

Nonviral follistatin gene therapy improves body composition and lowers epigenetic age in healthy adults

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Acknowledgements:

We would like to thank Mae Davis and Tristan Roberts for contributing to the collection and management of patient data, and Neville Kahsai for work performing the ELISAs. We would also like to thank Jessica Cheyane Parker.

Author contribution statement:

Walter Patterson and Mac Davis conceived, developed, and managed the project. Glenn Terry oversaw clinical application, study design, and data collection. Walter Patterson constructed the plasmid and performed ELISAs. Raven Garuda analyzed the data. Ryan Rossner helped with data analysis and results interpretation. Ryan Rossner and Mac Davis wrote the manuscript.

Data availability statement:

The raw data from this study are accessible through Zenodo at <https://zenodo.org/records/17718682?preview=1&token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6IjViODBkYjc5LTllODEtNDU3OC05OGZiLTljZjVIMDMxY2EzMSIsImRhdGEiOnt9LCJyYW5kb20iOiJiN2VjMmJhZGEzMmI1N2NkNmRjNzI0YTljZmE4MWIxYSJ9.OUQMia-xQtTTjeT1KmmuNtCd4hPVP9Rs7wsiexETlmWKd5xRm>. Data will be available indefinitely and include deidentified participant data and a data dictionary.

Conflicts of Interest:

All authors except Glenn C. Terry are employees of Minicircle Inc. GARM Clinic, at which Glenn C. Terry is a practicing physician, operates in commercial partnership with Minicircle Inc.

Funding:

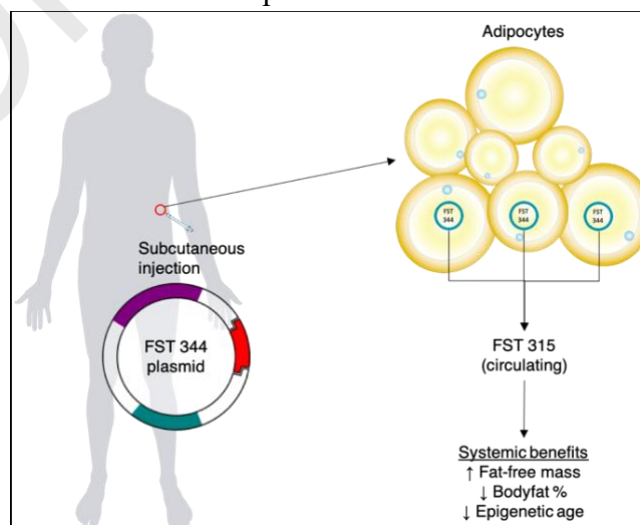
This research was sponsored by Minicircle Inc.

Abstract

Background: Despite the fact that age is one of the strongest predictors of frailty and frailty-related diseases, current treatment protocols for older adults target symptoms instead of the root biological mechanisms underlying age-related frailty. Follistatin gene therapy has so far been used to regulate and increase muscle in patients with degenerative muscle disorders, usually with a modified adeno-associated virus (AAV) vector. The goals of the present study were two-fold: 1) To test whether follistatin gene therapy can improve the health and epigenetic age of adults who do not have any diseases, and 2) to test this question using a nonviral plasmid delivery method, given the potentially favorable safety profile of plasmids relative to viral vectors. **Method:** We injected 50 μg plasmid delivering human follistatin (FST) 344 cDNA into subcutaneous abdominal fat of 42 adult human volunteers and tracked their health over 3 months. Pre- and post-administration measurements included body composition, inflammation, epigenetic age, and serum follistatin levels, as well as a background hormonal and metabolic health panel to ensure that health effects occurred within the context of broader physiological stability (final n per variable = 23-32). **Results:** We found that serum follistatin levels were significantly elevated 3 months post-injection and that body composition and extrinsic epigenetic age improved, especially in older participants. As expected, the background hormonal/metabolic panel remained relatively stable, although a few participants showed elevated LDL. No moderate or severe adverse events were reported. **Takeaway:** The present study shows that follistatin gene therapy has potential to be a novel, powerful, safe way to combat age-induced frailty. Our findings also provide evidence that nonviral plasmid DNA delivery can be efficacious and safe. This is important given that, in addition to being cheaper to manufacture than viral vectors, nonviral methods are free from replication-competent virus concerns and come with a much lower risk of insertional mutagenesis, which are important advantages for clinical safety.

Key words: follistatin, muscular dystrophy, adeno-associated virus (AAV), plasmids, gene therapy, aging, sarcopenia, osteoporosis

Graphical abstract



Introduction

With the population of adults over age 65 expected to double by 2050 (United Nations, 2020), frailty is a growing global health concern. This is important because age is one of the strongest risk factors for frailty-related diseases and conditions, including sarcopenia, osteoporosis, and metabolic dysfunction (Fulop et al., 2010). While frailty creates an array of health issues, studies estimate that falls alone cost the US healthcare system \$80 billion per year (Haddad et al., 2024). It is undeniable that we need strategies to support older adults' health. However, despite advances in geroscience, current therapies manage symptoms instead of directly addressing the biological mechanisms that underlie age-related frailty. Gene therapy targeted at follistatin (FST) has the potential to mitigate these risks significantly. The present study aimed to understand the effects of FST administration as a potential gene therapy for aging and frailty in healthy adults.

Follistatin

Frailty increases with age and is driven by rapid declines in muscle size, muscle strength, and bone mineral density (BMD) (Ferruci & Fabri, 2018; Hoogendijk et al., 2019; Palliyaguru et al., 2019). FST has anti-inflammatory effects and has shown promise for combating physical frailty (Al-Zaidy et al., 2015; Kota et al., 2009; Lee & McPherron, 2001). The mechanisms by which it mediates these effects are reasonably well understood (Rodino-Klapac et al., 2009). FST binds to Transforming Growth Factor beta ($TGF\beta$) proteins in circulation, primarily Activin and Myostatin, and subsequently prevents their binding to and activation of the Activin Receptor Type II B (ActRIIB) cell surface receptor (Zhang et al., 2018). FST improvement of lean mass and strength requires both Activin and Myostatin inhibition (Gilson et al., 2009; Lee & McPherron, 2001), but FST-mediated improvement of inflammation is thought to primarily require Activin inhibition (Hedger et al., 2011). In both human and animal trials, FST has a consistent safety profile and has been shown to be effective at improving healthy longevity (Guha, Pichavant, & Calos, 2019; Kota et al. 2009; Mendell et al. 2015).

