Immune gene expression analysis indicates the potential of a self-amplifying Covid-19 mRNA vaccine

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Remarkable potency has been demonstrated for mRNA vaccines in reducing the global burden of the ongoing COVID-19 pandemic. An alternative form of the mRNA vaccine is the self-amplifying mRNA (sa-mRNA) vaccine, which encodes an alphavirus replicase that self-amplifies the full-length mRNA and SARS-CoV-2 spike (S) transgene. However, early-phase clinical trials of sa-mRNA COVID-19 vaccine candidates have questioned the potential of this platform to develop potent vaccines. We examined the immune gene response to a candidate sa-mRNA vaccine against COVID-19, ARCT-021, and compared our findings to the host response to other forms of vaccines. In blood samples from healthy volunteers that participated in a phase I/II clinical trial, greater induction of transcripts involved in Toll-like receptor (TLR) signalling, antigen presentation and complement activation at 1 day post-vaccination was associated with higher anti-S antibody titers. Conversely, transcripts involved in T-cell maturation at day 7 post-vaccination informed the magnitude of eventual S-specific T-cell responses. The transcriptomic signature for ARCT-021 vaccination strongly correlated with live viral vector vaccines, adjuvanted vaccines and BNT162b2 1 day post-vaccination. Moreover, the ARCT-021 signature correlated with day 7 YF17D live-attenuated vaccine transcriptomic responses. Altogether, our findings show that sa-mRNA vaccination induces innate immune responses that are associated with the development of adaptive immunity from other forms of vaccines, supporting further development of this vaccine platform for clinical application.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has exerted a heavy toll on human lives, healthcare systems and socioeconomic well-being worldwide1. Fortunately, accelerated vaccine development and emergency use approval has now begun to change what was once a potentially life-threatening infectious disease into mostly asymptomatic infection and mild febrile illness2. Amongst these vaccines are the newly deployed mRNA vaccines, which have been consistently shown to be the most effective in reducing the global burden of the ongoing COVID-19 pandemic. An alternative form of the mRNA vaccine is the self-amplifying mRNA (sa-mRNA) vaccine. Such a vaccine is built using an alphavirus genomic backbone, with the gene encoding the antigen of interest, in this case the SARS-CoV-2 spike (S) transgene. However, early-phase clinical trials of sa-mRNA COVID-19 vaccine candidates have questioned the potential of this platform to develop potent vaccines. We examined the innate immune response to a candidate sa-mRNA vaccine against COVID-19, ARCT-021, and compared our findings to the host response to other forms of vaccines. In blood samples from healthy volunteers that participated in a phase I/II clinical trial, greater induction of transcripts involved in Toll-like receptor (TLR) signalling, antigen presentation and complement activation at 1 day post-vaccination was associated with higher anti-S antibody titers. Conversely, transcripts involved in T-cell maturation at day 7 post-vaccination informed the magnitude of eventual S-specific T-cell responses. The transcriptomic signature for ARCT-021 vaccination strongly correlated with live viral vector vaccines, adjuvanted vaccines and BNT162b2 1 day post-vaccination. Moreover, the ARCT-021 signature correlated with day 7 YF17D live-attenuated vaccine transcriptomic responses. Altogether, our findings show that sa-mRNA vaccination induces innate immune responses that are associated with the development of adaptive immunity from other forms of vaccines, supporting further development of this vaccine platform for clinical application.

RESULTS

Humoral and cell-mediated responses to ARCT-021 are weakly correlated

Total RNA was extracted from the whole blood of 106 healthy volunteers who provided written informed consent for participation in a phase I/II randomised, placebo-controlled clinical trial that assessed the safety and immunogenicity of ARCT-021 (clinicaltrial.gov NCT04480957). Gene transcript levels were determined at baseline (pre-dose 1), day 2, 3, and 8 for all vaccinees. In addition, gene expression was profiled at day 29 (pre-dose 2), 30 and 36 among vaccinees in the two-injection cohort. The magnitude of antibody responses to ARCT-021 was
measured by the Luminex immuno-assay against the full-length recombinant S protein, and the S-reactive T-cell responses were assessed using IFNγ ELISPOT assay following stimulation with overlapping S protein peptide pools. The effects of vaccine dose and subject demographics on antibody and T-cell responses has been previously reported. Among the ARCT-021 vaccinees, there was variation in S-binding IgG titers (Fig. 1a) and T-cell IFNγ responses (Fig. 1b) of >100 fold. We found low or weak correlation between S-binding IgG titers and T-cell IFNγ responses (Fig. 1c), although both measurements approximate to a Gaussian...
Early innate immune responses to ARCT-021

