Plasmid delivery of Follistatin gene therapy safely improves body composition and reverses epigenetic age estimates in sex- and age-diverse adult human subjects.

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Walter Patterson and Mac Davis conceived, developed, and managed the project. Walter Patterson constructed the plasmid and performed ELISAs. Walter Patterson and Ryan Rossner wrote the manuscript. Raven Garuda analyzed the data. Glenn C. Terry administered the therapy.

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Abstract

We injected polyethyleneimine (PEI)-complexed plasmid delivering the Follistatin (FST) 344 gene to 43 adult human volunteers of both sexes, age 23-88, to test the safety and efficacy of this delivery method and target gene as an anti-frailty longevity intervention. Patients received subcutaneous injection into abdominal fat of 50 μg FST in a plasmid vector. We evaluated several metrics immediately prior to administration and at or through three months post-therapy: serum FST, body composition, blood biomarkers of inflammation, glucose metabolism, and lipid metabolism, and epigenetic age estimates. Serum FST, measured by enzyme-linked immunoassay , increased over two-fold from a baseline mean of 8.58 ng/ml to 24.03 ng/ml, a largely supraphysiological value. Body composition, measured by dual energy x-ray absorptiometry scans, improved, with a mean fat-free mass increase of 1.69 lbs and a mean bodyfat reduction of 0.80%. High-sensitivity C-reactive protein and homocysteine, two common markers of inflammation, showed signs of trending towards improvement. Glucometabolic markers increased slightly, but these changes often failed to reach statistical significance and were largely inconsistent across groups. Lipid panel changes were primarily driven by a large, statistically significant increase in low-density lipoprotein (LDL) that was largely conserved across groups. High-density lipoprotein increased and triglycerides decreased, but these changes were smaller, less significant, and less consistent across groups compared to the increase in LDL. Intrinsic and extrinsic epigenetic age both decreased dramatically, with mean decreases of 6.03 and 8.18 years, respectively, and maximum decreases of 21.08 and 27.91 years. Telomere length increased modestly, and DunedinPACE rate of aging trended towards a slight increase, but this change was not statistically significant. Of paramount importance, no adverse effects related to the gene therapy were reported. This clinical trial establishes PEI-complexed plasmid delivery of FST as a potentially safe, anti-frailty longevity therapy for both male and female human subjects across a near maximal adult age range.

Keywords: gene therapy, plasmid, Follistatin, frailty, longevity

Introduction

DNA, the human genome, and gene therapy

After the discovery of the structure of DNA in 1953 [1] and the sequencing of the human genome in the early 2000s [2, 3], attention naturally turned to developing gene therapies [4–6]. There have been three major innovations in gene therapy delivery: viral vectors [7], CRISPR-Cas9 [8], and plasmids [9]. The tremendous promise of such therapies, however, was disrupted by early setbacks including a child's death in a viral vector gene therapy trial and broad safety and ethical concerns [10–13]. Our analysis of this situation lead us to develop plasmids as the therapeutic mode with the most favorable risk:reward ratio.

Plasmid gene therapy

Plasmids were conceptually described in the 1920s [14] and named in the 1950s [15]. They were first isolated in 1969 [16] and first engineered in the early 1970s [17, 18]. By the late 1990s, plasmids were viewed as safe but ineffective [19]. In 1997 and 2003, smaller, higher expression [20], and longer-lasting [21] plasmids called "minicircles" were developed. Nanoplasmids [22] and mini-intronic plasmids [23] further developed this strategy over the next ten years. Despite these advances, plasmids remained out of favor generally. All FDA-approved gene therapies to date utilize viral vectors [24], and only 12% of gene therapy trials worldwide use plasmids [25].

Localized linear polyethyleneimine transfection

Polyethyleneimine (PEI) is a cationic polymer used to transfect nucleic acids into mammalian cells. PEI's positive charge enables interaction with cell membranes, subsequent endocytosis, and ultimately interaction with DNA [26]. PEI can be linear (LPEI) or branched (BPEI), and a range of molecular weights are possible for both types. There are safety concerns associated with PEI [27], but we make the case that localized LPEI in a 4:1 PEI:DNA ratio is both safe and efficacious in vivo. First, the toxicity associated with PEI is mostly limited to BPEI and is dependent on free PEI [28, 29]. We used a 4:1 PEI:DNA ratio that minimizes free PEI. LPEI hydrochloride has been shown to produce neither proinflammatory cytokines nor alterations to hepatic enzyme levels in vivo [30, 31]. PEI-DNA administration additionally has been demonstrated efficacious in human patients in transgenic vaccination for B-cell lymphoma [32]. Safety and efficacy advantages of LNP > PEI in vitro have been reversed in vivo [26]. Thus, localized LPEI at low dosages in a 4:1 ratio with DNA could be reasonably expected to be safe and efficacious.

Target genes and Geroscience

Countless genes are candidate targets for gene therapy [33]. Our approach was to develop gene therapies targeting longevity. A paradigm shift has taken place in the last ten years towards a strategy called Geroscience that treats aging itself as the biggest risk factor for the major killer diseases [34–36]. Instead of targeting each disease individually, we target their biggest shared risk factor, aging. Significant progress has been made towards understanding the molecular mechanisms of aging [37], making the Geroscience paradigm actionable.