FST gene therapy has so far been researched as an intervention for muscle degeneration diseases. For example, one research group injected FST using adeno-associated virus (AAV) delivery in six participants with Becker muscular dystrophy (Mendell et al., 2015). The researchers found that it improved walking distance in four participants by 29-125 meters. The FST treatment also resulted in reduced endomysial fibrosis, central nucleation, and improved fiber size distribution, with no adverse effects reported within one year of follow-ups. A different clinical trial also found favorable effects for sporadic inclusion body myositis (Mendell et al., 2017).

Although it has therapeutic potential for muscle degeneration, FST may also be beneficial in the absence of disease, particularly for increasing and regulating muscle mass/strength. In one study (Kota et al., 2009), researchers administered an FST intramuscular injection to nonhuman

primates that did not have a degenerative muscle disease. There were significant increases in strength as well as muscle size, and follistatin levels remained elevated at a 15-month post-injection blood draw. At 4-week health assessments for the duration of the 15 months of follow-up, health measures remained within normal limits (i.e, organ function, reproduction, and muscle integrity). These findings add to the evidence that follistatin is safe and could have globally useful effects even in the absence of a specific disease.

Plasmids

Plasmids offer a potentially safer and cheaper option than viral vectors for DNA therapies and vaccines (Hardee et al., 2017). However, they have not been widely adopted so far, with their main limitation being concerns about their effectiveness. Viral vectors, on the other hand, are highly effective at transfecting cells and have been more widely adopted by clinical practice. However, even with tight regulation surrounding viral vector manufacturing, the risks of innate immunity and/or insertional mutagenesis remain and have been reported in hundreds of clinical trials (Flotte, 2007).

Plasmids are nonviral, non-integrating DNA molecules that do not insert into the host genome, which mitigates the risk of insertional mutagenesis. Plasmids are also less likely to provoke an immune response than viral vectors (Bessis, GarciaCozar, & Boissier, 2004). Plasmids were conceptually described in the 1920s (Griffith, 1928) and named in the 1950s (Lederberg, 1952). They were first isolated in 1969 (Cohen & Miller, 1969) and first engineered in the early 1970s (Cohen et al., 1973). By the late 1990s, plasmids were viewed as safe but ineffective (U.S. Department of Health and Human Services, 1996). In 1997, plasmids with the bacterial elements excised, called "minicircles" were developed (Darquet et al., 1997), and in 2003, they were demonstrated to be long-lasting (Chen et al., 2003). Nanoplasmid technology (Luke, 2009) and Mini-intronic plasmids (MIPs) (Lu, 2013) and further developed this strategy over the next ten years. As of 2017, nearly 200 clinical trials had been conducted on plasmid-based therapeutics, including gene therapies and DNA vaccines (see Hardee et al., 2017 for review).

The main limitation and hesitancy to adopt plasmids has traditionally been driven by concerns about their effectiveness. That is, plasmids have poor transfection on their own. However, when combined with a dependable delivery method, transfection can be done efficiently. This has been demonstrated for many DNA-based therapeutics, including FST (Guha, Pichavant, & Calos, 2019).

Because plasmid DNA alone is a poor transfection agent, it must be combined with a delivery method. Cationic polymers have been widely employed in gene delivery applications (Al-Dosari & Gao, 2009). One such cationic polymer, called polyethylenimine (PEI), is understood to be one of the most effective agents for transfecting nucleic acids into mammalian

cells. PEI's mechanism of action and toxicity profile are well-understood. PEI's positive charge enables interaction with cell membranes and subsequent endocytosis (Boussif et al., 1995). The main concern with PEI is toxicity and inflammation (Hall et al., 2017); however, these problems are generally limited to conditions that are modifiable. PEI can be linear (LPEI) or branched (BPEI), and a range of molecular weights are possible for both types. The toxicity associated with PEI is mostly limited to BPEI and is dependent on free, non-complexed PEI (Erbacher et al., 2004; Godbey et al., 1999). LPEI has lower toxicity (Zakeri et al., 2018) and accumulates less in tissues than BPEI (Jeong et al., 2007). The 4:1 PEI:DNA ratio used in the present study minimized free PEI (see Method). LPEI hydrochloride has been shown to produce neither proinflammatory cytokines nor alterations to hepatic enzyme levels in vivo (Bonnet, Erbacher, & Bolcato-Bellemin, 2008; Kawakami et al., 2006). Additionally, cytotoxicity is directly related to molecular weight and dose (Godbey, Wu, & Mikos, 1999). Given that PEI molecular weights generally range between 25-800 kDa (Bieber and Elsässer, 2001), the PEI in the present study was on the low end of this range (40 kDa).

PEI-DNA administration has been demonstrated as efficacious with low-grade or no side effects in human patients in transgenic vaccination for B-cell lymphoma (Meleshko et al., 2022; see also Ohana et al., 2004). This is in line with findings from animal models that suggest that PEI delivery of plasmids is well-tolerated and less inflammatory than viral vectors like adenovirus (Oh et al., 2007).

The Present Study

The objective of the present study was to investigate follistatin as a gene therapy for healthy longevity using a (nonviral) plasmid delivery method in healthy adult humans. A paradigm shift has taken place in the last decade toward a strategy called geroscience that treats aging itself as the biggest risk factor for major killer diseases (Kaeberlin et al., 2015; Kennedy et al., 2014; Sierra, 2016). However, our medical system lacks strategies to directly address the underlying biological mechanisms of aging and frailty, with current approaches generally only addressing symptoms. At the time of this paper, there were only two previously published studies that delivered FST gene therapy in humans, and both used a viral delivery method (Mendell et al., 2015, 2017). The present study's objective was accomplished by administering FST to healthy adults and then, across a 3-month period, monitoring serum follistatin levels, body composition, inflammation markers, and biological age measurements. It was hypothesized that at a 3-month follow-up, serum FST levels would be elevated and body composition, inflammation markers, and biological age measurements would improve. Evidence in favor of this hypothesis would support the possibility of FST as a gene therapy for longevity that healthy humans could benefit from, even in the absence of a disease.

Methods

Participants

To be included in the study, participants had to be adults over the age of 23 without any genetic disorders or autoimmune diseases. Participants were excluded if they were unable to travel, were pregnant or lactating, or had a genetic disorder or autoimmune disease. Forty-two participants were recruited across the United States (age range 23-88 years; 72% males, 28% females; Table 1). Some participants failed to complete a follow-up measurement (like DEXA scans or blood draws) and in such cases were dropped on a per-variable basis, resulting in sample sizes per measure ranging between 23 and 32 (Table 2).

Table 1

Descriptive statistics

Group	n	Age		
		Median	Min	Max
All	42	48	27	88
Male	28	42	28	88
Female	14	57	27	86

This study was registered at clinicaltrials.gov as #NCT06411366. Ethics approval was obtained from the Global Alliance for Regenerative Medicine (GARM) IRB committee. Written informed consent was obtained from all participants. Participants were volunteers who did not receive any financial compensation. Participants were asked to pay for their own lab work and travel, but financial support was provided to participants in need. The test treatment was provided at no cost to participants.

