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Gene Suppression Therapy in Hereditary Cerebellar Ataxias

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Gene Suppression Therapy in Hereditary Cerebellar Ataxias

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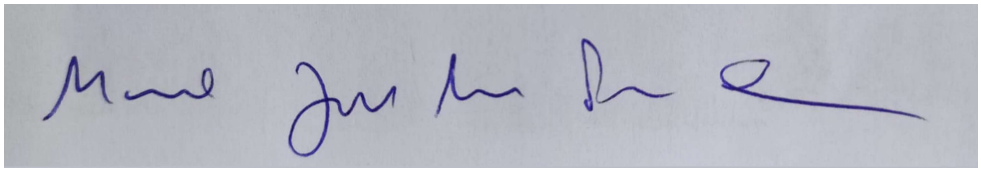

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ABSTRACT

BACKGROUND: Hereditary Cerebellar Ataxias (HCA) are a heterogenous group of neurodegenerative diseases associated with severe disability. Treatment options, in most HCA's subtypes, are still limited to symptomatic approaches leading to a poorer prognosis. During the last decades, there has been extensive research on gene suppression therapies (GST) that present a new hope as disease-modifying treatments of a variety of disorders, including HCAs.

OBJECTIVE: To perform a systematic review on *in vivo* studies investigating the efficacy and safety profile of GST in HCAs.

METHODS: A structured PubMed® search on GST in HCAs from January 1993 up to October 2020 was performed. Inclusion and exclusion criteria were defined, and the selection process was done accordingly. The screening process was carried out, independently, by two authors, initially based on title and abstract reading, followed by a full-text reading. Risk of bias assessment was conducted following SYRCLE's risk of bias tool. A data extraction sheet was created to extract relevant information from each article selected.

RESULTS: The initial search yielded 262 papers, of which 238 were excluded. An additional article was obtained from reference scrutiny, resulting in a total of 25 articles for final analysis. Most studies were not clear on the used tools to assess bias. In MJD/SCA3, SCA7, SCA1 and SCA2, iRNA and ASO therapy proved to be well tolerated and effective in suppressing mutant protein levels, improving neuropathological features and motor phenotypes. In SCA6, disease phenotypes were improved, but no investigation on adverse effects was performed. In FRDA, only suppression efficacy through electroporation of CRISPR-Cas9 system was tested and confirmed.

CONCLUSION: The literature reviewed suggests that GSTs are well tolerated and effective in suppressing the targeted genes, improving neuropathological features and motor behavior phenotype *in vivo.* Nonetheless, there is no guarantee that these results are free of bias. Moreover, further investigation is still needed to clarify GSTs effect in each HCA, particularly, FRDA, SCA6 and SCA2.

RESUMO

INTRODUÇÃO: As Ataxias Cerebelosas Hereditárias (HCA) são um grupo heterogéneo de doenças neurodegenerativas associadas a incapacidade severa. As opções de tratamento, na maioria dos subtipos de HCA, são ainda limitadas a abordagens sintomáticas que não alteram o prognóstico natural da doença. Durante as últimas décadas, tem havido extensa investigação em terapias de supressão genética (GST). Estas representam uma nova esperança como tratamento modificador da evolução de uma variedade de doenças, incluindo HCAs.

OBJETIVO: Realizar uma revisão sistemática de estudos *in vivo* sobre a eficácia e segurança das GST em HCAs.

METODOLOGIA: Foi efetuada uma pesquisa estruturada na PubMed® sobre GST em HCAs, no período de janeiro de 1993 a outubro de 2020. Os critérios de inclusão e exclusão foram previamente definidos e o processo de seleção feito em conformidade. A seleção foi realizada, de forma independente, por dois autores, inicialmente a partir da leitura do título e resumo, seguido da leitura integral do artigo. Foi criada uma folha de extração de dados, para introdução da informação relevante de cada artigo. A avaliação de risco de viés foi conduzida de acordo com a ferramenta SYRCLE.

RESULTADOS: Da pesquisa inicial resultaram 262 artigos, dos quais 238 foram excluídos. Um artigo adicional foi obtido pela análise de referências, resultando num total de 25 artigos para análise final. A maior parte dos estudos não detalharam as ferramentas utilizadas para avaliar o viés. Na MJD/SCA3, SCA7, SCA1 e SCA2, a terapêutica com iRNA e ASO revelou ser bem tolerada e eficaz na supressão dos níveis de proteína mutante, bem como na melhoria dos fenótipos neuropatológico e motor. Na SCA6, a terapêutica com iRNA mostrou melhorar o fenótipo da doença, no entanto, os efeitos adversos associados não foram estudados. Na FRDA, apenas a eficácia de supressão por eletroporação do sistema CRISPR-Cas9 foi testada e confirmada.

CONCLUSÕES: A literatura revista sugere que as GSTs são bem toleradas e eficazes *in vivo* na supressão dos genes alvo e na melhoria dos fenótipos neuropatológico e motor das HCAs. No entanto, não há garantia de que estes resultados estejam isentos de viés. É também ainda necessária investigação adicional para esclarecer o efeito dos GSTs em cada HCA, particularmente, FRDA, SCA6 e SCA2.

LIST OF ABREVIATIONS

ASO- Anti-sense Oligonucleotides

CRISPRS/Cas9 system- Clustered Regularly Interspaced Short Palindromic Repeats Associated with Cas 9 Enzyme System

GST- Gene Suppression Therapy

HCA- Hereditary Cerebellar Ataxia

iRNA- RNA Interference

SCA- Spinocerebellar Ataxia

WT- Wild Type

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INTRODUCTION

BACKGROUND

Hereditary cerebellar ataxias (HCA) are a heterogeneous group of neurodegenerative disorders that mainly affect the cerebellum, its afferent and efferent pathways.[1] Several classifications may be identified in the literature, with the one more frequently used being in accordance with the inheritance pattern: autosomal dominant, autosomal recessive, mitochondrial and X-linked.[1] Autosomal dominant ataxias are usually referred to as spinocerebellar ataxias (SCA) followed by a number, which is assigned according to the order of detection of the genetic locus involved.[2] The most frequent are polyglutamine disorders, arising from repeat-expansion triplets (SCA 1, SCA2, MJD/SCA3, SCA6 and SCA7).[3] The expansion size is partially responsible for the age of onset, severity and progression of the disorder.[3] The most common autosomal recessive ataxia worldwide, Friedreich Ataxia, is also a triplet expansion disorder, as well as one of the most typical X-linked ataxias: fragile X tremor ataxia syndrome.[4]

HCAs treatment can be divided into symptomatic and disease-modifying strategies.[5] The last is currently available for a few hereditary ataxias, such as ataxia with vitamin E deficiency, ataxia associated with glucose transporter type 1 deficiency and Niemann-Pick disease type C.[5] In the remaining, the only treatment remains symptomatic.[5]

Prognosis is generally poor, leading to severe disability and premature death.[6] Ataxia's progression rate depends on a great variety of factors, from age of onset to repeat expansion size.[7] In a study comprising 466 patients with cerebellar ataxias, the median time for patients with Friedreich Ataxia to require a wheelchair was eleven years.[8] In the same study, SCA 1 displayed a faster progression rate than SCA 2 and SCA 3, with the median age of death being 56 years old.[8] Therefore, the need for continuing research in disease modifying therapies is crucial.[6]

