (DMBA) or N,N-diisopropylethylamine (DMEA) for ion pairing. Similarly, the determination of mipomersen in rat plasma was achieved on a C18 column using triethylamine (TEA) and hexafluoropropan-2-ol (HFIP) as mobile phase additives.[101] Comparing HILIC-, IP-RP-LC, and ion-pair reagent-free RP-LC, only very recently Studzinska et al. reported the superiority of IP-RP-LC-MS for the analysis of nusinersen and its metabolites in serum samples. They found a higher number of metabolites as well as higher signal intensities when using IP-RP-LC-MS with DMBA and HFIP in water and methanol.[115] The same ion-pairing reagents are also mentioned in the review of Fekete et al.[102] For improved separation of up to 50-mer oligonucleotides, Donegan et al. used hydrophobic alkylamines in combination with acetic acid or HFIP. When HFIP was used instead of acetic acid 2–3-fold higher signal intensities were observed with the highest peak capacities with octylamine and dihexylamine.[116] Furthermore, LC-MS/MS analysis of fomivirsen, a 21-mer antisense oligonucleotide, was conducted for blood samples collected by applying volumetric adsorptive microsampling (VAMS).[117] The authors achieved a quantitative recovery that was independent of the samples' hematocrit with an optimized impact-assisted extraction using an aqueous solution containing the nonionic surfactant Nonidet P40. Finally, IP-RP-LC-MS/MS analysis was performed after hybridization extraction, which included proteinase K digestion and purification using Streptavidin C1 magnetic beads. The data reported therein may open the perspective of also using dried blood spots (DBS) as specimens for oligonucleotide detection. Similarly, Leuenberger et al.[118] mentioned DBS for RNA analysis, however, discussed them as biomarkers for substance misuse rather than as drugs themselves. Paßreiter et al. showed recently the detection of sgRNA in mouse serum samples using Specific High Sensitive Enzymatic Reporter Unlocking (SHERLOCK) with a polyacrylamide gel electrophoresis-based analysis of the previously reverse-transcribed and amplified RNA.[119]

Only few methods are reported for targeting oligonucleotide therapeutics in urine samples. The analysis of rat urine samples after treatment with two potential myostatin-inhibiting siRNAs was reported by Thomas et al.[120] These nonapproved 21-mers contained several modifications in their sequences. Their detection as intact and hydrolyzed compounds was performed using LC-MS partially involving previous separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and in-gel hydrolysis. For oligonucleotides, LC-MS analysis was performed with ammonium acetate and acetonitrile as mobile phase without ion-pairing reagents on a C18 column, while fully hydrolyzed samples were separated on a phenyl-hexyl column. They reported urine as a suitable specimen while considering blood samples as unpromising.

5.3 | Sample preparation

General aspects of sample preparation methods for oligonucleotides are reviewed by Nuckowski et al.[121] Putting special emphasis on biological specimens as the matrix, further careful considerations regarding sample pretreatment are needed in addition. As oligonucleotides are highly prone to protein association, the sample pretreatment needs to allow for the disconnection of these aggregates. Fast and easy options may be simple protein precipitation. However, due to low recoveries and limited efficiency for purification from other matrix compounds, it is only rarely applied as a single sample pretreatment.[121] In contrast, the dilution of samples with an aqueous solution of a nonionic detergent (Nonidet P-40) resulted in high recoveries with surprisingly low matrix interferences.[122] As the authors used UVdetection after either HPLC or capillary electrophoresis separation, they were not affected by interferences with the added detergent. In contrast, signal suppression in mass spectrometric detection is expected.[123] Thus, the value of this sample pretreatment option seems reduced for use in that case and an effective detergent removal may therefore be required. This was successfully performed by ethyl acetate extraction in the case of Nonidet.[124] Alternatively, the liberation of the oligonucleotides from their protein binding may be achieved by digestion of the sample with proteinase K.[121] However, this procedure is controversially evaluated and