Plasmid construct

Follistatin isoform. A plasmid was constructed delivering the 344 amino acid secreted isoform of the human follistatin gene. Transgene expression was driven by a CMV promoter. FST 344 was used to increase circulating FST 315 based on specific characteristics of FST 344 as well as prior research groups' results (Kota et al., 2009; Mendell et al., 2015). FST is initially produced in two isoforms, FST 317 and FST 344 (Rodino-Klapac et al., 2009) (Figure 1). Both are secreted then subsequently cleaved from their secretory signal peptide, resulting in FST 288 and FST 315, respectively. FST 344 and FST 315 contain a C-terminal acidic region that prevents FST 315 from binding to the plasma membrane's extracellular surface, subsequently allowing FST 315 to be released into circulation. FST 317 and FST 288 lack this C-terminal feature. Therefore, FST 288 is not the major circulating form of FST.

FST is a secreted protein, meaning it is made in one cell, exported to the cell surface, cleaved of its signal peptide, and released into systemic circulation via the blood (Rodino-Klapac et al., 2009). This means we could transfect a small number of cells and have them become

export factories for FST. Thus, we injected the plasmid subcutaneously to transfect a small number of adipocytes capable of subsequently creating systemic effects.

Vector Purification and Characterization. The sequence was verified by third party synthesis post-oligo ligation via m-13 reverse and forward primers as well as next-generation sequencing by GeneWiz. Endotoxin concentration was determined to conform to FDA Guidance for Industry recommendations (U.S. Department of Health and Human Services, 2007) via kinetic LAL assay performed by third-party Charles River Laboratories.

Transformation Technique. Transfection was achieved by means of 40 kDa linear polyethyleneimine (Polysciences catalog no. 24765-1) coadministration with plasmid. This relatively low molecular weight PEI was chosen to reduce cytotoxicity (possible ranges are 25-800 kDa; Bieber and Elsässer, 2001). A 4:1 ratio of transfection agent to plasmid was used per guidelines by the manufacturer, Polysciences.

Measurements

The overarching hypothesis – that biological indicators of health and longevity would improve following FST administration – was operationalized by measuring body composition (fat-free mass, body fat percentage, and bone mineral density), inflammation markers (high-sensitivity C-reactive protein (hsCRP) and homocysteine (Hcy)), and biological age (intrinsic/extrinsic epigenetic age, DunedinPace, and DNA methylation-based estimates of telomere length). A secondary panel of metabolic and hormonal background markers was also collected in order to characterize participants' overall health status and ensure that any observed effects could be interpreted in the context of broader physiological stability: follicle-stimulating hormone (FSH), luteinizing hormone (LH), insulin, leptin, glucose, hemoglobin A1c (HbA1c), total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), albumin-globulin (A/G) ratio, and triglycerides.

Dual-energy x-ray absorptiometry (DEXA). The following variables were measured using DEXA scans: fat free mass, body fat percentage, and bone mineral density.

Blood biomarkers. The following biomarkers were measured via blood draw: serum FST, hsCRP, Hcy, A/G ratio, HbA1c, FSH, LH, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, leptin, insulin, and glucose.

Biological aging. The following were calculated by TruDiagnostic (Lexington, KY, USA) using proprietary algorithms applied to DNA methylation data from whole blood: Intrinsic Epigenetic Age (IEA), Extrinsic Epigenetic Age (EEA), DunedinPACE, and DNA methylation-based estimates of telomere length (DNAmTL). These measures are derived from established DNA methylation clocks, with IEA and EEA being based on first generation clocks originally

described by Horvath (2013) and Hannum (2013) and refined into a second generation clock by Levine et al. (2018). DunedinPACE is based on the pace of aging model described in Belsky et al. (2022). DNAmTL is a methylation-based estimate of telomere length as defined by Lu et al. (2019) that has been found to predict health outcomes such as time to death, time to heart disease, and smoking history.

Procedure

FST gene therapy injections were administered at the GARM clinic in Próspera ZEDE, Honduras. Two sterile vials, one containing plasmid and one containing PEI, were mixed and incubated at room temperature for 30 minutes prior to administration. After incubation, 400 μ l of saline solution containing 50 μ g of plasmid was injected into participants' abdominal subcutaneous fat. The primary physician observed patients for 15 minutes post-injection in case of allergic reactions or other complications.

Serum follistatin was measured prior to administration as well as three months post-administration. The FST 344 concentration was determined using the Invitrogen Follistatin (FST) Human ELISA Kit. This assay kit is a colorimetric sandwich-type ELISA provided by ThermoFisher, catalog #EHFST.

Biomarkers were also measured prior to FST administration and three months post-administration. For bloodwork, participants were directed to their local Quest Diagnostics clinics. For bone scans, they were directed to a clinic local to them that could provide DEXA, such as BodySpec or a university medical center.

Analyses

Data privacy. To protect privacy, participant names were anonymized with multi-character letter/number codes. The code key was stored in a secure cloud storage service separately from the data.

Study design. This longitudinal study used a within-subjects pre-post design to analyze baseline and follow-up measurements.

Statistical Analysis. To analyze a measurement for significant changes, only participants with a baseline and a month 3 measurement were included. Distributions were checked for normality and extreme outliers (≥ 3.0 SD from the mean). Shapiro-Wilk test was performed on change from baseline (month 3 - baseline) measurements, where $p < .05$ indicates a violation of the assumption of normality. A one-sample Student's t-test was conducted to compare baseline to month 3 measurements in normally distributed change-from-baseline measurements with no extreme outliers. A one-sample Wilcoxon signed-rank test (V) was performed on change-from-baseline measurements that violated at least one of the assumptions. The following variables

violated the assumption of normality: fat free mass, bone density, EEA, DunedinPACE, FST, FSH, LH, total cholesterol, HDL cholesterol, triglycerides, insulin, and hsCRP, and Hcy. All other variables were normally distributed with no extreme outliers.

All p-values for primary variables (Table 2) were FDR adjusted within their domain (body composition, inflammation markers, or biological age) except for FST, which was the only variable tested in its domain. Variables measured as part of a background metabolic/hormonal panel (Table 3) were left uncorrected in order to minimize the type II error rate, since the primary concern for them was safety. The exception was LDL, for which multiple FDR-corrected tests were conducted on its subgroups. The two-sample *t*-test used to compare the LDL response groups' baselines used Welch's correction for unequal variances.