Gene therapies are considered a promising approach to several human disorders, from inherited to acquired diseases.[9,10] In the past decades, there has been an extensive research in this novel methods, evolving from viral vectors (which only act by gene addition) to engineered and bacterial nucleases (acting by gene ablation and correction), amplifying treatment possibilities.[9] Nonetheless, there are still significant concerns such as genotoxicity, off-target genome editing and immune reactions.[9]

Gene suppression therapies (GST) have been showing relevant progresses in the treatment of neurodegenerative disorders such as Huntington's disease (HD) and hereditary transthyretin amyloidosis (ATTR).[11,12] As its name suggests, the main effect consists on lowering the expression

of specific genes and, being these genes responsible for mutant proteins, these techniques will also reduce mutant protein levels.[11] GST include RNA interference (iRNA), anti-sense oligonucleotides (ASOs), catalytic nucleic acids, zinc finger proteins (ZFPx) and clustered regularly interspaced short palindromic repeats associated with Cas 9 enzyme system (CRISPR/Cas9 system).[11]

To further enlighten the progress of gene therapies in neurodegenerative disorders, the ATTR is a great example. In 2018, the drugs Inoserten (ASOs) and Patisiran (RNAi) have been approved in Europe for the treatment of neuropathy in ATTR.[12,13] They have demonstrated to reduce the levels of the mutated and wild-type TTR protein in 50-96%,[12] delaying and halting the progression of neurological symptoms.[12,14] Concerning Huntington's disease, in the beginning of 2021, there were three ongoing clinical trials assessing three different promising ASOs. However, all three have been precociously terminated: two failed to lower huntingtin protein levels and the third has not yet shared information regarding the reasons behind the trial cessation. Nonetheless, there are projections on a new clinical trial in 2021 testing a chemically improved ASO.[15]

HCAs, particularly triplet expansion disorders, have been subject of intense disease modifying investigation for the past years. GST have been designed and tested, with very promising results.[3,9]

OBJECTIVE

The aim of this work was to perform a systematic review on studies investigating the efficacy and safety of GST on HCAs.

METHODS

The methodology protocol for this study was elaborated according to the Prisma guidelines.

ELIGIBILITY CRITERIA FOR INCLUSION

Eligibility criteria for inclusion were studies assessing GST effects on HCA. Inclusion criteria comprised studies performed either on animal models or human population. Articles regarding GST in disorders other than HCAs, articles on other therapies for HCA and *in vitro* only studies were excluded. Review papers were also excluded to decrease the risk of data duplication, as well as studies reported in languages other than English or Portuguese.

SEARCH AND SCREENING STRATEGY

A structured PubMed search from January 1993 (year of the first description of the underlying genetic defect of HCA), up to October 2020 was performed. The search strategy included the following MeSH terms: “Hereditary Spinocerebellar Degenerations”, “Friedreich Ataxia”, “Olivopontocerebellar Atrophy”, “Spinocerebellar Ataxia”, “Machado Joseph Disease”, “Gene Silencing” and “RNA Interference”; and the following all fields terms: “Gene Suppression”, “Genome editing”, “Catalytic Nucleic Acids”, “Antisense Oligonucleotides” and “CRISPr-Cas 9 System”. The search was performed as follows: [“Hereditary Spinocerebellar Degenerations” OR “Friedreich Ataxia” OR “Olivopontocerebellar Atrophy” OR “Spinocerebellar Ataxia” OR “Machado Joseph Disease”] AND [“Gene Silencing” OR “RNA Interference” OR “Gene Suppression” OR “Genome editing” OR “Catalytic Nucleic Acids” OR “Antisense Oligonucleotides” OR “CRISPr-Cas 9 System”].

Peer reviewed articles in English or Portuguese were included, and references were checked to guarantee maximal coverage. The screening process was independently carried out by two authors (CS and SF) and, when divergences were identified, a third author (JD) was consulted. All articles were screened by heading and abstract, followed by full-text screening of the remaining articles.

RISK OF BIAS ASSESSMENT AND DATA EXTRACTION

Since all selected studies were performed on animal models, the risk of bias assessment was conducted using the SYRCLE's risk of bias tool, an adapted version of the Cochrane risk of bias tool for animal studies.[16]

A standardized data sheet was created to extract relevant information from each article: authors, year, type of HCA, type of GST aim of the study, intervention and control groups, outcomes, results and key conclusions (Supplementary table I).

RESULTS

SEARCH OUTCOME

Pubmed search generated a total of 262 articles without duplicates. The initial screening, based on heading and abstract reading, excluded a total of 196 articles: 141 didn't concern gene therapy, 38 were review articles and seventeen didn't concern HCAs. After full text reading an additional 41 articles were excluded: 34 *in vitro* only studies, two editorials, four didn't concern GST, one didn't concern HCAs, and another consisted of study methods description. An additional article was included in this review since its results were thoroughly described in a previous selected article. Hence, the number of selected studies for analysis in the present systematic review was 25: eleven concerning MJD/SCA3, six regarding SCA1, three on SCA7, two on SCA6, one on SCA2, one on Friedreich ataxia and another on ataxia telangiectasia. A schematic representation of the search results is depicted on figure 1. A summarized data extraction sheet for each HCA is presented in the supplementary material (Tables II-V).

Regarding risk of bias assessment, most studies were not clear on the categories analysed by the risk of bias tool recommended for animal studies (figure 2).

MACHADO-JOSEPH DISEASE

Machado-Joseph disease (MJD), also referred to as spinocerebellar ataxia type 3 (SCA 3), is the most common autosomal dominant spinocerebellar ataxia worldwide.[17] Eleven articles on GST in MJD animal models were included: nine on iRNA therapy and two on ASO therapy.[18-28]

Suppression efficacy

Suppression efficacy was evaluated in all selected articles, except one by Clévio Nóbrega et al, on a specific iRNA that had already proven to be effective in previous studies.[25] Seven articles assessed mutant ataxin-3 transcript and/ or protein levels; one analysed mutant ataxin-3 expression on immunohistochemistry*;* another the levels of ASO targeted exons and the last analysed Relish expression and Relish-dependent anti-microbial peptides (AMPs) levels.[18-24,26-28]

Regarding the first seven articles mentioned, all authors identified significant decreased levels of mutant ataxin-3 mRNA and/ or decreased mutant ataxin-3 protein when comparing the treated to the control group.[19-21,24,26-28] The extent of the reduction in mutant transcript levels varied from 32% to 92% and both extremes were attained with iRNA therapy.[20,26] Likewise, with respect to mutant protein levels, the values varied from 32% to 91%, with the highest reduction having been reached with ASO therapy.[20,24]

In addition, Sandro Alves *et al* only quantified the reduction in mutant mRNA and protein levels *in vitro*.[18] In a mouse model, the authors confirmed iRNA's suppression efficacy by verifying a decrease in the expression of mutant ataxin-3 in immunohistochemistry.[18]

Maria do Carmo Costa *et al* analysed mutant ataxin-3 levels at end stage of life (9-10 months after iRNA injection) and identified a reduction of mutant ataxin-3 levels up to 40% of the corresponding levels in control mice, demonstrating lifelong suppression efficacy.[21]