Follistatin (FST) safety and efficacy

Follistatin (FST) was our first choice because, in both animal and human trials, it not only has an exceptionally good safety profile [38], but it has also been exceptionally effective at improving healthy longevity [39]. Frailty, in particular, increases with age, driven largely by late-life rapid and catastrophic declines in muscle size, muscle strength, and bone mineral density (BMD) [40–42]. FST is particularly good at combating physical frailty [43–45].

Our system and FST secretion and expression

Additionally, FST is amenable to our plasmid delivery system. FST is a secreted protein, meaning it is made in one cell, exported to the cell surface, cleaved of its secretory sequence, and released into sys-

temic circulation via the blood [46]. 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 (Fig. 1). FST counterintuitively increases with age [47], but this increase is clearly insufficient to combat aging-induced frailty.

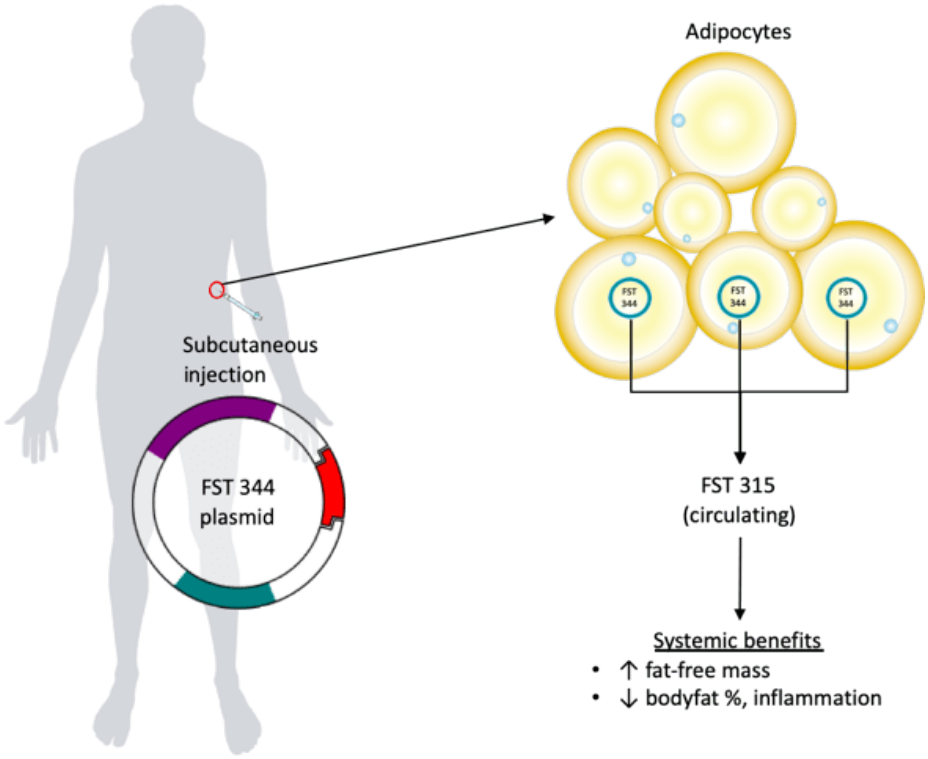

Figure 1: Injectable plasmid therapeutic strategy. Plasmid administration via subcutaneous injection into abdominal fat leads to FST export into circulation from local site and subsequent systemic benefits.

FST isoforms

FST is initially produced in two isoforms, FST 317 and FST 344 [46] (Fig. 2). 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. Based on this and prior groups' research results [38], we used FST 344 to increase circulating FST 315.

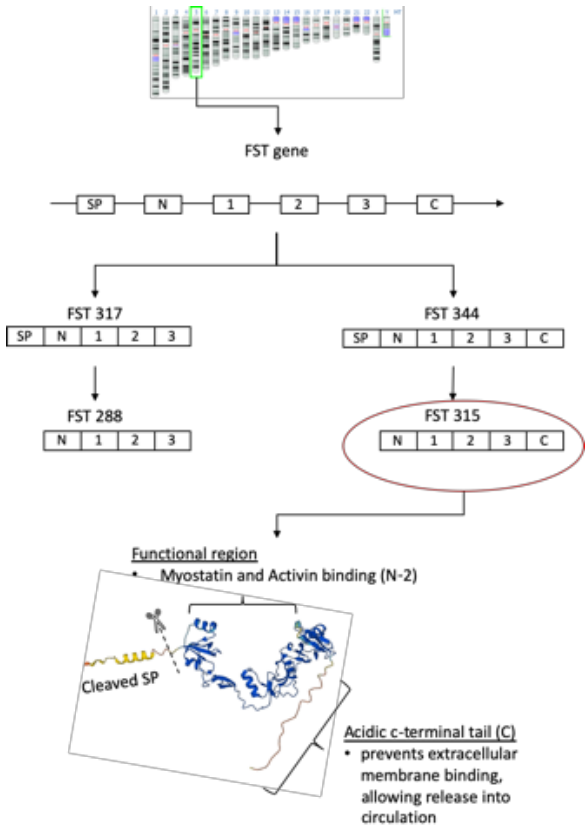

Figure 2: FST isoforms and domain functions.