Individual Data. Each participant had a set of measurements per variable: pre-treatment (months -1 and 0) and treatment (month 3). Months were numbered relative to the date of FST administration. For body composition and bloodwork, baseline values were calculated as the mean of the two pre-treatment timepoints when both were available; if only one pre-treatment value was available, that measurement was used as baseline. Other domains included only month 0 as baseline. Change from baseline was calculated by subtracting the baseline value from the treatment value.

Longitudinal studies always carry a risk of attrition or loss of follow up measures. In cases where a participant failed to get one of their follow up measurements within the given timeline, data were removed on a per-variable basis. In some cases, a participant got their DEXA scan done at a location that missed one or more variables, in which case data were again removed on a per-variable basis. Data from a cohort of 42 participants were used, with the final *n* per ranging between 23 and 32 for any given variable (see Table 2).

Results

Primary analyses

Serum follistatin was significantly higher at the 3-month mark compared to baseline, with a median increase of 12.1 ng/mL ($V = 22$, $p < .001$; Figure 2). Significant improvements were found in two body composition markers, with fat-free mass showing a median increase of 1.40 lbs ($V = 238$, $FDR\text{-corrected } p = .004$) and body fat percentage showing a mean decrease of 0.9% ($t(22) = -2.80$, $FDR\text{-corrected } p = .016$; Figure 3). Inflammatory markers (hsCRP and Hcy) did not show significant changes, nor did DunedinPACE. Extrinsic epigenetic age (EEA) improved significantly, with a median decrease of 5.9 years ($V = 45$, $FDR\text{-corrected } p = .004$; Figure 5). Intrinsic epigenetic age (IEA) only trended towards significance ($t(24) = -2.18$, $FDR\text{-corrected } p = .079$; Figure 6). Changes in DNAmTL were not significant. All statistical results have been summarized in Table 2.

Within variables, visual interpretation revealed that EEA, IEA, and DNAmTL effects were potentially more robust in older adults, but only DNAmTL reflected this at a statistical level (see Figures 4, 5, and 6 respectively). That is, a Kruskal-Wallis test indicated significant changes by age group ($\chi^2(2) = 7.52, p = 0.023, \varepsilon^2 = 0.25 [-0.03 - 0.69]$). Post hoc Dunn's tests showed that participants aged 61+ exhibited significantly greater telomere lengthening from DNAmTL compared to those aged 21-40 (*FDR-corrected* $p = .020$).

Table 2

Summary of results

Domain	Variable	Unit	<i>n</i>	Mean Δ [SE]	Median Δ [IQR]	Min Δ	Max Δ	Test statistic (df), adjusted p-value, effect size [95% CI]
FST	FST	ng/mL	32	+15.44 [2.13]	+12.10 [9.54]	+1.67	+59.51	$V = 22, p < .001^{***}, r = .82 [0.76 - 0.86]$
Body composition	FFM	lbs	23	+1.87 [3.0]	+1.4 [2.6]	-2.57	+12.2	$V = 238, p = .004^{**}, r = .64 [0.36 - 0.84]$
	BF%	%	23	-0.9% [0.3%]	-0.8% [2.1%]	-3.8%	+1.5%	$t(22) = -2.80, p = .016^*, d = -0.58 [-1.08 - -0.22]$
	BMD	g/cm ²	22	+0.006 [0.005]	+0.004 [0.016]	-0.049	+0.074	$V = 166, p = .085, r = .37 [0.03 - 0.72]$
Inflammation	hsCRP	mg/L	22	-0.3 [0.4]	0.0 [0.2]	-6.6	+2.9	$V = 34.5, p = .463, r = .18 [0.01 - 0.58]$
	Hcy	μmol/L	22	-2.4 [3.3]	+0.3 [2.0]	-71.7	5.7	$V = 144, p = .290, r = .33 [0.02 - 0.70]$
Biological aging measures	EEA	years	25	-8.2 [2.2]	-5.9 [6.9]	-37.7	+10.2	$V = 45, p = .004^{**}, r = .63 [0.34 - 0.86]$
	IEA	years	25	-4.3 [2.0]	-1.85 [7.55]	-27.7	+18.4	$t(24) = -2.18, p = .079, d = -0.44 [-0.81 - -0.04]$
	DNAmTL	kB	25	+0.065 [0.037]	+0.040 [0.170]	-0.270	+0.55	$t(24) = 1.77, p = .120, d = 0.35 [-0.04 - 0.73]$
	DP	years	25	0.00 [0.02]	+0.02 [0.09]	-0.39	+0.21	$V = 178, p = .432, r = .17 [0.01 - 0.58]$

p-values were FDR-corrected within domain. FST, follistatin; FFM, fat-free mass; BF%, body fat percentage; BMD, bone mineral density; hsCRP, high sensitivity c-reactive protein; Hcy, homocysteine; EEA, extrinsic epigenetic age; IEA, intrinsic epigenetic age; DNAmTL, DNA methylation-based estimates of telomere length; DP, DunedinPACE.

Background health panel

We administered a metabolic and hormonal panel so that we could interpret the above-mentioned changes in the context of general physiological and health stability. These variables were measured to ensure that in the presence of “healthy” changes, other health markers were not getting affected. A/G, BMD, Glucose, HbA1c, insulin, leptin, TC, HDL, and TG did not change (Table 3).

Table 3*Background metabolic and hormonal panel*

Variable	Unit	<i>n</i>	Mean Δ [SE]	Median Δ [IQR]	Min Δ	Max Δ	Test statistic (df), adjusted p-value, effect size [95% CI]	
A/G	-	22	-0.02 [0.02]	-0.01 [0.08]	-0.18	+0.15	$t(21) = -1.08, p = .292,$ $d = -0.23 [-0.68 - 0.21]$	
FSH	mIU/mL	22	+2 [1.7]	+0.8 [2.7]	-14.2	+27.7	$V = 161, p = .276,$ $r = .24 [0.02 - 0.61]$	
LH	mIU/mL	22	+1 [1]	+0.9 [2.4]	-16.2	+10.6	$V = 192, p = .033^*,$ $r = .45 [0.05 - 0.80]$	
Glucose	mg/dL	23	+1 [2]	+2 [12]	-17	+28	$V = 121.5, p = .848,$ $r = .07 [0.01 - 0.50]$	
HbA1C	%	17	+0.1 [0]	+0.1 [0.2]	-0.3	+0.4	$t(26) = 1.25, p = .228,$ $d = 0.29 [0.02 - 0.73]$	
Insulin	μIL/mL	22	+0.6 [0.8]	+0.9 [4.1]	-9	+7.2	$t(21) = 0.8, p = .431,$ $d = 0.30 [-0.19 - 0.84]$	
Leptin	ng/mL	18	+0.4 [0.3]	+0.3 [1.3]	-2.4	+3.2	$t(17) = 1.55, p = .139,$ $d = 0.37 [-0.10 - 0.97]$	
TC	mg/dL	17	+6 [6]	+6 [34]	-28	+55	$V = 92, p = .478,$ $r = .18 [0.02 - 0.59]$	
HDL	mg/dL	22	+2 [2]	+2 [6]	-11	+32	$V = 132, p = .322,$ $r = .22 [0.01 - 0.58]$	
LDL	mg/dL	22	Refer to Table 4			-21	+53	Refer to Table 4
TG	mg/dL	16	-5 [8]	-2 [21]	-100	+36	$V = 63, p = .816,$ $r = .06 [0.01 - 0.53]$	

A/G, android/gynoid fat ratio; FSH, follicle-stimulating hormone; LH, luteinizing hormone; HbA1c, hemoglobin A1C; TC, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglycerides.

