Antisense oligonucleotide (ASO) therapy is a novel genetic therapy approach with the potential to revolutionize the treatment of numerous human disorders. ASO therapy has already delivered remarkable results in select neurological disorders, but this progress took years to achieve. This proposal aims to leverage the numerous splice site variants observed in the genes of patients with autism spectrum disorder (ASD) to assess the potential to rapidly design and validate multiple personalized ASO therapies. This could result in a potential treatment for many ASD cases and greatly decrease the development time for future genetic disorders treatments.

Background: Autism Spectrum Disorder (ASD) is a phenotypically heterogenous disease, described by impairments with social interactions and communication[1], affecting about 1.5% of births in the United States (about one million children in the USA).[2] Furthermore, it is estimated that more than 20,000 military dependents have ASD[3]. Twin and family studies have demonstrated that ASD is one of the most heritable complex disorders, strongly suggesting that genetic factors are the major cause.[4–6] Multiple studies have shown that highly deleterious *de novo* variants, i.e. variants newly arising in a child and not present in either parent, confer significant risk to ASD in about at least 10% of cases.[7–11] These rare *de novo* variants have the potential to confer larger effects on ASD risk because they have not yet passed through reproductive fitness filters[12]. In ASD cases, deleterious *de novo* variants often cluster at specific loci across multiple cases, while similar clustering is not observed in controls. Using the controls to define how unexpected these clustering patterns are, allows us to identify specific ASD risk genes. At present, 99 genes have been implicated using this approach (false discovery rate ≤ 0.1).[13]

Despite the frequency of ASD and the suffering it leads to in individuals and families, as well as the cost to society[14], there are no therapies that modify the core ASD symptoms. Intensive behavioral therapies have formed the backbone of ASD treatment, limited effects in a small number of cases.[15] While recent progress has provided key insights into many of the genes involved in ASD, we are far from the sophisticated understanding of ASD pathology that would be necessary to design therapies. However, in the ~10% of ASD cases with an ASD risk gene disrupting *de novo* variants, therapeutic strategies that aim to reverse the genetic deficit may offer a therapeutic "shortcut" by treating the underlying cause directly.

Of the *de novo* variants observed in individuals with ASD, the majority have a loss-of-function effect, disrupting one copy of the gene and consequently decreasing protein levels.[7,13] This raises the possibility that restoring the expression of these genes to their native expression levels, for example via genetic therapies, could improve symptoms. This notion is supported by the landmark observation that genetic rescue in mature animals of loss-of-function variants in *MECP2*, which causes Rett's syndrome, a syndrome closely related to ASD, resulted in substantial improvements in mortality and cognitive function.[16]

Several classes of variants result in loss-of-function effects, including nonsense, frameshift insertions or deletions, and splice site variants. This later class of variants disrupt the native activity of the spliceosome complex, which coordinates the precise removal of introns, and subsequent ligation of exons to form mature RNA. In the presence of a splice site variant, an intron may be retained or the exon may be skipped, both of which usually prevent the synthesis of functional protein.

At present, there are two mature genetic therapy methods with precedent in treating loss-of-function variants in humans: ASOs to modify splicing[17] and gene replacement by adeno-associated virus (AAV).[18] However, most of the genes disrupted by *de novo* variants in ASD are too large to fit in an AAV vector. Therefore, at present, it is the splice site variants that are most tractable to treat in ASD. Further increasing our interest in splice site variants, is our recent advancement in computational genomics has enabled the accurate and rapid detection of splice site variants outside of the canonical two nucleotide splice sites flanking an exon[19]. Using this approach, it is estimated that up to 11% of ASD cases have a splice site variant amenable to ASO therapy.[19]

ASOs are highly specific, RNA-modulating therapeutics that hybridize to the pre-mRNAs via antisense orientation. This creates competition between the ASO and the spliceosome, with the potential to preventing

incorrect splicing. ASO therapy has already made a significant impact in neurological disease[17], specifically spinal muscular atrophy (SMA) and Batten's disease. SMA is an autosomal recessive disorder characterized by severe muscle weakness resulting in respiratory failure and death, often in the first year of life, caused by loss-of-function variants in the gene *SMN1*. The closely related gene *SMN2* is non-functional due to a premature stop codon. ASO therapy has been used to "skip" the exon in SMN2 with the premature stop codon, resulting in a functional protein that can replace the protein made by *SMN1*. The exon-skipping ASO targeting *SMN2* has been approved by the Federal Drug Administration (FDA) for therapy (Nusinersen®) following a Phase 3 clinical trial that demonstrated dramatic clinical improvements, with survival extended beyond one year of life of in 84% (67/80) of infants treated compared to 61% (25/41) of untreated controls.[17]