To examine the early innate immune gene signatures induced by ARCT-021, we conducted NanoString profiling using the nCounter Human Immunology v2 Panel (NanoString Technologies), which allows quantification of 579 immune-related transcripts with high precision16. The greatest number of differentially expressed genes (DEGs) (fold change >1.3; false discovery rate [FDR]-adjusted p-value < 0.05, Benjamini-Hochberg step-up procedure) was detected at day 2, and the number of DEGs decreased three-fold at day 3 (Fig. 1d). Secondary vaccination generated similar changes in transcriptional responses at day 30 relative to day 2 (Fig. 1d), with strong correlation observed for expression of transcripts between these 2 time points (Supplementary Fig. 1b).

Notably, the most upregulated genes comprised cytokines, chemokines and interferon (IFN)-stimulated genes, including CXCL10, CCL2, CCL8, LAMP3, IFIT2 and MX1 (Fig. 1e, Supplementary Fig. 1c). Visualisation of gene networks by Ingenuity Pathway Analysis (IPA) revealed a tight interacting network of transcription factors responsible for the induction of these cytokines and chemokines, including IRF3, IRF7, STAT-1 and STAT-2 (Supplementary Fig. 2).

To dissect the molecular pathways induced by ARCT-021, we utilised the blood transcription modules (BTMs), a curated database comprising of an integrated large-scale network of publicly available human blood transcriptomes17. We found that BTMs related to activation of myeloid cells, antigen presentation, cell cycle and interferon signalling were significantly enriched on days 2, 3 and 30. The majority of transcripts returned to baseline levels at day 8 and day 36 (Fig. 1d, f-g). The upregulated transcripts in the enriched in monocytes (II) (M11.0) module included Toll-like receptors (TLR1-8), leukocyte immunoglobulin-like receptors and inflammatory mediators (Fig. 1g, Supplementary Fig. 3a), indicating that ARCT-021 vaccination leads to an early activation of innate immune responses. Furthermore, the majority of upregulated transcripts related to antigen presentation constitute the MHC-I signalling pathway, including induction of transcripts involved in peptide degradation and processing (LMP2, LMP7), peptide loading (TAP1, TAP2, TPN, CLIP) and MHC-I molecules, which could facilitate antigen presentation to CD8 T-cells (Supplementary Fig. 3b). Taken together, our data showed that vaccination with ARCT-021 triggers an early and strong induction of the innate immune response, leading to upregulation of genes involved in pattern recognition receptor signalling, cytokine and chemokine signalling and MHC-I antigen presentation.

Expression of BSIG transcripts 1 day post-vaccination correlates with humoral responses to ARCT-021

An unsupervised hierarchical clustering of the 231 upregulated DEGs at day 1 post-ARCT-021 vaccination indicated 3 distinct clusters: placebo group, C1 (purple) and C2 (yellow) (Fig. 2a). The top 10 differentially regulated genes between C1 and C2 were related to complement activation (C1QB, C2, SERPING1) and inflammatory response (CCL2, IL1RN, IDO1, TNFAIP6) (Fig. 2b, Supplementary Fig. 4a-b). The majority of participants in C1 were younger adults who received at least 3 μg of ARCT-021 (Fig. 2c). In contrast, participants in C2 consisted of either older adults or those who received lower doses (1µg-3µg) of ARCT-021 (Fig. 2c), indicating that the transcriptional responses to ARCT-021 in older adults are more blunted.