Low density lipoprotein (LDL) cholesterol responses showed a clear bifurcation, with participants separating into responder and non-responder subgroups. Baseline LDL levels were comparable between groups (Figure 7). Over the 3-month intervention, non-responders (64%) demonstrated modest decreases in LDL, while responders (36%) exhibited marked increases (Table 4). These distinct response patterns were not explained by age or sex and warrant further investigation.

Table 4*LDL cholesterol responses*

Group	Baseline LDL (mg/dL, mean $\pm$ SE)	Month 3 LDL (mg/dL, mean $\pm$ SE)	Δ LDL (median, IQR)	Test statistic (df), adjusted p-value, effect size [95% CI]
Non-responders (<i>n</i> = 14)	101 $\pm$ 7	94 $\pm$ 8	-7 [-12 to +2]	$V = 14, p = .045^*, r = .57 [0.14 - 0.84]$

Responders ($n = 8$)	106 ± 12	140 ± 14	$+30 [+26 \text{ to } +35]$	$V = 36, p = .023^*,$ $r = .89 [0.89 - 0.90]$
Between groups at baseline	-	-	-	$t(10.8) = -0.33, p = .748,$ $d = -0.15 [-1.07 - 1.12]$

We tested whether follicle-stimulating hormone (FSH) or luteinizing hormone (LH) changed as an effect of sex and found that changes were driven by women. FSH showed an average increase of 4.0 mIU/mL (SE = 3.4) in women and 0.1 mIU/mL (SE = 0.5) in men. LH showed an average increase of 1.4 mIU/mL (SE = 2.1) in women and 0.5 mIU/mL (SE = 0.4) in men. It is necessary to note that FSH and LH levels change substantially throughout the menstrual cycle, so from the present data it is not possible to tell whether these changes were treatment-related or due to natural biological fluctuations.

Discussion

The goal of this study was to understand the effects of a nonviral plasmid-delivered follistatin gene therapy in healthy adults. This goal was achieved by administering the gene therapy to a final cohort of 32 adults and tracking their health for three months. Post-intervention measures showed increased FST levels, improved body composition and extrinsic epigenetic age, but no improvement in the two inflammatory markers that were measured. No severe side effects were reported. The results from this study provide evidence in favor of the hypothesis that plasmid-delivered follistatin is a viable and effective way of administering follistatin gene therapy.

Serum follistatin

One of the principal concerns with nonviral plasmids is their ability to effectively transfect cells and to have a lasting impact. In the present study, participants showed an increase in serum follistatin three months post-intervention that was significantly higher than their baseline values (Figure 2). Specifically, serum follistatin showed a median increase of 12.10 ng/mL at 3 months relative to baseline ($V = 22, p < .001$). This finding is important because plasmid delivery systems have been traditionally viewed as safe but having poor transfection efficiency and duration, which led to an industry-wide focus on development of viral vectors.

Although viral vectors have become the current status quo for DNA therapy and vaccines, the question of whether nonviral plasmids are a viable route for DNA administration should be revisited. By the 2000's, research had largely turned away from developing nonviral plasmids and instead focused on developing viral vectors. At the time, viral vectors showed more promise in terms of transfection efficiency and duration of action. However, many advances in science have been made in recent decades, and the original reasons for which plasmid development was more or less abandoned may no longer apply. Namely, plasmids can be reliably efficacious at penetrating cells if they are combined with a transfection agent (Al-Dosari & Gao, 2009), and other scientific advances provide options to increase their duration

substantially (i.e., Mini-Intronic Plasmids (MIPs) (Lu et al., 2013), scaffold/matrix-attached region- (S/MAR-) containing episomal plasmids (Jenke et al., 2002), and minicircles (Chen et al., 2003; Darquet et al., 1997)).

It is worth noting that, compared to viruses, plasmids are easier to manufacture, free from replication-competent virus concerns, and can be cleared naturally over time, which makes them well-suited for transient gene expression needs. These features make plasmid delivery particularly attractive for applications where repeated dosing, tissue-specific expression, or reduced immunogenicity are essential.

Body composition

The goal of follistatin gene therapy is to improve body composition and healthy longevity. In line with the second hypothesis, body composition improved considerably, with median fat-free mass gain of 1.40 lbs ($V = 238$, $p = .004$) and mean body fat percentage loss of 0.9 % ($t(22) = -2.80$, $p = .016$; Figure 3). Although changes in bone mineral density appeared to trend towards significance, any changes observed here were likely too small to surpass the expected precision error for DEXA¹. With that said, bone grows slowly, so it is possible that later time points or therapy re-administration would lead to quantifiable BMD improvements. Such changes would be in line with the literature describing a correlation between follistatin and bone mineral density (Jürimäe et al., 2023).

The improvements seen in fat-free mass and body fat percentage fit with research by Kota and colleagues (2009), which found that follistatin gene therapy led to improvements in strength and muscle size in healthy nonhuman primates without any degenerative muscle diseases. Given that age-related frailty can tremendously decrease quality of life and increase risk of catastrophic adverse health events, body composition is especially important for older adults (Hoogendijk et al., 2019; Palliyaguru et al., 2019). Falls that lead to fractures and other injuries are especially harmful (Cruz-Jentoft & Sayer, 2019) and are estimated to cost the US healthcare system at least 80 billion dollars each year (Haddad et al., 2024). FST gene therapy has the potential to be a novel, powerful, and safe way to combat age-related frailty, with useful effects even in the absence of a disease.

Inflammation and biological aging markers

Among the biological aging markers tested, EEA showed a significant decrease ($V = 45$, $p = .004$), while IEA only trended toward decrease and DunedinPACE (DP) did not show a significant change. The main difference between EEA and other biological aging markers is that

¹Precision error generally ranges between 1.0-1.5% at the spine and hip (Blake & Fogelman, 2010). Although precision error varies slightly across machines and across time, given that BMD is expressed as grams of mineral existing within one cm², this value tends to come out to approximately 0.01-0.015 g/cm² (see Baim et al., 2005 and Blake & Fogelman, 2010 for further details).