Melvin M. Evers *et al* tested an ASO targeting the exons responsible for the CAG repeat expansion in the mutant protein.[22] They verified, *in vitro*, the appearance of a modified ataxin-3 protein lacking the polyQ repeat, while total ataxin-3 protein levels were unaltered.[22] When evaluating the suppression efficacy *in vivo,* they measured the levels of the targeted exons that were, in fact, reduced comparing with controls.[22]

Y. X. Li *et al* assessed whether knockdown of Relish, an astrocyte-specificNF-kB transcription factor, or Relish-dependent AMPs could attenuate eye degeneration in MJD/SCA3 Drosophila

models. Indeed, knockdown efficiency was confirmed by assessing Relish expression and Relish-dependent AMPs levels.[23]

Neuropathology

CAG repeat expansion in *ATXN3* confers a gain of function to ataxin-3, leading to the formation of neuronal mutant ataxin-3 inclusions, neuronal dysfunction, and degeneration.[25,27] These neuropathological features are mostly observed in the substantia nigra, dentate nuclei, pontine nuclei and striatum.[19,25] Effects of GST on neuropathology were studied in eight publications: seven on iRNA and one on ASO.[18-20,23,24,26-28]

The methodology used to evaluate the efficacy on neuropathological studies were: quantitative and qualitative analysis of neuronal ataxin-3 or ubiquitin positive inclusions, nuclear accumulation of mutant ataxin-3 protein, Purkinje cell count, cerebellar granular and molecular layer thickness, DARPP-32 immunoreactivity volume depletion, neuronal cells with pycnotic nuclei, firing frequency in Purkinje neurons, autophagy impairment.[18-20,23,24,26-28]

Neuronal mutant ataxin-3 inclusions were assessed in all 8 publications.[18-20,23,24,26-28] Apart from Y. X. Li *et al,*[23] all studies showed a decrease in neuronal inclusions.[18-20,23,24,26-28] X. Li *et al* did not study an iRNA construct directed at ataxin-3 but at Relish and Relish dependent AMPs.[23] Relish pathway is associated with neurodegeneration in MJD/SCA3.[23] The authors did not find lower mutant ataxin-3 aggregates in the treated group, despite confirming an improved neuronal and eye phenotype.[23] Thus, they concluded that phenotype improvement was not reached thanks to clearance of protein aggregates.[23] Sandro Alves *et al*, comparing anti ataxin-3 iRNA and vehicle treated mice found a relative decrease to 48,2% in number and 12,7% in size of mutant ataxin-3 positive inclusions.[18] Similarly, Clévio Nóbrega *et al* revealed a significant reduction of the number of inclusions per 100 Purkinje cell: 4.30 in iRNA treated mice to 19.5 in control treated mice.[27] Hayley S. McLoughlin *et al,* proved ASO treatment to be effective in reducing neuronal nuclear ataxin-3 accumulation to vehicle treated WT levels in pontine and deep cerebellar nuclear neurons.[24] This reduction was verified until at least 22 weeks of age. The control group, receiving a control ASO not targeted at mutant ataxin-3, also presented at 16 weeks, a significant reduction in neuronal and non-neuronal nuclear accumulation of ataxin-3, when compared to vehicle injected mice.[24] The authors believe this to be a result of a non-specific effect of ASO therapy requiring further investigation.[24]

Neuronal loss and dysfunction analysis was evaluated in seven articles,[18-20,23,24,26-28] of which six demonstrated significant neuronal preservation in the intervention group.[18-20,24,26,27] DARPP-32 immunoreactivity analysis, a sensitive marker of early neuronal dysfunction, was performed in five papers on iRNA therapy.[18-20,26,27] All authors found higher DARPP-32 marker expression in the treated group, translating neuronal preservation with iRNA administration.[18-20,26,27] Sandro Alves *et al* revealed the greatest DARPP-32 immunoreactivity rescue, with a ~70% preservation of DARPP32 expression.[18] The same authors also verified a marked decreased number of degenerating neurons and atrophic nuclei in the iRNA treated mice and, consequently, no signs of striatal atrophy were identified in contrast to control treated mice.[18] In addition, although two articles have analysed the Purkinje cell count,[20,26] only Clévio Nóbrega *et al* verified a significant preservation of Purkinje cells in the iRNA treated group: 11.85 to 7 in the control group.[26]

Another important readout for neuronal degeneration is cerebellar molecular and granular layer thickness.[20,26,27] Cerebellar granular layer thickness was evaluated in three articles, and all three showed a significant atrophy attenuation in the iRNA treated mice.[20,26,27] Likewise, the two papers that also studied the molecular cerebellar layer also detected a significant larger layer in the iRNA treated mice.[26,27] For instance, Clévio Nóbrega *et al* identified a 145.1 molecular layer thickness in the intervention group and a 116.3 in the control group.[26,27] In addition the authors also verified a preservation of dendritic arborization in the molecular layer of iRNA treated mice.[26,27]

Hayley S. McLoughlin *et al* have focused on the evaluation of ASO therapy effects on Purkinje cell function by assessing the firing frequency in these cells.[24] In some ataxia models, it has been suggested a slower firing frequency. In fact, there was a rescue of this phenotype in the intervention group, translating a rescue of Purkinje cell neuron function.[24] In addition, this same study also assessed the autophagy impairments that have been linked to age and disease related neuronal dysfunction.[24] The autophagy process is thought to be responsible for the clearance of misfolded proteins, and WT ataxin-3 has been suggested to be a stabilizer of Beclin1, an essential autophagy adapter protein.[24] Reduction of ataxin-3 levels by gene silencing, or a mutant ataxin-3 protein, could lead to decreased levels of Beclin1 and, thus autophagy impairment.[24] Notwithstanding, the authors detected decreased levels of Beclin1 only in the pons and no difference between groups in the cerebellum, suggesting a region-specific autophagic impairment in MJD/SCA3.[24] Moreover, ubiquitin-binding adapter protein p62 localizes to protein aggregates and is thought to have a protective role in the autophagic clearance of polyglutamine disease proteins.[24] Nonetheless, ASO therapy only prevented intranuclear accumulation of p62 in pontine neurons which might be related to the decreased ataxin-3 aggregates following ataxin-3 silencing.[24]

Motor behavior

MJD clinical features includes cerebellar ataxia, pyramidal and extrapyramidal signs, peripheral neuropathy, oculomotor abnormalities and sleep disorders.[17] Motor behavior was assessed in five studies: four on iRNA therapy and one on ASO therapy.[20,21,24,26,27] The methodology adopted was similar between studies: beam walking, hind limb dragging, swimming performance, footprint pattern analysis, accelerating rotarod test, locomotor and exploratory activities.[20,21,24,26,27]

All studies showed significant improvement of motor behavior when comparing to untreated MJD/SCA3 mice models and/ or similar performances when comparing to wild type mice.[20,24,26,27] Clévio Nóbrega *et al* found iRNA treated mice to have a significant better performance on accelerating rotarod test, higher stride length and a better footprint overlap as well as increased movement, velocity and longer distance traveled when monitoring behavior in an activity box.[27]