Next, we examined if the C1-C2 dichotomy affected anti-S IgG titers and S-specific T-cell IFNγ responses. While no significant differences were seen in T-cell responses (Fig. 2d, Supplementary Fig. 4c), anti-S IgG titers at day 15, 29, 43 and 57 were significantly lower in C2 compared to C1 (Fig. 2e, Supplementary Fig. 4d). To verify that the gene expression differences between C1 and C2 were indeed correlated with anti-S IgG titers, we calculated the arithmetic mean of the log2 fold change values from the top 10 transcripts that most distinctly separate C1 and C2 (termed as BSIG score) for each sample, and performed a correlation analysis with anti-S IgG titers. Indeed, the BSIG score was significantly correlated with anti-S IgG titers (Fig. 2f), thus supporting an association between the magnitude of induction of the BSIG transcripts at 1 day post-vaccination with humoral responses to ARCT-021.

A random forest regression model (Supplementary Fig. 5a) was used to identify the immune transcripts that could most accurately predict anti-S IgG titers. 75% of the samples were used as training data and the remaining 25% as test data to evaluate the accuracy of our machine learning model. Our first model generated a decision tree that was able to predict anti-S IgG titers with 84.8% accuracy, with root mean square error (RMSE) of 1.43. The relative feature importance of the individual genes involved in generation of the decision tree is as depicted in Supplementary Fig. 5b. After hyperparameter tuning, we ascertained that the random forest regression model based on the log2 fold-change values of the top 6 genes (KLRB1, CCL2, FCER1G, ITGA6, MSR1 and GPR183) further improved the accuracy to 96.0% (RMSE = 1.70) (Supplementary Fig. 5c). Notably, many of these genes are involved in leukocyte migration (GO:0060900, FDR = 0.0179), highlighting the pivotal role of chemotaxis in generation of humoral immune responses to ARCT-021.

Since the magnitude of BSIG upregulation is associated with higher antibody response, we further examined upregulated immune transcripts that best differentiate responders from non-responders. Responders were defined by subjects that seroconverted with at least a 4-fold rise in antibody titers15. In agreement with the random forest regression model (Supplementary Fig. 5b), we identified MSR1 and FCERT1G to be most significantly increased
in responders (Supplementary Fig. 6a-c). Both MSR1 and FCER1G were also significantly increased in responders compared to non-responders among the older adults (Supplementary Fig. 6d). However, the magnitude of upregulation for MSR1 and FCER1G was not associated with severity of side effects (Supplementary Fig. 6e-f). Likewise, BSIG score was not found to be associated with severity of side effects (Supplementary Fig. 6g), demonstrating that the transcriptomic signatures for ARCT-021 immunogenicity and reactogenicity are distinct.

Next, we performed pathway enrichment for downregulated genes following ARCT-021 vaccination. Negatively enriched BTMs were mostly related to T-cells and NK cells (Fig. 3a), with genes that encode for surface receptors on T-cells and NK cells being significantly downregulated at day 2 and day 30 (Fig. 3b). However, expression of downstream T-cell signalling transcripts remained unchanged (Supplementary Fig. 7), suggesting that

**Immune signatures at day 8 correlated with spike-specific T-cell response to ARCT-021**

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ARCT-021 vaccination did not lead to global suppression of T-cell activity. Instead, the downregulation of these T-cell transcripts could indicate increased migration of T-cells out of the peripheral blood, and likely to lymphoid organs. In addition, the majority of downregulated NK cell-related genes encoded for inhibitory receptors (KLRB1, KLRC1, KLRD1), suggesting an early activation of NK cells post-vaccination (Fig. 3b). To ascertain if the differences in downregulated DEGs post-vaccination could predict differences in T-cell responses, we performed an unsupervised hierarchical clustering, which generated 3 distinct clusters: placebo group, T1 (blue) and T2 (pink) (Supplementary Fig. 8a). However, the T1-T2 dichotomy was unable to discriminate subjects based on vaccine-induced T-cell response (Supplementary Fig. 8b). Instead, gene expression signatures at day 8 identified transcripts that were associated with T-cell responses. Interestingly, a total of 18 transcripts (6 positively correlated, 12 negatively correlated) were found to be significantly correlated with CD8 T-cell responses (Fig. 3c). We calculated a TSIG score for each sample by subtracting the arithmetic log2 fold change values of the 12 negatively correlated genes from that of the 6 positively correlated genes. The TSIG scores were significantly correlated with T-cell response, with correlation coefficient of 0.566 (p < 0.0001) (Fig. 3d), indicating that the expression levels of these 18 transcripts at day 8 could discriminate subjects with strong from weak T-cell response. Of note, many of the positively correlated transcripts were associated with T-cell maturation and expansion (CD27, LEF1, CD44, XB1), as well as proteasome degradation (PSMB5)18-22. Conversely, negatively correlated transcripts were mostly associated with complement activation (MASP2, C1QA, CFB), T-cell proliferation (PDCC1LG2), dendritic cell activity (TNFSF11, CD209, IFNA1/13) and type I interferon response (IFNA1/13).