FST mechanisms (Myostatin, Activin, ActRIIB)

FST has an excellent safety and efficacy record in animal and human trials, but the mechanisms of its regulation and function are only partially understood [48]. FST binds to Transforming Growth Factor beta (TGFβ) 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 [49]. FST improvement of lean mass and strength requires both Activin and Myostatin inhibition [43, 50], but FST-mediated improvement of inflammation is thought to primarily require Activin inhibition [48].

Materials & Methods

Plasmid Construct

A plasmid was constructed delivering the 344aa secreted isoform of the human follistatin gene. Transgene expression was driven by a CMV promotor. Scaffold/Matrix attachment regions (S/MARs) were included in order to enhance tethering of the plasmid to the nuclear matrix, thus promoting nuclear retention.

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 determined to conform to FDA Guidance for Industry recommendations via kinetic LAL assay performed by third-party CRO Charles River [51].

Transformation Technique

Transfection was achieved by means of 40kDA linear polyethyleneimine coadministration with plasmid. A 4:1 ratio of transfection agent to plasmid was used as suggested by the manufacturer, Polysciences.

Plasmid administration

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 μl of of saline solution containing 50 μg of plasmid was injected into subjects' abdominal subcutaneous fat. The primary physician observed patients for 15 minutes post-injection in case of allergic reactions or other complications.

Serum FST ELISA

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. GDF8 concentration were determined by means of Invitrogen HumanGDF-8/Myostatin ELISA Kit, ThermoFisher catalog #EH215RB. Serum was isolated prior to administration as well as three months post administration.

Cohort selection

Subjects volunteered for the trial. Informed consent and IRB were handled with GARM.

Body composition

DEXA scans were used to measure body composition of subjects.

Blood biomarkers

Blood draws were performed according to a defined panel of desired values, and providers directly emailed to study organizers.

Individual Data

Each participant has a set of measurements per parameter: pre-treatment (months ≤ 0) and treatment (months > 0). A baseline measurement b is calculated by taking the mean of the pre-treatment measurements. This, along with its standard error, is reported in the table under each participant's timepoint data. An individual's change in baseline measurement is calculated by subtracting the baseline from the last recorded timepoint.

Group Trends

A trend analysis is then conducted for a given group of participants. In order to perform a meaningful analysis on a given parameter, it is important that only participants with at least one pre-treatment measurement and at least one treatment measurement be included, so those not meeting those criteria are immediately excluded. This number of viable participants is reported (n). The absolute data of these

participants is then graphed as a line chart. A dashed horizontal line is included to show the mean of all the participants' baselines. This chart is to serve as a visual aid of the general behavior of this parameter across viable participants in the group. The participant data for months 0 – 3 is then prepared for a statistical analysis as follows: a participant's month 0 is fixed at 0, and the baseline measurement is subtracted from the treatment measurements (months 1 – 3). The means and standard errors of this adjusted data set are then graphed, and the mean/SE of the month 3 adjusted measurements are then reported as "Change from Baseline/SE" for the group. The Kendall tau test for monotonicity is performed on the adjusted data set and the *p*-value reported, with $p < 0.050$ indicating a significant increase or decrease in the data, $p \geq 0.050$ indicating no significant increase/decrease.

Comparisons between groups

Comparisons between groups are always conducted with regards to "change from baseline." To ensure meaningful reporting, only participants with a baseline measurement and a month 3 measurement are included. The mean/SE of each group is reported and graphed, and a statistical test (Student's *t*-test for two groups, or ANOVA (Tukey's post-hoc) for three or more groups) is performed and its *p*-value reported, with $p < 0.050$ indicating a significant difference between two groups, and $p \geq 0.050$ indicating no significance between two groups. Significant differences between groups are noted using a marker on the graph.

Results

Supraphysiological serum FST without adverse effects

We measured serum FST by enzyme-linked immunoassay (ELISA) immediately prior to plasmid administration and three months post-administration. Mean serum FST increased over two-fold (Fig. 3), the latter value being largely supraphysiological. Importantly, despite the large increase, across 43 adult human subjects of both sexes, age 23-88 (Table 2), no adverse effects related to therapy were reported.

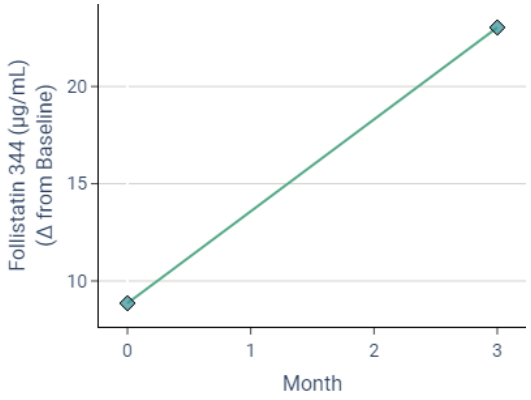

Figure 3: Supraphysiological FST increase.