One of the most relevant aspects was timing of therapy administration. Only one study investigated the administration in pre-symptomatic mice, concluding that iRNA therapy prevented deterioration of balance, motor coordination, gait, and hyperactivity.[26] Hayley S. McLoughlin *et. al* showed that ASO administration in early-symptomatic mice fully rescued locomotor activity until 29 weeks of age, when the last evaluation was performed.[24] Clévio Nóbrega *et al* studied the effect of iRNA administration in mice with severe symptoms.[27] iRNA treated mice showed improvement in the different motor behavior readouts, but with the performance being far from the identified in wild type mice.[27] Nevertheless, the preservation of motor behavior was considered significant.[27] Maria do Carmo *et al* demonstrated that, despite the effective lifelong suppression of mutant ataxin-3 and the apparent widespread delivery to the cerebellum, iRNA treatment failed to improve motor impairment during the whole duration of the study (48 weeks of age).[21] The authors suggested the motor phenotype might not be solely due to cerebellar dysfunction, with other CNS regions being also involved.[21] The authors also discuss the timing of treatment administration, with a late administration probably not being sufficient to revert symptoms that were already present.[21]

The route of administration GST was a crucial aspect of these therapies. Four of the selected papers assessed the effects of intrathecal administration: in all there was good cerebellar distribution of the GST and good performance in motor behavior parameters.[21, 24, 26, 27] Conceição et al tested a noninvasive approach, by peripheral injection of lentiviral vectors encoding iRNA.[20] Significant positive results in beam walking and footprint pattern analysis were obtained, and a

tendency for better swimming performance was also observed. These were very promising results, on the efficacy of peripheral administration of GST.[20]

Wild type protein

The function of normal ataxin-3 protein has not been fully understood, being suggested that it might have a protective role in neuronal function and viability[18,19] In fact, homozygous MJD/SCA3 patients present a more severe phenotype than heterozygous individuals.[18] Moreover, previous studies in *Drosophila* models have shown that WT ataxin-3 decreases the neurotoxicity of mutant ataxin-3 by reducing mutant ataxin-3 levels and nuclear inclusions.[18,19] It is thought that this apparent protective role may be associated with the involvement of WT ataxin-3 in protein surveillance pathways.[19]

Two of the articles selected have focused on assessing the benefits of keeping or overexpressing the WT protein in MJD/SCA3 mouse models.[18,19] Sandro Alves *et al* demonstrated that allele specific silencing of mutant ataxin-3 resulted in reduced levels of mutant ataxin-3 protein without affecting WT protein levels, as well as attenuation of neuropathological phenotype when compared with control treated MJD/SCA3 mice.[18] The same authors, in another work, concluded that overexpression of WT ataxin-3 did not protect against MJD/SCA3 neuropathological phenotype.[19] In fact, expression of both WT and mutant ataxin-3 was associated with a larger DARPP-32 depleted region, suggesting an increased toxicity when comparing with rats expressing only mutant ataxin-3.[19] This study also assessed the effects of knocking down ataxin-3 in WT rats: there were no differences in neuropathological features between WT rats, with lower levels of ataxin-3 protein,, and the control group.[19]

Safety profile

Safety profile was evaluated in five studies. The measures used were lifespan, neurotoxicity, glial activation markers, pro- and anti-apoptotic protein quantification, off target effects and saturation of the endogenous iRNA processing machinery.[21,23-25,28]

Lifespan was evaluated on two articles.[21,23] Maria do Carmo Costa *et al* didn't find significant difference in lifespan between iRNA treated and control treated mice; detecting similar average

age of death at 58 weeks.[21] On the contrary, Y. X. Li *et al* found an extended lifespan in the iRNA treated flies compared to control group.[23]

Maria do Carmo Costa *et al* and Clévio Nóbrega *et al* evaluated the possible neurotoxicity associated with iRNA therapy administration.[21,25] For this analysis they evaluated the DARPP-32 immunoreactivity and/ or NeuN expression.[21,25] At 10 weeks post injection, Maria do Carmo Costa *et al* found no differences between treated or untreated mice relatively to NeuN positive cells distribution and density.[21] Clévio Nóbrega *et al* concluded that the surgical injection, effectively, led to a loss of DARPP-32 and NeuN expression measured at 2 weeks post injection. However, at 8 weeks post injection, neuronal recovery was far more pronounced in the iRNA treated than in the control vector injected hemisphere.[25]

Four studies assessed the effects on glia activation, using Gfap and microglial activation marker Iba1.[21,24,25,28] On three studies there were no differences of glia activation markers between intervention and control groups.[21,24,25,28] Nevertheless, Clévio Nóbrega *et al* detected glia activation 2 weeks post injection, either with control vector or vector containing iRNA.[25] However, at 8 and 20 weeks post injection, no differences in Gfap and Iba1 markers, respectively, were found between the injected and non-injected mice, meaning that the injection procedure, effectively, lead to an inflammatory response that resolved at 20w.[25]

Clévio Nóbrega *et al* studied the potential off target effects and saturation of endogenous iRNA processing machinery associated with iRNA administration.[25] Relative to off target effects, the authors measured the levels of endogenous transcripts Taok-1 and Tnem-106b.[25] These transcripts have sequences that pair the seed region of the iRNA tested in this study.[25] At 2 and 20 weeks post injection, the quantitative analysis revealed no differences between injected and non-injected mice.[25] Relative to the saturation of endogenous iRNA processing machinery, if present it could be responsible for disrupting the biogenesis and function of miRNAs. In fact, Grimm *et al* described a casual-effect relationship between oversaturation of iRNA pathways and mice death.[25] Hence, Clévio Nóbrega *et al* analyzed Drosha, Dicer and exportin-5 mRNA levels at 2 and 20 weeks post injection.[25] No significant difference was found between injected and non-injected mice, discarding iRNA pathway saturation.[25]

Finally, to assess possible cellular toxicity with administration of increasing doses of ASO, Haly S. McLoughin *et al* evaluated the ratio of pro- and anti-apoptotic proteins (BAX/BCL2).[24] At four weeks post injection, when BAX and BCL2 were quantified, there was no increase in the ratio BAX/BCL2 with increasing ASO doses in the intervention group relative to WT or control MJD mouse models.[24] Thus, authors concluded that there was no cellular toxicity at tested doses.[24]

SPINOCEREBELLAR ATAXIA TYPE 1

Six papers on SCA1 and GST were included: five on iRNA therapy and one on ASO therapy.[29-34]

Suppression efficacy

Suppression efficacy was assessed in all articles and, overall, the tested therapies were effective in reducing ataxin-1 mRNA and/ or protein levels.[29-34] Megan S. Keiser et al showed that iRNA was capable of sustained suppression of ataxin-1 transcripts: at 3-weeks after injection, iRNA-treated mice had 30% of the total ataxin-1 transcripts present in control-treated mice, and had 75% at 35-weeks after injection.[31] Jillian Friedreich et al demonstrated that a single ASO injection reduced ataxin-1 transcript levels from two to eighteen weeks post-injection.[29] However, when measuring ataxin-1 protein levels, the pons was the only region to show sustained reduction until eighteen weeks post-injection, having ataxin-1 protein levels returned to untreated levels in medulla, cerebral cortex and cerebellum.[29]