Given that the top 3 positively correlated genes (CD27, LEF1 and PSMB5) were previously demonstrated to be critical for CD8 T-cell expansion, maturation and MHC-I presentation18-20,22, we examined if the random forest regression model based on log2 fold-change values of these genes could accurately predict spike-specific T-cell response. The accuracy of the model based on CD27, LEF1 and PSMB5 was found to be 70.43% (RMSE = 2.11) (Supplementary Fig. 8c), suggesting that the induction of these transcripts is important in inducing spike-specific T-cell responses.

**Comparison of ARCT-021 with other vaccines**

To understand how the host response to sa-mRNA vaccination fares against those to other vaccines, we performed a comparative analysis of our data with other published vaccine trials (Supplementary Table 1). Immune-related genes induced by different vaccines were visualized using a correlation matrix showing pairwise correlations of the mean gene log2-transformed fold changes between vaccines. When we compared gene expression at 1 day post-vaccination, ARCT-021 exhibited a strong correlation with live viral vectors (MRKAd5-HIV and rSVS-ZEBOV vaccines), adjuvanted vaccines (HepB + AS01B/AS01E and HSN1 + AS03) and mRNA vaccines (BNT162b2).

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peptide degradation and processing, peptide loading and MHC class I, which can in turn promote CD8 T-cell survival and responses. Two possible reasons may account for why T-cell signatures were more apparent at a later time point. One possibility is that the early migration of T-cells from peripheral organs, which may have hindered the detection of transcripts in tonsils or lymph nodes after sa-mRNA vaccination. Future studies that evaluate gene expression in tonsils or lymph nodes after sa-mRNA vaccination related to T-cell memory. Future studies that evaluate gene expression in tonsils or lymph nodes after sa-mRNA vaccination related to T-cell memory. Future studies that evaluate gene expression in tonsils or lymph nodes after sa-mRNA vaccination related to T-cell memory. Future studies that evaluate gene expression in tonsils or lymph nodes after sa-mRNA vaccination related to T-cell memory. Future studies that evaluate gene expression in tonsils or lymph nodes after sa-mRNA vaccination related to T-cell memory. Future studies that evaluate gene expression in tonsils or lymph nodes after sa-mRNA vaccination related to T-cell memory. Future studies that evaluate gene expression in tonsils or lymph nodes after sa-mRNA vaccination related to T-cell memory. Future studies that evaluate gene expression in tonsils or lymph nodes after sa-mRNA vaccination related to T-cell memory. Although the early transcriptomic signatures of BNT162b2 and ARCT-021 were largely similar after the first dose, an interesting observation was that the innate immune activation was not of a higher magnitude after the second dose of ARCT-021. This is in contrast to BNT162b2 and adjuvanted vaccines where the second dose resulted in greater innate immune activation. A plausible explanation could be that a single dose of the sa-mRNA vaccine is sufficient to induce significant up- or down-regulation of immune genes as self-replication results in double-stranded RNA intermediates with immunostimulatory properties that can likely prolong and intensify the ensuing innate immune response. However, over-activation of innate immune response may block translation and amplification of sa-mRNA, and potentially reduce vaccine immunogenicity. We observed that the initial stimulation of innate immune response following ARCT-021 vaccination resolves by day 8. Moreover, downregulation of interferon-alpha at day 8 was associated with better T cell responses.