appears to require a combination with further purification procedures to effectively remove interfering matrix components.[121] To achieve this, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are widely used in bioanalysis. Due to the high polarity of oligonucleotides, LLE is most frequently used to eliminate matrix compounds, rather than extracting the oligonucleotides into the organic layer.[125,126] However, the use of phenol or a mixture of guanidinium thiocyanate with phenol as additives enables LLE as introduced for the extraction of RNA from cells and/or tissues.[122,127,128] These reagents are valued for their parallel protein precipitation, allowing for additional clean-up of the samples. In addition, they are also reported for the successful extraction of RNAs from lipid nanoparticles, which represent a frequently applied formulation for oligonucleotides.[126] Especially if guanidine thiocyanate is added, efficient denaturation of the oligonucleotides is observed due to its chaotropic properties.[129] Frequently, the phenol extractions are used in combination with subsequent precipitation of the RNA by ethanol addition for further purification, which, however, reportedly results in low recoveries.[130] Additionally, the involved chemicals, especially phenol and chloroform, are classified as harmful and are therefore desirably avoided.[126] Thus, SPE is more frequently applied using either polymeric Oasis HLB sorbents or weak anion exchanger (WAX) materials.[121] The latter are also used for native RNA purification using a fast chromatographic procedure.[131]

In addition, the limited stability of oligonucleotide drugs, which does not only raise challenges for the shelf-life stability and stability during sample pretreatment of the drug itself, may also result in rapid decay during body passage, thus reducing detectability if parent compounds are considered as analytical targets. As one example of incorporating metabolites for bioanalysis, Studzinska et al. reported the inclusion of metabolites of nusinersen as target analytes besides the parent drug.[114] In their investigation, most of the metabolites were truncated at both ends (e.g., assigned to 5′N-4+3′N-11, 5′N-10+3′N-5, 5′N-11+3′N-4, and 5′N-15+3′N-1 for the most abundant signals in the reported serum sample chromatogram). Minor amounts of metabolites only truncated at either 5′N or 3′N (postulated as 5′N-16 and 3′N-14) were also detectable. Similarly, only shortmers (tri- or tetramers) were detected in serum after nusinersen administration as reported in Vosahlova et al.[97] Applying HILIC-MS they found the separation of the mixture of nusinersen metabolites challenging, mainly due to their similarities. IP-RP-LC, challenging in separation as well, showed the additional disadvantage of including ion-pairing reagents. No intact drug was found in the extract of the serum samples collected in the above-mentioned investigations, which they report to contrast the data provided by the drug manufacturer.[114] In their documents,[132] low plasma concentrations of the intact drug are reported with medium plasma T_{max} ranging from 1.7 to 6.0 h with renal elimination as the primary clearance route. However, at 24 h, only 0.5% of the dose was recovered in the urine as parent drug plus metabolites after intrathecal administration. This may reflect challenging detection in doping control samples, where urine represents the majority of samples collected at present.

6 | CONCLUSION

In conclusion, the interest in oligonucleotide therapeutic methods has increased during the last two decades resulting in the approval of 16 drugs by now. With the ongoing research on potential new therapeutics, the availability of oligonucleotide-based or in general gene therapeutic material for misuse by athletes is conceivable. Hence, there is a need for analytical approaches for the detection of oligonucleotide administration. Besides methods based on recombinase polymerase amplification or polymerase chain reaction for the detection of exogenous nucleic acids, mass spectrometric methods with or without prior digestion can also be applied. Modified nucleic acids may be used as analytical targets for effective initial screening procedures, at least for the majority of oligonucleotides that bear these modifications. Hydrolysis of the monomeric nucleotides will help reduce analytical complexity for these types of analyses. In the case of the detection of these modified nucleotides, non-preselected oligonucleotides may also be traced back. However, confirmation procedures will later also require testing at the oligomer level. Investigations on ideal setups for oligonucleotide drug analysis in doping control need to be addressed in the near future.

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CONFLICTS OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Maria K. Parr http://orcid.org/0000-0001-7407-8300
Annekathrin M. Keiler http://orcid.org/0000-0002-2157-4711

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