Body composition improved in all age groups and both sexes

We measured body composition via dual-energy X-ray absorptiometry (DEXA) scans before and after treatment at one month intervals through three months. By three months, mean fat-free mass (FFM) increased (Fig. 4(A)), and mean body fat % (BF%) decreased (Fig. 4(B)). Android to gynoid fat ratio (A/G) was trending downward, towards improvement, by three months (Fig. 4(C)). Bone mineral density (BMD) was trending upward slightly (Fig. 4(D)).

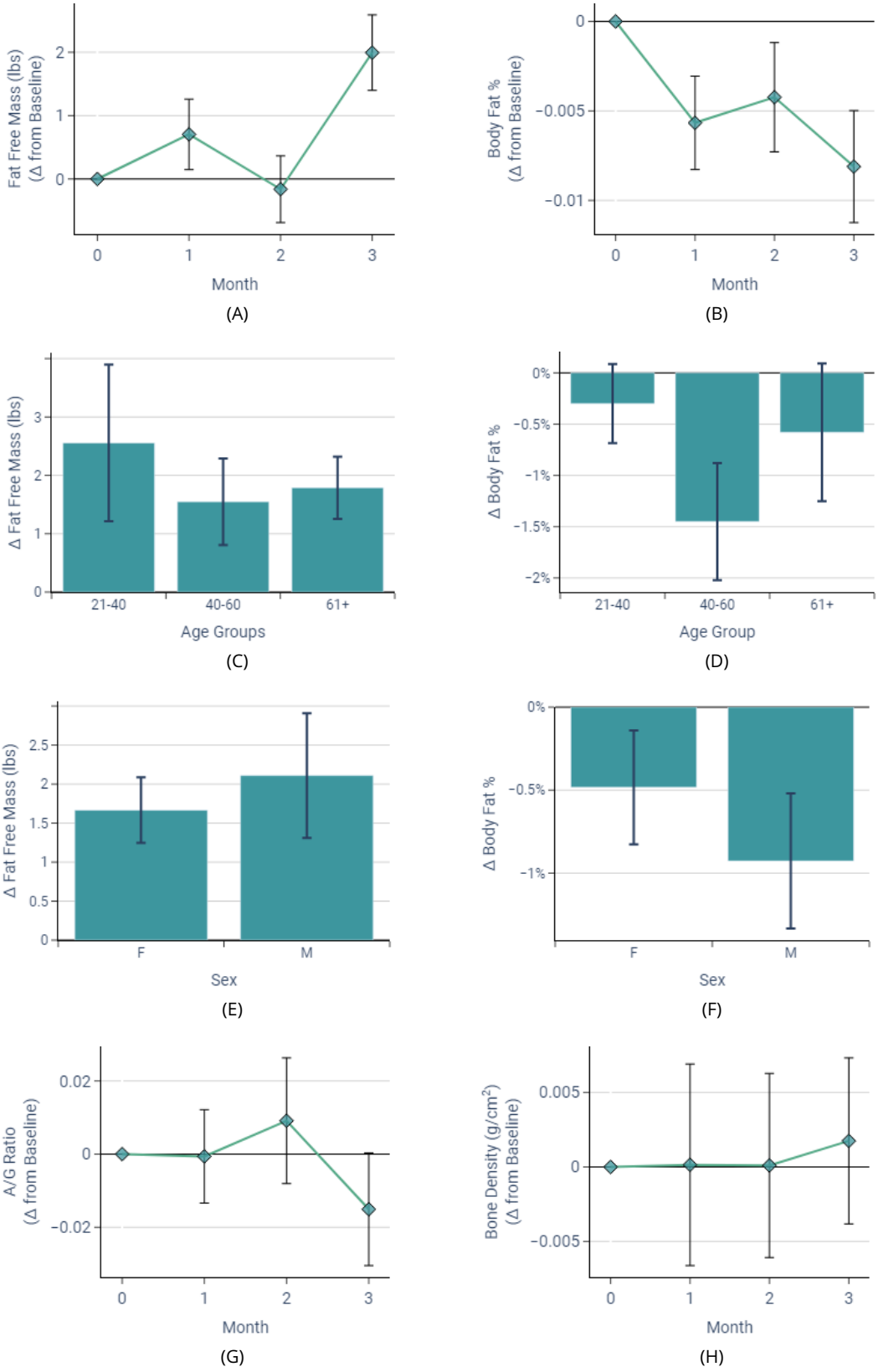

Figure 4: Body composition improved in all age groups and both sexes. (A) FFM increased. (B) BF% decreased. (C) FFM increase was statistically significant in all age groups. (D) BF% decreased in all age groups, but was only statistically significant in the middle-aged group. (E) FFM increased in both sexes. (F) BF% decreased in both sexes. (G) A/G ratio was trending downward, towards improvement, at three months. (H) BMD was trending upward very slightly at three months.

Markers of inflammation trended downwards

High-sensitivity measurement of C-reactive protein (hsCRP) decreased by a small amount, though this change failed to reach statistical significance (Fig. 5(A)). Homocysteine (Hcy) also decreased (Fig. 5(B)), though this change also failed to reach statistical significance.