Neuropathology

Neuropathology was assessed on four articles.[30,31,33,34]

Concerning cerebellar molecular layer thickness, all four studies demonstrated that iRNA anti ataxin-1 treated mice had wider molecular layer, when compared to control or saline treated mice, and no significant differences to WT mice.[30,31,33,34] In fact, Haibin Xia et al found a width of 162µm in the intervention group and 158µm in the WT group.[34] Megan S. Keiser et al proved iRNA to rescue this neuropathological feature when therapy was administered either before or after symptoms onset.[33]

Haibin Xia et al also studied the effect of iRNA anti ataxin-1 on intra-neuronal inclusions formation.[34] SCA1 mice model presented inclusions in approximately 50% of Purkinje cells at 16 weeks.[34] When analyzing the iRNA treated mice, the authors found complete resolution of inclusions in the transduced Purkinje cells.[34] At last, Megan S. Keiser et al found a significant rescue of Purkinje cell number, as well as a reduction of ectopic Purkinje cells.[31]

Motor behavior

Motor behavior was assessed in five articles.[29-31,33,34] Different methods were used to evaluate motor activity, namely, rotarod test performance, gait and beam walking analysis, ledge test and hindlimb clasping analysis.[29-31,33,34] Overall, the treated groups performed better than control treated or untreated SCA1 group. [29-31,33,34] Megan S. Keiser et al found that iRNA treated mice showed sustained longer strides and wider hindlimb stances at 30 and 40 weeks of age when compared to control treated mice.[30]

Regarding administration timing, Megan S. Keiser et al found that iRNA therapy administration, before and after symptoms onset, led to improvement in rotarod test performance compared to control or untreated SCA1 mice.[33] In fact, when administered in pre-symptomatic mice, iRNA treated mices' performance was similar to WT littermates; in contrast to administration after symptoms onset that resulted in a performance still far from WT littermates.[33] Moreover, Jillian Friedreich et al found ASO injection in a very early stage of disease to improve performances on rotarod test and beam walking analysis, whereas administration in an early mid stage of disease only led to significant differences on the beam walk and not on the rotarod test.[29]

Safety profile

Safety profile was evaluated by assessing microglia and astrocytic activation markers (Iba1 and Gfap, respectively) expression in four articles.[30-33] Megan S. Keiser et al found no differences between iRNA anti ataxin-1 and saline treated mice, regarding Iba1 or Gfap expression at injection site or cerebellar lobules.[30,31] The same authors, in another study, identified a slight enhancement of both markers on the injected cerebellar cortex hemisphere, when compared with the untreated hemisphere.[32] Another study found a slight enhancement of astrocytic marker at the injection site in all injected mice and no difference in microglia activation marker expression when compared to control group.[33] Nonetheless, all authors concluded that GST were well tolerated.[30-33]

SPINOCEREBELLAR ATAXIA TYPE 7

Three articles on spinocerebellar ataxia type 7 (SCA7) and GST were selected: two on iRNA and one on ASO.[35-37] Since SCA7 is characterized by retinal degeneration, two of these studies have focused on analyzing retinal degeneration with GST administered via subretinal injection or injection into vitreous humor.[35,36]

Suppression efficacy

All three articles assessed suppression efficacy, with all identifying decreased mutant ataxin-7 transcript and protein levels, when compared to placebo or untreated SCA7 mice.[35-37] Chenchen Niu et al showed a >60% reduction in ataxin-7 mRNA levels 6 weeks after ASO retinal injection.[35] Similarly, Pavitra S. Ramachandran et al, identified a sustained reduction of 50% in ataxin-7 mRNA levels and 35% in ataxin-7 protein levels, at 33 weeks after intrathecal iRNA injection, demonstrating sustained ataxin-7 suppression.[37]

Neuropathology

Neuropathology was assessed in two studies.[35,37]

Chenchen Niu et al observed that ASO anti-ataxin-7 administration at the time or after symptoms onset resulted in a significant reduction in ataxin-7 intraneuronal inclusions.[35] In this paper the authors demonstrated that ASO targeted at the CAG repeat, had a less clear reduction in ataxin-7 aggregates than anti-ataxin-7 ASO.[35] Pavitra S Ramachandran et al demonstrated that anti-ataxin-7 iRNA intrathecal injection resulted in an approximate 80% reduction in neuronal inclusions in transduced Purkinje cells when compared to control iRNA or saline treated mice.[37]

Molecular cerebellar layer thickness was assessed in one article.[37] Pavitra S Ramachandran et al, identified an increase of 10% of molecular cerebellar layer thickness in the iRNA anti-ataxin-7 treated mice relatively to control iRNA or saline treated mice.[37]

Motor behavior

Motor behavior was assessed in one paper. Pavitra S Ramachandran et al found that anti-ataxin-7 iRNA treated mice compared to control iRNA or saline treated SCA 7 mice, presented a significant improvement in the hindlimb clasping score, ledge score, rotarod test performance and stride length.[37]

Retinal degeneration

Retinal degeneration occurs in SCA7 due to cone-rod dystrophy, with two papers assessing photoreceptors' function via electroretinogram (ERG).[35,36] Chenchen Niu et al proved that anti-ataxin-7 ASO injection before symptoms' onset led to increased cone and rod photoreceptor

function from 4 to 6 weeks post injection, when compared to vehicle or control-ASO treated mice.[35] However, when ASO treatment was administered after symptoms onset, only cone receptor function was significantly better at 9 weeks post injection.[35] Moreover, ASO targeting the CAG repeat led to no visual function improvement when compared to vehicle treated at 6 weeks post injection.[35] The authors' also assessed ASO effect on retinal histology.[35] In fact, SCA7 leads to thinning of retinal layers and ASO treatment administered before symptoms' onset improved all retinal layers thickness, when compared to vehicle treated mice.[35] However, when ASO was administered after symptoms' onset, no improvement in retinal segments was observed.[35] Pavitra S. Ramachandran et al found no difference between iRNA anti-ataxin-7 and saline treated mice regarding either mixed rod-cone response or isolated cone response, at 30 weeks of age.[36]

Also, when assessing the optokinetic tracking response, there was no difference between iRNA anti-ataxin-7 and wild type mice.[36]

Safety profile

Safety profile was assessed by both articles on iRNA treatment.[36,37] Overall, both concluded there was no toxicity associated with iRNA delivery.[36,37] iRNA anti-ataxin-7 subretinal injection led to no differences in retinal thickness or gliosis when compared to saline injected eyes.[36] iRNA anti-ataxin-7 intrathecal administration led to no astrocytic, neither microglial, activation when compared to wild type mice.[37]

OTHER HCAS

In this chapter we will analyze the search results concerning SCA6, Friedreich Ataxia (FRDA), SCA2 and Ataxia Telangiectasia (A-T). The search and selection process combined, resulted in two articles related to SCA6 and one article per each one of the other HCAs.[38-42]