EEA specifically incorporates methylation-based estimates of immune cell composition. IEA adjusts these immune-related markers out. DP was trained on 19 different biomarkers to predict pace of aging, meaning that although immune/inflammation changes may indirectly affect it, a substantially smaller portion of the model would be affected by such changes, especially when compared to EEA. The finding that only EEA changed suggests that changes in epigenetic age were driven partly by methylation related to immune system processes.

This finding makes sense in light of research demonstrating a direct relationship between FST and activin A, a cytokine that suppresses immune cell processes and limits their activation. It has been well established that both endogenous and exogenous follistatin obstruct activin A (Hedger et al., 2011). Activin A has been implicated in the immune cell processes that EEA incorporates in its calculation, specifically T cells (Morianos et al., 2019), B cells (Ogawa et al., 2008), NK cells (Rautela et al., 2019), and granulocytes (Wiley et al., 2024). Based on this information, a likely explanation for the decrease in EEA is that FST-mediated inhibition of activin A affects immune cell composition and attenuates chronic inflammatory signaling.

If FST inhibits activin A to reduce chronic inflammation, then at first glance it is surprising that the blood inflammation markers we tested (hsCRP and Hcy) did not show evidence of change. One reason for the lack of change in these blood markers may be that hsCRP and Hcy alone did not provide a comprehensive enough picture of inflammation to see effects. Although hsCRP is a widely used measure of systemic inflammation and Hcy is a known indicator of metabolic and cardiovascular stress, there are other blood markers of inflammation that could have been more sensitive to systemic changes. Possibilities include interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α), and interleukin-1 beta (IL-1 β). Future studies should consider examining these cytokine-based markers. Given that sarcopenia-induced frailty itself might be a rheumatic inflammatory condition (Gupta et al., 2018), there is a strong case for follistatin action at the intersection of frailty and inflammation.

As seen in Figures 4, 5, and 6 respectively, changes in EEA, IEA, and DNAmTL appeared to have more robust effects in older adults. Although only DNAmTL showed an effect of age group at a statistically significant level, it is reasonable to expect a ceiling effect in which younger adults show less change because they are already towards the upper limit of a measure. It is not unlikely that the benefits of follistatin gene therapy are more profound in older adults.

Background health panel and safety

No treatment-related severe or moderate adverse events were reported during the 3-month study duration among participants. Some participants reported a mild adverse effect, which was temporary irritation at the injection site. With a couple exceptions that will be discussed in this section, analyses of the background metabolic and hormonal panel (see Method) did not reveal significant changes (Table 3).

Although HDL and TG did not show any significant change, there was a bifurcation in LDL levels, where some participants showed increased LDL and others showed a slight decrease or no change (Figure 7). Post-hoc analyses tested whether this effect was driven by age or sex but did not find either to be a driving factor. While it is possible that there was an increase in appetite in participants with the LDL increase, it is unlikely given the previously mentioned increase in fat-free mass and decrease in body fat percentage. Another possibility is that the anabolism we observed in the form of lean mass gain required cholesterol export from the livers of subjects. The phenomenon of increase was large enough to warrant some caution, but it is possible that diet, exercise, and/or prophylactic medication could attenuate the observed increase. Future studies should explore the role of individual differences in response to gene therapy.

It is also worth noting that the changes in follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were driven by women. However, this study was limited by the fact that timing of menstrual cycles in relation to blood draw was not tracked. FSH and LH levels vary widely throughout the menstrual cycle. FSH has a range of approximately 3-15 mIU/mL during the follicular phase up to 5-23 mIU/mL mid-cycle, and back down to about 1-9 mIU/mL during the luteal phase⁵⁵. This means that nothing conclusive can be drawn from the present FSH/LH findings, but future studies should prioritize tracking menstrual phase when blood draws occur in order to accurately characterize how FST gene therapy interacts with female physiology.

Limitations and future directions

This study was limited due to the fact that there was no control group. We recommend that future studies use a placebo-controlled design. Although we instructed participants to avoid changing anything about their exercise routine for the study duration, future studies should control for exercise experimentally with a pre-post placebo-controlled design. Groups could include placebo/no exercise, placebo/exercise intervention, FST treatment/no exercise, FST treatment/exercise intervention.

As with all early-phase, single-site studies, the present findings should be interpreted in light of the need for independent replication. Conflict of interest is a known limitation in privately funded research and has been a concern for decades, especially in pharmaceutical-sponsored studies; similar considerations are relevant here. Given that only a few studies exist on follistatin gene therapy in humans, replication by independent groups and data transparency will be critical moving forward, especially with larger participant cohorts.

A potential limitation of this study is that open-ended adverse event reporting forms were used in place of a checklist of symptoms. While checklists can create a reporting bias (due to the power of suggestion) and can lead to participants to self-limit to only the options provided, open-

ended questionnaires can result in underreporting if participants forget symptoms or assume they are irrelevant. Future studies should consider a hybrid approach that combines open-ended reporting and checklists. Additionally, while the PEI used here had a low molecular weight (40 kDa on a scale of 25-800 kDa) which is known to be safer and less toxic than higher weights, future studies should test PEI with an even lower molecular weight as well as biodegradable options.

The present study's findings on FSH and LH were inconclusive. These variables are worth examining more deeply in the future to best characterize how gene therapy interacts with female physiology. The present study's findings on FSH and LH were inconclusive – these variables are worth examining more deeply in the future to best characterize how gene therapy interacts with female physiology. Future studies should also measure objective functional outcomes (strength, gait, etc.) and subjective measures (fatigue, perceived energy, etc.). Additional future directions include testing multiple dosages, a longer study window of 12-24 months, and potential benefits in patients with inflammatory conditions like rheumatoid arthritis and/or inclusion body myositis.

Conclusion

The present study shows that follistatin gene therapy carries potential as a safe, powerful, and novel way of preventing age-induced frailty. Beyond FST, our results establish a potential path for human trials utilizing the same delivery system with other candidate genes. If such additional plasmid gene therapies are safely and effectively developed, administration would likely take place yearly. A shortcoming of this system is that it is currently limited to secretory proteins, but this limitation is something we seek to eventually overcome. The future of gene therapy likely involves utilizing all delivery modes according to their complementary strengths and weaknesses to optimize overall therapeutic safety and efficacy.