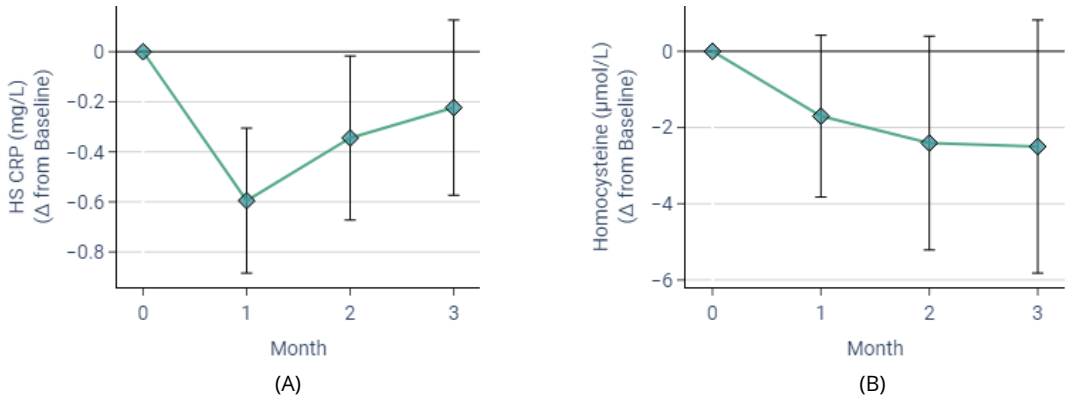

Figure 5: Markers of inflammation trended downward. (A) hsCRP and (B) Hcy both decreased, though neither change reached statistical significance.

Slightly elevated glucometabolic markers

We measured fasting serum levels of glucose, Hemoglobin A1c (HbA1c), insulin, and leptin, and observed small mean increases in each by three months post-therapy. Fasting glucose increased slightly (Fig. 6(A)), though this change was not statistically significant and was inconsistent across groups. HbA1C, a measure of average blood glucose over the 2-3 months prior to measurement, also increased slightly (Fig. 6(C)). This change was statistically significant but was inconsistent across groups. Fasting insulin also increased slightly (Fig. 6(C)), but this change was neither statistically significant nor consistent across groups. Leptin increased (Fig. 6(D)), and this change was both statistically significant and consistent across age groups (Fig. 6(E)) and both sexes (Fig. 6(F)).