SCA6 results from a CAG repeat expansion in the voltage-gated calcium channel gene *CACNA1A*.[40] This gene encodes both α1A protein (pore-forming protein of Cav2.1 Calcium channel) and α1ACT protein (transcription factor that regulates several genes).[40] A polyQ-expanded α1ACT protein is the responsible for the SCA6 pathogenesis whereas α1A protein is essential to life.[40] Therefore, selective silencing of α1ACT would be the desirable approach, in contrast to full CACNA1A gene silencing.[40] Provided that α1ACT is translated through an internal ribosome entry site (IRES), in

contrast to α1A cap-dependent translation, Parvis Pastor *et al,* selected a miRNA that would silence these sequences within the CACNA1A IRES region. The authors tested a miRNA in a hyperacute SCA6 mouse model.[40,42] Regarding suppression efficacy, the miRNA treated group showed lower mutant α1ACT protein levels, while keeping similar α1A levels when compared to control treated.[40,42] When assessing neuropathological features, the authors identified a protective role in cerebellar molecular layer thinning, decreased dendritic tree density and decreased number of Purkinje cells.[40,42] In addition, motor deficits were improved in the treated group, when compared to control: better performances on rotarod test, greater distances in the open field assay and improved gait instability in all four limbs.[40,42]

FRDA is the most common autosomal recessive hereditary ataxia and results from a GAA triplet expansion in *frataxin* gene which leads to a deficiency in frataxin protein, essential to mitochondrial function.[41] On one study on CRISPR-Cas9 system, the authors demonstrated its suppression efficacy *in vivo*, verifying GAA repeat excision through electroporation into *tibialis anterior* muscle of FRDA mouse model.[41]

Scoles et al studied the administration of ASO therapy in two SCA2 mouse models: one containing 127 CAG repeats (ATAXN2-Q127) and another, closer to the human disease features, containing 72 CAG repeats (BAC-Q72).[39] Regardless of the mouse model used, the authors found ASO intrathecal injection, at the time of symptoms onset, to be effective in suppressing human and mouse ataxin-2 transcript levels: 75% and 20%, respectively, in the Q127 mouse model.[39] Concerning the safety profile, there was no significant astrocytic or microglial activation.[39] On motor behavior, both mouse models showed better performances at accelerating rotarod test, when compared to SCA2 saline treated group.[39] Nonetheless, the performance was still far from the one found in WT mice.[39] In addition, related to neuropathological features, Purkinje cell function was assessed via firing frequency analysis.[39] Once more, both models showed a near complete restoration of normal Purkinje cell firing frequency.[39] This restoration is thought to be part of the explanation for better motor performances in the treated mice.[39]

A-T is a HCA that along with neurodegeneration is also associated with higher cancer risk, immunodeficiency and radiosensitivity.[38] Previous studies had shown low *in vivo* correction efficiency and systemic delivery with antisense morpholino oligonucleotides (AMOs) alone. [38] In

an attempt to address this issue, Liutao Du et al conjugated AMOs with arginine rich cell penetrating peptides (CPPs).[38] These CPPs had previously proven to promote nuclear delivery of AMOs in cell cultures.[38] After confirming the correction efficiency of selected splicing mutations *in vitro,* the authors demonstrated that systemic administration of AMOs-CPPs in mice reached satisfactory delivery to brain and, particularly, Purkinje cells.[38] In fact, Purkinje cells had an apparent higher fluorescence intensity and the authors suggest that the large cell size may make them easier targets for AMOs-CPP.[38] These are promising results since Purkinje cells play a critical role in A-T pathogenesis.[38] Nonetheless, authors call for the need of following studies focused on off target effects as well as immune responses associated with AMOs-CPPs *in vivo*.[38]

DISCUSSION

One of the main aspects to consider was that most of the articles were not clear on the parameters used to assess the risk of bias. Thus, there was no guarantee that results and conclusions were free of bias. The lack of clarity on these parameters enhances the need for methodology improvement on animal studies in this area.

Concerning the search and selection process results, this review included articles concerning MJD/SCA3, SCA7, SCA1, SCA6, SCA2, FRDA and A-T. Given the lack of studies on some HCAs, there were no articles assessing suppression efficacy in A-T; neuropathological features, motor behavior and safety profile assessment in A-T and FRDA. Further investigation on these HCAs would be valuable, particularly on FRDA, the most frequent autosomal recessive HCA.

All articles selected proved to be effective in suppressing *in vivo* the targeted genes. The silencing magnitude varied from study to study, but they all achieved significant values either when testing iRNA, ASO or CrisprCas9 system.

In general, when assessed, all studies demonstrated that GST were effective in improving neuropathological features associated with HCAs, including decreasing neuronal inclusions, neuronal dysfunction, and neuronal loss.

With respect to motor phenotype, every study that assessed motor behavior, except one, proved GST to halt and improve motor performance in the intervention group. Indeed, on this one exception the authors found iRNA treatment to be effective in suppressing the targeted gene, as well as improving neuropathological features.[19] Thus, they concluded that either the administration timing was too late to revert symptoms and/ or pathogenesis was not limited to the cerebellum.[19]

Relatively to administration timing, GST administered either before or after symptoms onset proved to be effective in improving motor performance in MJD/SCA3 and SCA1 and eye degeneration in SCA7. When administered before symptoms onset, or in early symptomatic mice, motor performance was similar to WT mice and eye degeneration was more attenuated than in the group treated after symptoms' onset. These results suggest that the earlier the administration, the more effective the therapy will be.

Another relevant aspect to analyse was the benefit associated with WT protein. This review includes only two studies on iRNA therapy in MJD/SCA3 mice that focus on this aspect. Their results suggest no apparent benefit in specific silencing the mutant allele. Nonetheless, authors

do not deny the possibility that WT protein at higher levels than the ones tested could be beneficial. Thus, further investigation is still needed.

Addressing the concerns about adverse effects associated with GST: the most common adverse effects found and assessed were glial and astrocytic activation. This non-neuronal response, when present, was related to the injection of the therapy tested. Indeed, most studies opted for intrathecal administration and this injection led to glia and astrocytic activation limited to injection site that resolved over time. These findings suggest that these gene therapies are safe. Nevertheless, further investigation on a less invasive via of administration would be desirable. In fact, two articles demonstrated that intravenous administration was also effective. However, not every article assessed adverse effects and, more importantly, there might be other adverse effects that, for not being measured, were not identified.

CONCLUSIONS

GST represent a new hope for the treatment of genetic neurodegenerative disorders, including HCAs. This systematic review on *in vivo* studies of GST on HCAs presents promising results. GST demonstrated to be effective in suppressing the targeted genes, improving neuropathological features, as well as motor phenotype. In addition, the adverse effects identified were related to the intrathecal injection and resolved over time. Nonetheless, further investigation is still needed due to the lack of studies on some HCAs.