In addition, our findings also suggest the potential of sa-mRNA vaccines to be further engineered to elicit the innate immune responses and consequently the immunogenicity associated with LAVs. Indeed, the association of sa-mRNA signatures with the YF17D day 7 signatures, as well as the delayed expression of the YF17D day 7 signatures, as well as the delayed expression of the
ARCT-021 gene signatures are correlated with live viral vectors, adjuvanted and mRNA vaccines. a Correlation matrix showing pairwise correlations of the mean gene log2-transformed fold changes between the different vaccines at time-points with peak transcriptional responses. Transcriptional responses peaked on day 7 for live-attenuated vaccines and Pneumovax, day 3 for H5N1 and day 1 for all other vaccines. Size and intensity of dots are proportional to the magnitude of correlation coefficient. b Pearson correlation of log2-transformed fold-changes of transcripts in subjects receiving rVSV-ZEBOV vs ARCT-21 at day 1 post-vaccination\(^{26,27}\). r = correlation coefficient, p = significance of correlation.

correlates of T cell responses at day 8 suggests that a longer period of antigen presentation may favour more robust B and T cell development. This could be achieved by introducing mutations in the sequence of non-structural proteins encoding the replicase to either slow down mRNA replication kinetics or through sequence optimization of S' and 3’ untranslated regions that may alter RNA secondary structures to avoid prematurely triggering the host immune response for enhanced translation of the sa-mRNA\(^{36,37}\). Consequently, further investigation of the interactions between sa-mRNA and host proteins would be warranted to strategically avoid a sharp but short surge of innate immune activity to prolong sa-mRNA replication and translation for enhanced antigen presentation and vaccine immunogenicity.

In conclusion, we identified gene expression signatures that correlated with humoral and T-cell responses to ARCT-021 vaccination and showed that sa-mRNA is able to trigger the innate immune responses observed in viral vector vaccines, AS01 and AS03 adjuvanted vaccines as well as BNT162b2. The molecular insights underpinning induction of adaptive immune response to ARCT-021 can guide the development of next-generation sa-mRNA vaccines for improved immunogenicity.

METHODS
Clinical trial design
ARCT-021-01 is a randomized, double-blinded, placebo (0.9% saline) controlled study, evaluating a range of doses of ARCT-021 relative to placebo in younger (21 to 55 years) and older (56 to 80 years) adult participants\(^{16}\). A total of 106 healthy participants were randomized into single and two-injection cohorts. In single injection cohorts, ARCT-021 was given as an intramuscular injection to younger (1 µg, 5 µg, 7.5 µg and 10 µg ARCT-021 versus placebo) and older adults (7.5 µg ARCT-021 versus placebo). In two-injection cohorts, two doses of ARCT-021 (3 µg and 5 µg ARCT-021 versus placebo) were administered 28 days apart to younger and older adults.

Immunogenicity assessments
Luminex immuno-assays were conducted to measure IgG against full-length recombinant spike protein at indicated time points following vaccination. Full-length recombinant SARS-CoV-2 spike protein (AcroBiosystems) was conjugated to Magpix Luminex beads using the ABC coupling kit (Luminex). Spike protein conjugated beads were blocked with 1% BSA in PBS with 0.05% Tween 20 and incubated with sera for 1 hour at 37 °C. Beads were then washed and incubated with PE-conjugated anti-human IgG secondary antibody (Invitrogen) for 30 minutes at 37 °C. Spike-binding antibodies were measured as median fluorescence intensity (MFI) using a Magpix instrument. SARS-CoV-2 specific T-cell responses were assessed using IFNy ELISPOT assay following stimulation with overlapping S protein peptide pools. ELISPOT plates were coated with human IFNy antibody (Mabtech) overnight at 4 °C. 200,000 PBMCs were seeded in each well and stimulated overnight with the S protein peptide pools. Plates were incubated with human biotinylated detection mAb (7-B6-1) before streptavidin-ALP was added. Plates were developed using BCIP/NBT phosphatase substrate (Seracare) and results expressed in spot forming cells (SFC)/10° PBMCs.

NanoString profiling
NanoString profiling was performed using the nCounter Human Immunology v2 Panel (NanoString Technologies, Inc. Cat# XT-CSO-HIM2-12). 50 ng of RNA was mixed with 8 ul of reporter probeset and 2ul of capture probe set, and incubated at 65 °C for 24 h. Hybridised samples were loaded onto an nCounter Sprint cartridge and imaged on a nCounter Sprint system. Raw and normalised counts were derived with the NanoString nSolver 4.0 software as per manufacturer’s protocols. nCounter probe sets included negative and positive controls that were used for background thresholding, and normalizing samples for differences in sample input or hybridisation.