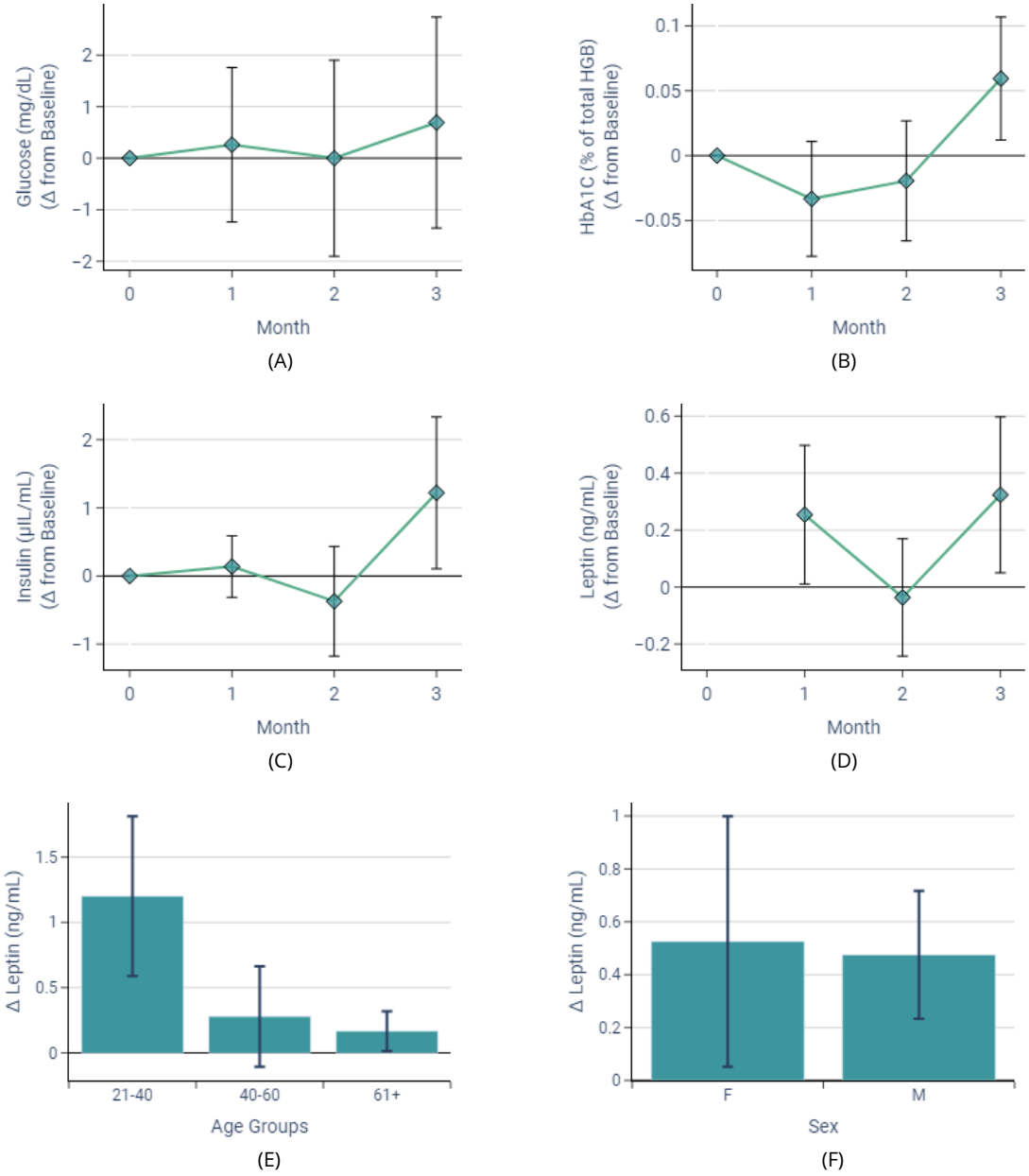

Figure 6: Slightly elevated glucometabolic markers. (A) Fasting blood glucose was trending upward by month three, and (B) HbA1c increased. (C) Fasting insulin and (D) leptin increased. Only leptin increased in all age groups (E) and both sexes (F).

Elevated LDL outweighs trends towards improvement to HDL and TG

Total cholesterol (TC) increased (Fig. 7(A)), a change driven almost entirely by increased low-density lipoprotein (LDL) (Fig. 7(B)). Increased LDL was consistent across all age groups (Fig. 7(C)) and both sexes (Fig. 7(D)). High-density lipoprotein (HDL) cholesterol increased slightly (Fig. 7(E)), and triglycerides (TG) decreased by a considerable amount (Fig. 7(F)), but neither change reached statistical significance. The decreased TG is potentially clinically significant due to effect size.