FIGURES

FIGURE 1- SEARCH RESULTS IN A FLOW DIAGRAM (ADAPTED FROM PRISMA 2009)

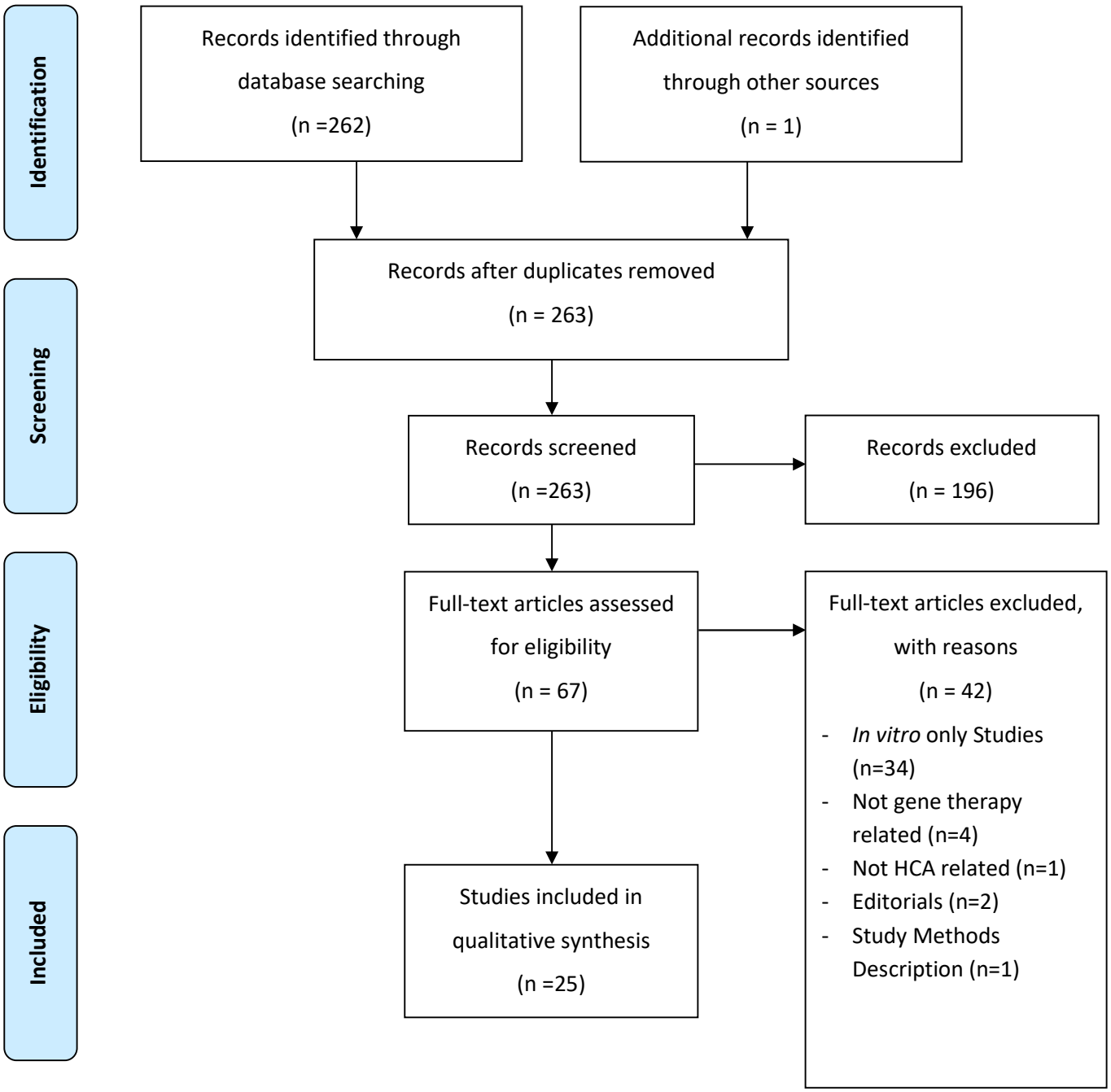
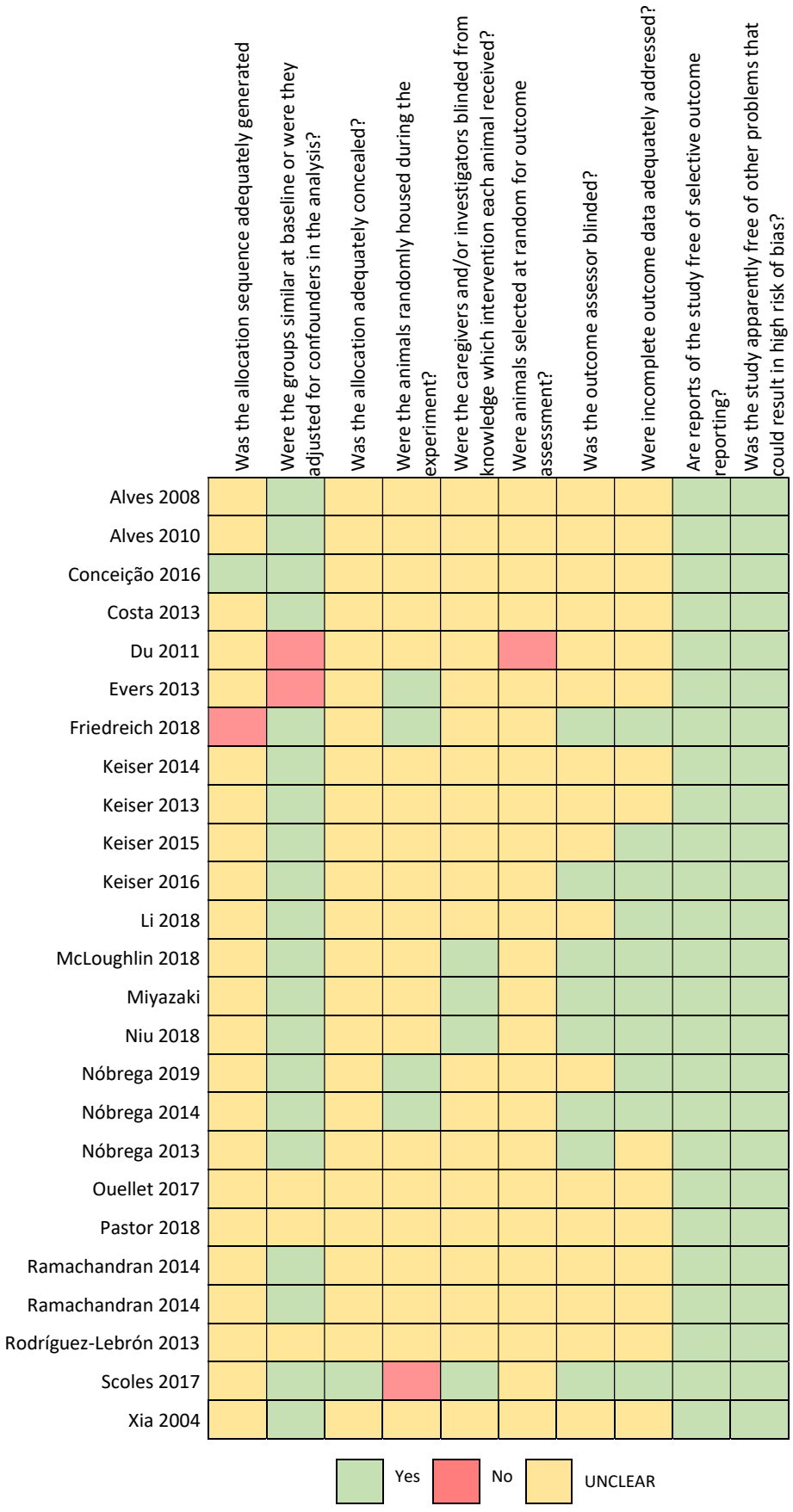