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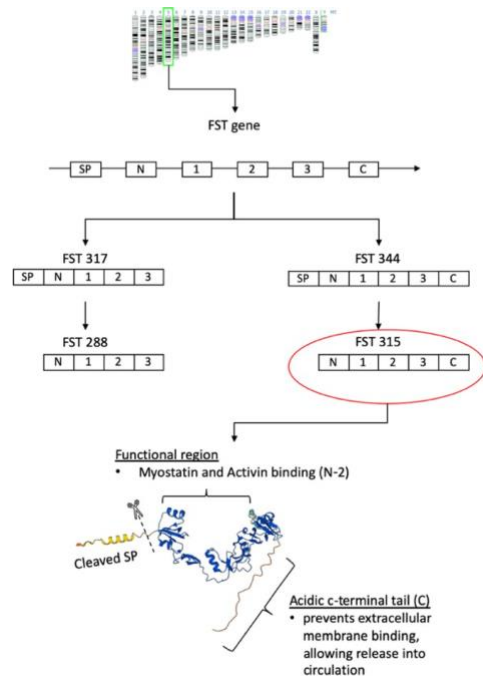
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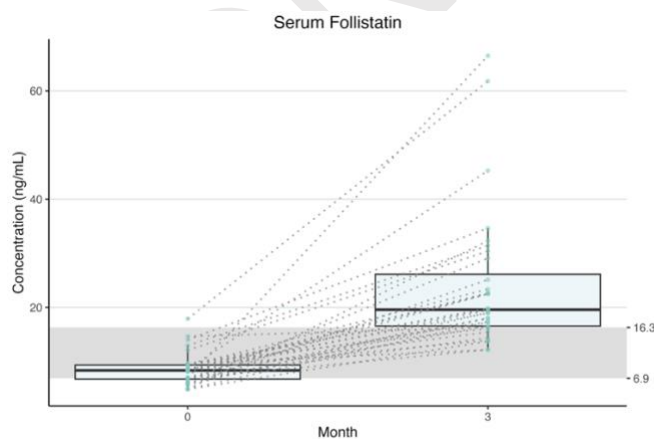
Zhang L, Liu K, Han B, Xu Z, Gao X. The Emerging Role of Follistatin under Stresses and Its Implications in Diseases. *Gene* **639**, 111–116. <https://doi.org/10.1016/j.gene.2017.10.017> (2018).

Figure 1
Follistatin isoforms and domain functions



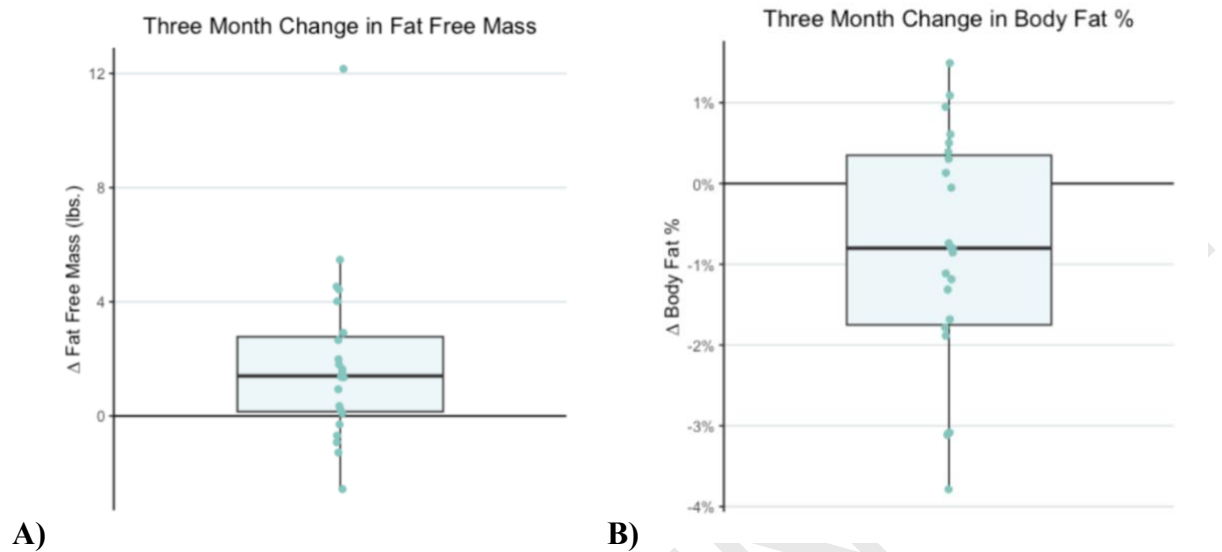
We used FST 344 to increase circulating FST 315. These two isoforms contain a C-terminal acidic region that prevents FST 315 from binding to the plasma membrane's extracellular surface, which allows FST 315 to be released into circulation. FST 317 and FST 288 lack this C-terminal feature.

Figure 2
Change in Serum Follistatin Levels 3 Months Post-Treatment



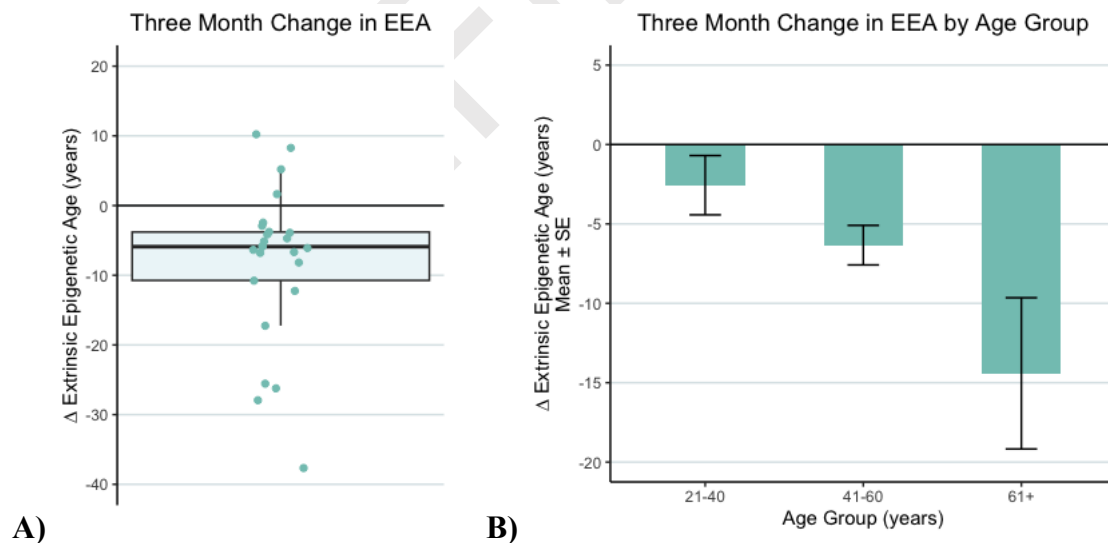
Serum follistatin increased significantly following intramuscular injection of a nonviral plasmid-based FST 344 gene therapy. Grey band shows the reference range for circulating follistatin in healthy adults ($Mean = 11.6$, $SD = 4.7$ ng/mL) from research by de Kretser et al. (2013).