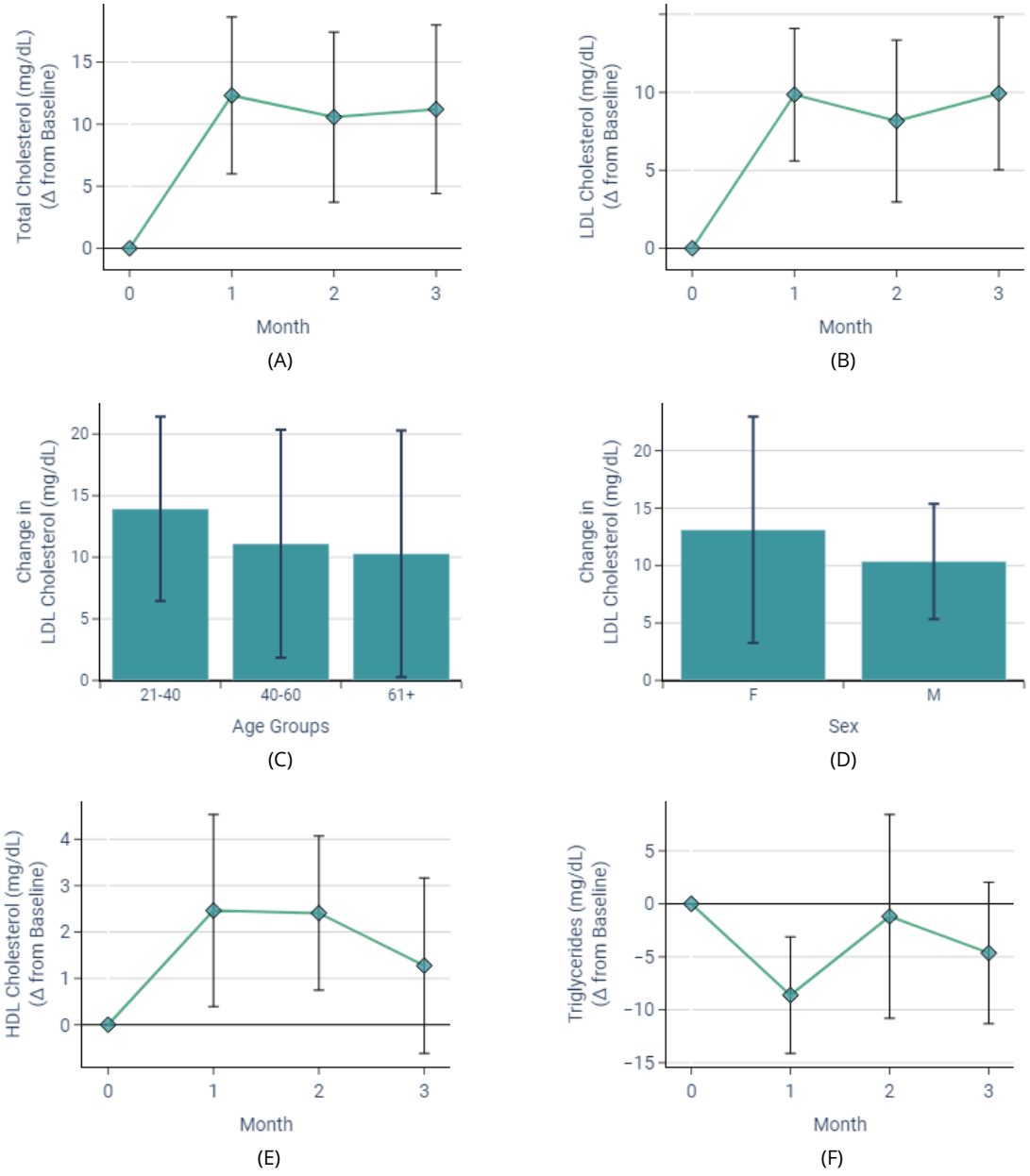

Figure 7: LDL increase, independent of age and sex, was the primary significant lipid alteration. (A) Total cholesterol increased, but this was driven almost completely by (B) increased LDL. LDL increased in (C) all age groups and (D) both sexes. (E) HDL was unchanged at three months. (F) Triglycerides decreased substantially, though this change was not statistically significant.

Dramatically decreased epigenetic age acceleration in all groups

Both intrinsic (IEA) and extrinsic (EEA) epigenetic age decreased dramatically (Fig. 8(A), Fig. 8(B)). Both changes were conserved across all three age groups (Fig. 8(C), Fig. 8(D)) and both sexes (Fig. 8(E), Fig. 8(F)). Average telomere length was increased slightly (Fig. 8(G)). Subjects' DunedinPACE rate of aging [52] experienced a small upward trend that did not reach statistical significance (Fig. 8(H)).

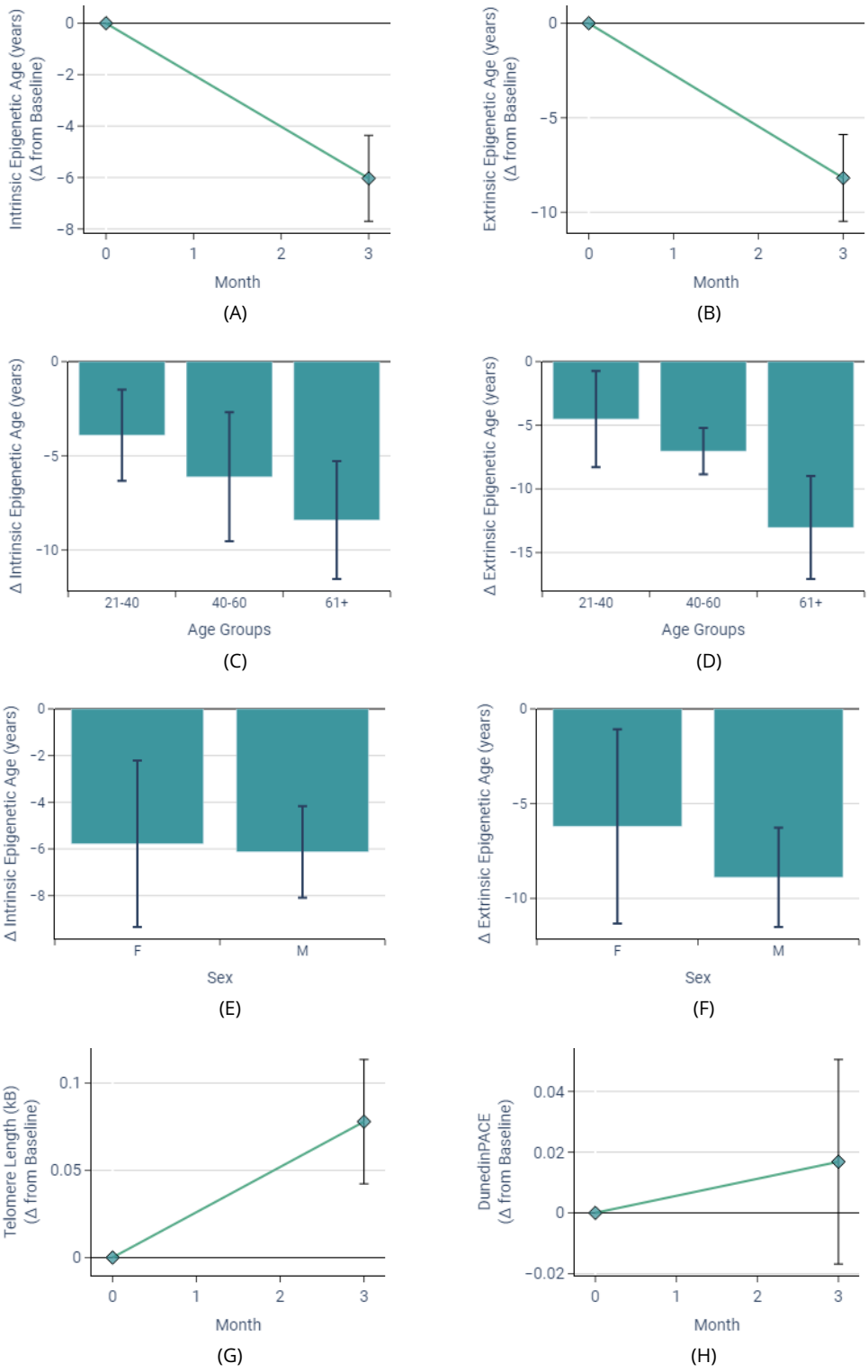

Figure 8: Dramatically decreased epigenetic age acceleration. (A) Intrinsic and (B) extrinsic epigenetic age acceleration both decreased dramatically. Both changes were conserved across all age groups (C,D) and both sexes (E,F). (G) Average telomere length increased slightly. (H) DunedinPACE rate of aging experienced a small, statistically insignificant increase.