More recently, Dr. Timothy Yu's lab described the clinical improvement with ASO therapy of a child with Batten's disease, a life threatening autosomal recessive metabolic disorder that leads to seizures, blindness, and developmental delay. Batten's disease is caused by loss-of-function variants in *MFSD8*/*CLN7*, a key lysosomal gene. This ASO therapy modified a loss-of-function splice site variant in one copy of the gene, resulting in dramatic improvements in seizure frequency and duration.[20] Furthermore, this work, which was made public at the American Society of Human Genetics Conference in October 2018, raise the possibility that ASOs can be made quickly and efficiently in a personalized manner.[20]

Therefore, because loss-of-function splice site variants are a major cause of ASD, and there is a new method to rapidly and accurately detect such variants in sequencing data, I would like to develop an approach to to rapidly design and validate ASOs against *de novo* splice site variants. I believe this could dramatically improve the splicing deficit in select cases, and potentially improve neurological conditions, as has been demonstrated in other diseases. The stage is set to assess whether ASOs could rescue the genetic defects that contribute to up to 11% of ASD cases.

This proposal aims to resolve two necessary steps to leverage ASO therapy as a treatment in ASD, each described as a specific aim. **Specific Aim 1:** High-throughput detection and validation of splice site variants**. Specific Aim 2:** Can ASO therapy resolve splicing deficits caused by variants detected in ASD cases? **Specific Aim 3:** Does ASO therapy cause off target effects that may pose safety concerns?

Specific Aim 1: High-throughput detection and validation of splice site variants. From a previous studies, the Sanders' lab has identified 170 *de novo* splice site variants in 2,501 cases of ASD,[7,10] of which 70 occur at canonical splice sites, and 100 occur at cryptic splice variants near exons, predicted by the novel machine-learning derived algorithm.[19] Further, we validated these variants by in patient specific lymphoblastoid cell lines (LCLs). Filtering for genes expressed in LCLs, we randomly selected 36 of these cryptic splice site variants and performed deep coverage RNA sequencing (RNA-seq; ~350-million reads per sample). Of the 28 genes that had sufficient coverage to assess splicing, we observed clear splicing disruption in 21 (75%).[19]

Subaim 1a: WGS analysis. To expand our pool of splice sites to test, I will apply the Sanders' Lab sequencing pipeline[8] to whole-genome sequencing data for the 36 cases with LCLs. Using the novel cryptic splice site detection algorithm,[19] I will identify additional *de novo* splice site mutations and validate these new splice site variants in the existing deep coverage RNA-Seq data, described above. **Subaim 1b: Generation of a simple splicing assay.** I will then design reverse transcription-polymerase chain reaction (RT-PCR) assays for each variant to detect the splice site variant in mRNA. This assay allows variation in splicing to be assessed rapidly, cheaply, efficiently.[21] For exon skipping defects I will use gel electrophoresis to compare the size of the RT-PCR product, while for cryptic splice sites I will design primers that only detect the novel mRNA. For size differences too small to detect with gel electrophoresis, I will confirm the cryptic splice site using Sanger sequencing.

Specific Aim 2: Can ASO therapy resolve splicing deficits caused by variants detected in ASD cases? I will design candidate ASOs and test whether they can correct the splicing deficit. While prior analyses have

focused on the splicing of single exons in single genes, I will design an assay to simultaneously test at least 21 variants, helping to establish the degree to which ASO design principles can be generalized across the genome.