Transcriptomics analysis
To identify DEGs, gene expression values were compared between vaccinated and placebo subjects. DEGs were identified based on fold-change >1.3 with respect to the placebo controls at the respective days, with false discovery rate adjusted p-value < 0.05 using the Benjamini-Hochberg step-up FDR-controlling procedure in Partek® Genomics Suite®. For pathway analysis, the identified DEGs were used as input data, and analysed against the blood
transcriptomics modules (BTMs) database. Enriched pathways were ranked and identified based on adjusted p-values < 0.05, using GSEApy, a Python wrapper for GSEA and Enrichr. Ingenuity pathway analysis was used to detect the gene networks and pathways that are differentially regulated after ARCT-021 vaccination. Unsupervised hierarchical clustering and heatmaps were plotted using Seaborn, which is a Python data visualisation library based on matplotlib. BSIG scores for each subject were tabulated by calculating the arithmetic mean of the log2-fold change values (day 2 vaccinated versus placebo controls) of IDO1, LAMP3, SERPING1, CCL2, IL1RN, TNFAIP6, C1QB, CEACAM1, TNFSF10 and C2, which are the top 10 genes that are most differentially expressed between C1 and C2 groups. TSIG scores for each sample was calculated by subtracting the arithmetic mean of the log2 fold change values of the 12 negatively correlated genes from the 6 positively correlated genes.

Analysis of gene expression and adverse events
Adverse events (AEs) were classified by system organ class according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0, and graded as mild, moderate or severe. For analysis of gene expression and side effects, subjects were grouped based on the highest grading of adverse events (AEs) reported. Subjects with no systemic AEs were scored as 0, mild AEs as 1, moderate AEs as 2 and severe AEs as 3.

Vaccine dataset meta-analysis
Datasets were obtained from Gene Expression Omnibus (GEO) via accession identifiers shown in Supplementary Table 1. For MRKAd5-HIV dataset, host transcriptomics responses were only analysed for the seronegative subjects, as the vaccine did not protect against HIV acquisition in seropositive subjects. CEL files were normalised and analysed with the Partek® Genomics Suite®. For RNAseq dataset, the normalised count matrix from GEO was used. Normalised log2 fold change values compared to baseline were computed for each vaccine, and the peak transcriptional responses were used for plotting of the correlation matrix. Correlation matrix was then visualised and plotted in R, using the corr() and corrplot() functions.

Statistics
Differentially expressed genes (DEGs) were defined as genes with fold-change > 1.3 and False Discovery Rate (FDR)-adjusted p-value < 0.05, Benjamini-Hochberg step-up procedure in Partek Genomics Suite. Ranking and identification of enriched pathways was based on adjusted p-values < 0.05, using GSEApy. Unpaired, two-tailed Student’s t-test was used for comparisons between vaccinated groups using Prism v.9.1 software (GraphPad Software Inc.).

Study approval
This study was approved by the SingHealth Centralized Institutional Review Board (CIRB/F 2020/2553) and the Singapore Health Sciences Authority. The trial was registered at ClinicalTrials.gov (NCT04480957). Written informed consent was obtained from all participants before enrolment.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY
The raw data and log, fold change values for NanoString profiling of immune responses are available at Array Express (E-MTAB-11315).
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AUTHOR CONTRIBUTIONS
E.Z.O., J.X.Y., A.S., R.d.A., S.C. and Y.S.L. performed the experiments. E.Z.O., J.X.Y., J.S.G.O. and K.R.C. conducted the statistical and data analyses. S.K., J.X.Y.S., Y.F.Z.C. and J.G.L. enrolled the subjects of the trial. R.S., B.M.S., K.L., S.B.S., P.C., S.G.H., J.G.L. and E.E.O conceived and designed the study. E.Z.O., J.G.L., K.R.C. and E.E.O. reviewed the data and wrote the manuscript.

COMPETING INTERESTS
The authors declare the following competing interests: this study was partially funded by Arcturus Therapeutics, Inc. Rose Sekulovich, Brian M. Sullivan, Kelly Lindert, Sean B. Sullivan, Pad Chivukula and Steven G. Hughes are employees of Arcturus Therapeutics, Inc.

ADDITIONAL INFORMATION
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