Figure 3
Body Composition Measures 3 Months Post-Treatment



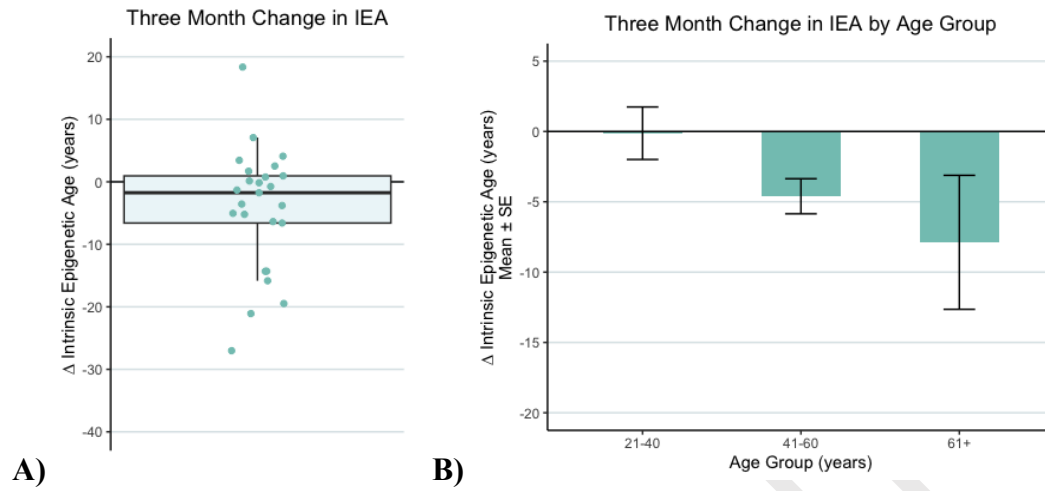
A) Fat free mass increased significantly post-administration. B) Body fat percentage decreased significantly post-administration.

Figure 4
Change in Extrinsic Epigenetic Age (EEA) 3 Months Post-Treatment



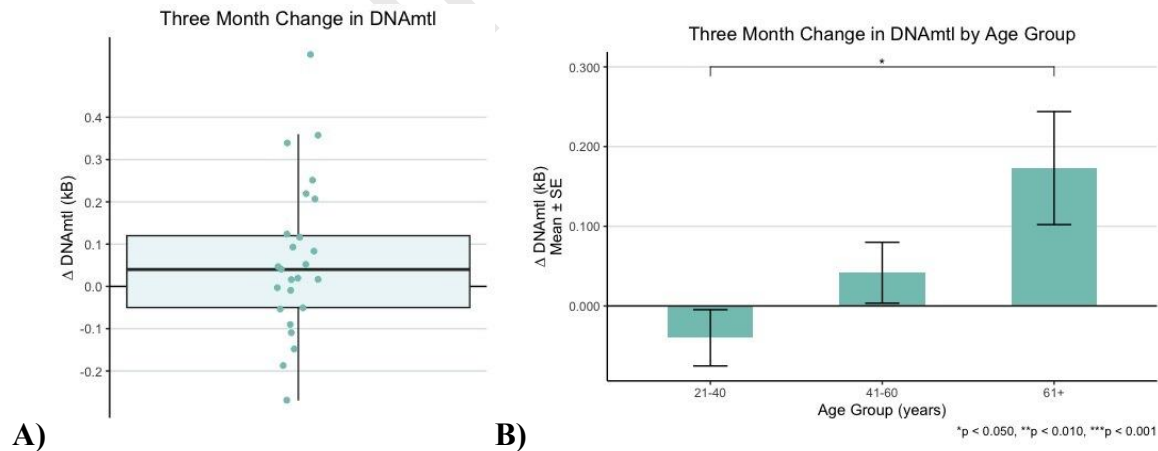
A) EEA decreased significantly following administration. EEA reflects DNA methylation at CpG sites chosen from the Hannum (2013) clock and includes methylation-based estimates of immune variation (as opposed to IEA). B) The biggest decreases in EEA were seen in older adults, likely due to there being more room for improvement than in younger adults.

Figure 5
Intrinsic Epigenetic Age (IEA) 3 Months Post-Treatment



A) Decreases in IEA only trended toward significance. IEA reflects DNA methylation at CpG sites chosen from the Horvath (2013) clock but specifically adjusts out immune-related markers. B) There was a trend towards decrease in IEA in older adults.

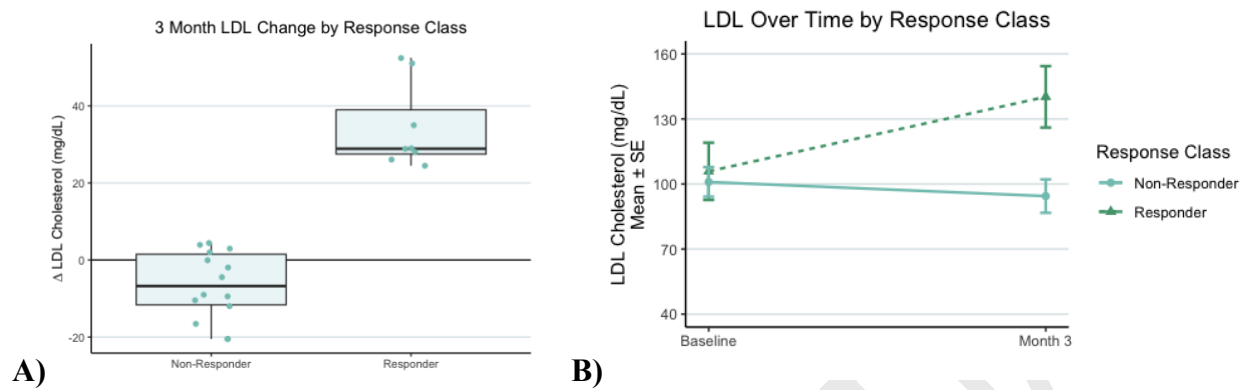
Figure 6
DNAmTL 3 Months Post-Treatment



A) Changes in methylation-based estimates of telomere length (DNAmTL) were not significant at the group level. B) Post hoc Dunn's tests showed that participants aged 61+ exhibited significantly greater changes in DNAmTL than those aged 21-40 (*FDR-corrected* $p = .020$), but differences between other age groups were not significant.

Figure 7

Bifurcation in Post-Treatment Low-Density Lipoprotein (LDL) Cholesterol Levels



LDL cholesterol responses were bimodally distributed. While non-responders showed no change, responders showing a clinically notable increase. Future studies should investigate the role of individual differences in gene therapy outcomes.