Subaim 2a: ASO design. For the 21 confirmed splice site variants, I will design an ASO to restore splicing function. Following on from the work in *SMN2,*[22] for intron retention variants, I will design ASOs to target 10-27 nucleotides upstream of the splice site, while for exon skipping variants I will design ASOs 10-27 nucleotides downstream of the skipped exon. Based on these designs, I will order 17-mer 2′-*O*-methyl RNA ASOs,[23] from Integrated DNA Technologies. Additionally, I will test a positive control (SMN2 exon skipping) and negative control (scrambled sequence that does not match any known human mRNA). **Subaim 2b: ASO testing.** I will add each ASO at varying concentration to the LCL culture medium and extract mRNA 48hrs later. Using the RT-PCR splicing assays developed in Aim 1b, I will assess whether the ASO is improving splicing activity. Based on the results of these splicing assays, I will identify the optimal ASO concentration to decrease the splicing deficit. The primary outcome will be the proportion of ASOs that successful resolve the splicing deficit. **Subaim 2c: Impact on protein concentration.** As we expect splice site variants to result in nonsense mediated decay of the mRNA, preventing protein translation. Consequently, if an ASO rescues the splicing deficit, we expect the concentration of protein to increase. For proteins with suitable antibodies, I will perform Western Blot analysis to demonstrate that ASOs not only rescue the splicing deficit, but that this results in increased protein levels.

Specific Aim 3: Does ASO therapy cause off-target effects that may pose safety concerns? One of the major concerns of CRISPR gene-editing technology is the potential for off-target effects that could have unanticipated consequences.[24] A similar concern exists for other genetic therapies, including ASOs.[25] The Sanders' lab pre-existing data and experimental design provide an unmatched opportunity to test for such effects. Having established the optimal ASO dose for each of the *de novo* splice site variants in the LCLs, described above, I will perform deep coverage RNA-sequencing (~350 million reads per sample). First, I will use this data to validate the splicing assay result at the locus of interest and assess whether there are new splicing deficits in the target gene. I will then examine differential splicing and differential expression genome-wide comparing RNA-seq data from ASO-treated LCLs versus untreated LCL controls. To quantify any off-target effects, I will perform two RNA-seq replicates of treated and control LCLs and assess whether there are more splicing and expression differences between samples with the same intervention status or between ASO treated and control samples. I will weight splice sites sequence similarity to the proposed ASO in our analysis. The primary outcomes will be the degree of splicing and expression differences in the presence of an ASO.

Future aims: Following the completion of this project, I will perform follow up experiments showing efficacy in neurons, from patient-derived iPSCs. I will also consider performing parallel experiments in mice engineered to have the same mutation. Since the resulting ASOs could improve symptoms in the ASD cases in which the *de novo* splice site variants were identified, we would approach the FDA regarding the possibility of clinical use of the ASO through the expanded access (compassionate use) pathway.

Significance: ASO therapy has the potential to treat millions of individuals with rare genetic disorders, including ASD. To date, ASOs have been designed for specific exons or variants; here I leverage a novel approach that will discover splice site variants across the genome, and then design and test multiple ASOs simultaneously. Furthermore, through deep coverage RNA-seq I will assess the extent and variation of off-target effects of different ASOs. Successful completion of this project will provide a method for rapidly developing a personalized therapy for numerous genetic disorders mediated by splicing variation, including ASD and many other diseases that affect military members and their dependents.

Innovation and novelty: ASO therapy is at the cutting edge of treatment for rare genetic disorders. To the best of my knowledge this is the first proposal applying ASO therapy in the context of ASD. The simultaneous design and treatment of multiple splice site variants is novel, and the application of RNA-seq to quantify off-target effects is not yet been performed. Additionally, this approach can easily be expanded to other diseases.