FIGURE 2- RISK OF BIAS ASSESSMENT- ADAPTED FROM SYRCLE'S RISK OF BIAS TOOL



SUPPLEMENTARY MATERIAL

TABLE I- STANDARDIZED SHEET FOR DATA EXTRACTION

Author	Year	Type of Cerebellar ataxia	Type of Gene therapy	Aim	Intervention					Categories Analysed			Outcome Results		Key conclusions of study authors
					Intervention	Control 1	Control 2	Control 3	Others	Suppression Efficacy	Motor Behaviour	Safety profile	Outcome	Overall results	

TABLE II- KEY CONCLUSIONS FROM DATA EXTRACTION SHEET- MJD/SCA3

AUTHOR	YEAR	TYPE OF GST	KEY CONCLUSIONS
Sandro Alves *et al*	2008	iRNA	Allele specific silencing of mutant ataxin-3 is effective and selective *in vivo* and decreases MJD associated neuropathological phenotype.
Sandro Alves *et al*	2010	iRNA	WT ataxin-3 does not reduce toxicity of mutant ataxin-3: WT overexpression did not protect against MJD neuropathology and knockdown of WT did not affect MJD neuropathology. Non-allele specific silencing of ataxin-3 reduced neuropathology.
Mariana Conceição *et al*	2016	iRNA	Intravenous administration is successful crossing de BBB. iRNA therapy was effective in mutant ataxin-3 knockdown *in vivo.* iRNA improved motor performance and rescued striatal and cerebellar associated neuropathology. No signs of toxicity were detected.
Maria do Carmo *et al*	2013	iRNA	Despite iRNA was effective in suppressing *ATXN3,* at the end of the study, at 48weeks of age, no improvement in motor impairment was detected: authors suggest that motor phenotype might not be solely due to cerebellar dysfunction or intervention was done later than ideal. No adverse effects were detected. No differences in lifespan were detected between groups.
Melvin M. Evers *et al*	2013	ASO	Intracerebral injection of ASO was effective in skipping exons targeted. No overt toxicity was observed *in vivo.*
Y. X. Li *et al*	2018	iRNA	Downregulation of Relish expression in astrocytes delayed neurodegeneration and extended lifespan in SCA3 flies model.
Hayley S. McLoughlin *et al*	2018	ASO	ASO achieved efficient silencing of mutant *ATXN3* and prevented nuclear accumulation of ataxin-3 protein. Administration in post-symptomatic mice fully rescued locomotor activity. No signs of an adverse immune response to treatment were detected.
Clévio Nóbrega *et al*	2018	iRNA	While evidence of neuronal dysfunction and gliosis were present at initial time-points, at 20 weeks post-injection no difference between the groups was found. No off-target effects or saturation of the endogenous iRNA processing machinery in the mouse striatum were detected.
Clévio Nóbrega *et al*	2014	iRNA	Effective gene silencing of *ATXN3* in pre-symptomatic mice led to clearance of mutant ataxin-3 from neuronal nuclei and prevented the development of motor impairments. There was no difference between groups concerning glial or astrocytic activation.
Clévio Nóbrega *et al*	2013	iRNA	iRNA proved to be effective in suppressing *ATXN3.* Its administration after symptoms onset prevented the development of MJD-associated motor-behavior and neuropathological abnormalities.

Edgardo Rodríguez-Lebrón	2012	iRNA	iRNA therapy was effective in suppressing *ATXN3.* Administration in pre-symptomatic mouse model prevented the development of neuropathological features and motor impairments found in the control group.

***TABLE III*- KEY CONCLUSIONS FROM DATA EXTRACTION SHEET- SCA1**

AUTHOR	YEAR	TYPE OF GST	KEY CONCLUSIONS
Jillian Friedrich et. Al	2018	ASO	Following a single ASO injection at 5 weeks of age, ataxin-1 transcripts remained reduced until 18w, but ataxin-1 protein remained at reduced levels only in pons at 18w. Nonetheless, mice demonstrated rescue of neuropathological and motor behavior phenotypes.
Megan S Keiser et. Al	2014	iRNA	Reduced ataxin-1 transcript and protein levels without overt neurotoxicity. It preserved cerebellar lobule integrity for over a year and preserved rotarod performance for months (30w).
Megan S Keiser et. Al	2013	iRNA	Silencing of mutant ataxin-1 using miRNAs rescued behavioral deficits and improved neuropathology. It is suggested that behavioral recovery does not require full rescue of all neuropathological aspects.
Megan S Keiser et. Al	2015	iRNA	Anti-ataxin-1 iRNA resulted in reduction of *ATXN1* mRNA and no signs of toxicity were detected.
Megan S Keiser et. Al	2016	iRNA	iRNA-mediated suppression of ataxin-1 mRNA alters disease progression, reverses motor symptoms, and normalizes cerebellar pathology, when delivered before and after symptoms onset.
Haibin Xia et. Al	2004	iRNA	iRNA reduced ataxin-1 transcript levels resulting in improved motor coordination, restored cerebellar morphology and resolved characteristic ataxin-1 inclusions in Purkinje cells of SCA1 mice.

***TABLE IV*- KEY CONCLUSIONS FROM DATA EXTRACTION SHEET- SCA7**

AUTHOR	YEAR	TYPE OF GST	KEY CONCLUSIONS
Chenchen Niu *et al*	2018	ASO	ASO therapy was effective in suppressing mutant ataxin-7 transcript and protein levels. ASO therapy improved visual function despite initiating treatment after symptoms onset. At the end of the study, ataxin-7 ASO therapy only ameliorated rod photoreceptor function. CAG repeat-targeting ASO was less effective than the Ataxin-7 ASO.
Pavitra S Ramachandran *et al*	2014	iRNA	Sustained reduction of ataxin-7 expression led to significant and robust improvements in the ataxic and neuropathological phenotypes as well as a delayed disease onset in SCA7 mice. No significant adverse effects were present.
Pavitra S. Ramachandran *et al*	2014	iRNA	Preservation of normal retinal function at 23 weeks post retinal injection and no adverse toxicity with reduction ataxin-7 transcript levels.

***TABLE V*- KEY CONCLUSIONS FROM DATA EXTRACTION SHEET- OTHER SCAS**

AUTHOR	YEAR	TYPE OF HCA	TYPE OF GST	KEY CONCLUSIONS
Daniel R. Scoles *et al*	2017	SCA2	ASO	Intracerebral injection of ASO led to reduced ataxin-2 transcript and protein levels resulting in delayed onset of SCA2 motor and neuropathological phenotypes without microglial activation.
Liutao Du *et al*	2011	A-T	ASO	Splicing correction efficiency of AMOs conjugated with CPPs was demonstrated *in vitro. In vivo,* systemic administration revealed efficient brain uptake, particularly in PC, without apparent signs of toxicity.
Parviz Daniel Hejazi Pastor *et al*	2018	SCA6	iRNA	iRNA therapy selectively inhibited alpha-1ACT mutant protein and kept alpha-1A normal levels. It also prevented the development of motor and morphological abnormalities.
Yu Miyazaki *et al*	2016	SCA6	iRNA	iRNA therapy targeted at alpha-1ACT prevented purkinje cell degeneration and motor deficits.

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