1. American Psychiatric Association. DSM-II. Diagnostic and statistical manual of mental disorders: DSM-IV-TR. *Am. Psychiatr. Publ.* (1968). doi:10.1111/j.1530-0277.2008.00750.x
2. Baio, J. *et al.* Prevalence of Autism Spectrum Disorder Among Children Aged 8 Years — Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2014. *MMWR. Surveill. Summ.* (2018). doi:10.15585/mmwr.ss6706a1
3. Life Journey Through Autism: A Guide for Military Families. *Autism Speaks* Available at: https://www.autismspeaks.org/support-military-families. (Accessed: 12th May 2018)
4. Tick, B., Bolton, P., Happé, F., Rutter, M. & Rijsdijk, F. Heritability of autism spectrum disorders: A meta-analysis of twin studies. *J. Child Psychol. Psychiatry Allied Discip.* (2016). doi:10.1111/jcpp.12499
5. Sandin, S. *et al.* The heritability of autism spectrum disorder. *JAMA - J. Am. Med. Assoc.* (2017). doi:10.1001/jama.2017.12141
6. Wang, K., Gaitsch, H., Poon, H., Cox, N. J. & Rzhetsky, A. Classification of common human diseases derived from shared genetic and environmental determinants. *Nat. Genet.* (2017). doi:10.1038/ng.3931
7. Sanders, S. J. *et al.* Insights into Autism Spectrum Disorder Genomic Architecture and Biology from 71 Risk Loci. *Neuron* (2015). doi:10.1016/j.neuron.2015.09.016
8. Werling, D. M. *et al.* An analytical framework for whole-genome sequence association studies and its implications for autism spectrum disorder. *Nat. Genet.* (2018). doi:10.1038/s41588-018-0107-y
9. De Rubeis, S. *et al.* Synaptic, transcriptional and chromatin genes disrupted in autism. *Nature* (2014). doi:10.1038/nature13772
10. Iossifov, I. *et al.* The contribution of de novo coding mutations to autism spectrum disorder. *Nature* (2014). doi:10.1038/nature13908
11. Levy, D. *et al.* Rare De Novo and Transmitted Copy-Number Variation in Autistic Spectrum Disorders. *Neuron* (2011). doi:10.1016/j.neuron.2011.05.015
12. Veltman, J. A. & Brunner, H. G. De novo mutations in human genetic disease. *Nature Reviews Genetics* (2012). doi:10.1038/nrg3241
13. Satterstrom, K., Kosmicki, J. & Daly, M. J. Novel genes for autism implicate both excitatory and inhibitory cell lineages in. *bioRxiv* (2018).
14. LeClerc, S. & Easley, D. Pharmacological therapies for autism spectrum disorder: a review. *P.T.* (2015).
15. Zwaigenbaum, L. *et al.* Early Intervention for Children With Autism Spectrum Disorder Under 3 Years of Age: Recommendations for Practice and Research. *Pediatrics* (2015). doi:10.1542/peds.2014-3667E
16. Guy, J., Gan, J., Selfridge, J., Cobb, S. & Bird, A. Reversal of neurological defects in a mouse model of Rett syndrome. *Science (80-.).* (2007). doi:10.1126/science.1138389
17. Finkel, R. S. *et al.* Nusinersen versus Sham Control in Infantile-Onset Spinal Muscular Atrophy. *N. Engl. J. Med.* (2017). doi:10.1056/NEJMoa1702752
18. Mendell, J. R. *et al.* Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *N. Engl. J. Med.* (2017). doi:10.1056/NEJMoa1706198
19. Jaganathan, K., Panagiotopoulou, S., McRae, J. F., Sanders, S. J. & Kai-How Farh, K. Predicting splicing from primary sequence with deep learning. *Cell* (2018).
20. Yu, T. W., Hu, C. & Berde, C. PgmNr 3570: Patient-customized oligonucleotide therapy for an ultra-rare genetic disease. in *American Society of Human Genetics* (2018).
21. Harvey, S. E. & Cheng, C. Methods for characterization of alternative RNA splicing. in *Methods in Molecular Biology* (2016). doi:10.1007/978-1-4939-3378-5_18
22. Hua, Y., Vickers, T. A., Okunola, H. L., Bennett, C. F. & Krainer, A. R. Antisense Masking of an hnRNP A1/A2 Intronic Splicing Silencer Corrects SMN2 Splicing in Transgenic Mice. *Am. J. Hum. Genet.* (2008). doi:10.1016/j.ajhg.2008.01.014
23. Altmann, K.-H. *et al.* Second generation of antisense oligonucleotides: from nuclease resistance to biological efficacy in animals. *Chimia (Aarau).* (1996). doi:10.1002/hyp.3360090305
24. Anderson, K. R. *et al.* CRISPR off-target analysis in genetically engineered rats and mice. *Nat. Methods* (2018). doi:10.1038/s41592-018-0011-5
25. Lindow, M. Assessing unintended hybridization-induced biological effects of oligonucleotides. *Nat. Biotechnol.* **30**, (2012).