Discussion

Safety

The paramount concern of any Phase 1 clinical trial is safety. This concern is elevated for gene therapy trials because of their novelty, intrinsic biological power, and historical adverse effects including child death. Thus, it is critical that none of our subjects, who represent both sexes and a near-maximal adult age range, reported adverse effects related to therapy.

Supraphysiological FST

Additionally, our plasmid was very effective at increasing serum FST to supraphysiological levels [47]. Human biological homeostatic systems have evolved over geological time, so it's not obvious that supraphysiological states would be well tolerated. Here, however, supraphysiological FST was well tolerated. Counterintuitively, FST increases naturally with age [47], but such increase is clearly insufficient to alleviate age-induced frailty. Thus, a supraphysiological increase is conceivably required to reap significant benefits.

Body composition

Our primary goal in terms of efficacy was improved body composition. As hoped, body composition improved considerably, with mean FFM gain of 1.99 lbs. and BF% of 0.81%. Importantly, both changes were consistent across both sexes and all age groups. Body composition is especially important for elderly people, because age-related frailty can tremendously decrease quality of life and increase risk of catastrophic adverse health events [40, 42]. Falls that lead to fractures and other injuries are often especially harmful [53]. FST gene therapy might be a novel, powerful, safe way to combat age-induced frailty.

Fat loss is intrinsically good until a healthy value is reached, but fat-free mass is a surrogate metric for the more functional metric, strength. Our trial produced extraordinary case studies of subjectively assessed strength improvement, but it will be essential to objectively measure strength in future FST trials.

We did not observe statistically significant changes in BMD, but FST can improve it [54]. Bone grows slowly, so it's possible later time points or therapy re-administration could lead to quantifiable BMD improvements even in elderly subjects. Again, there were extraordinary case studies in our trial in which BMD improved rapidly, but these will be discussed in a separate publication.

FST in mice has recently been shown to cause tibial fractures [55] and dramatically reduce bone quality [56], but these effects are secondary to extreme skeletal muscle growth [57] that is not seen in humans.

A/G ratio improved, which is indicative of a shift towards healthy fat distribution, away from excessive belly fat and/or visceral fat. FST can positively impact the closely related process of adipose beiging [58], consistent with FST having a net positive effect on metabolic status.

Inflammation

Our secondary goal in terms of efficacy was improvements to markers of inflammation and corresponding reports of subjective improvements in patients with inflammatory conditions, e.g. rheumatoid arthritis. Our results were directionally aligned with our expectations, but, unlike body composition changes, inflammatory markers hsCRP and Hcy only decreased slightly and did not reach statistical significance. Similarly to body composition results, however, we observed outstanding individual results that will be covered in separate publications. Mechanistically, sarcopenia-induced frailty, discussed above, itself might be a rheumatic inflammatory condition [59], strengthening the case for FST action at the intersection of frailty and inflammation.

Inclusion body myositis is an inflammatory condition that might be treatable with FST. Inclusion body myositis is the most common inflammatory myopathy in older adults, most commonly affecting men after age 50 [60]. Circulating levels of Activin A in IBM patients are higher than controls and circulating myostatin levels of IBM patients are higher than those of other neuromuscular disease patient groups

[61]. Because of this, FST may be able to address the unique pathology of IBM. Indeed, early results have shown improved ambulation and muscle regeneration as well as decreased fibrosis in IBM patients injected with follistatin gene therapy [62–64].

Small, inconsistent effects on glucometabolic markers

Literature offers mixed evidence about the effects of FST on glucose metabolism [39, 65, 66]. Thus, we were not completely surprised to see small changes consistent with worsened glucose metabolism. The changes we observed are unlikely to elevate the vast majority of patients above the healthy reference range but nonetheless are important to note.

Leptin, the "satiety hormone," was the lone glucometabolic elevation that was consistent across groups. Leptin deficiency in animals causes obesity [67], but obesity also increases leptin and leptin resistance can occur [68]. Thus, we interpret Leptin increase as a potentially positive effect that may counteract the small negative effects on glucose metabolism markers, but we remain open to further data. It is interesting that our subjects simultaneously increased Leptin while losing fat, opposite the common pattern.

Negative and positive lipid alterations

The main change to subjects' lipid values was an mean increase in LDL of 11.6 mg/dL. This increase in LDL is responsible for almost all of the increase we observed in total cholesterol. It is possible that the anabolism we observed in the form of lean mass gain required cholesterol export from the livers of subjects. This increase was larger than those seen in glucometabolic biomarkers, so it does warrant more caution.

HDL increased slightly, but TG decreased by 7.32 mg/dL, a decrease similar in magnitude to the observed LDL increase. TG decrease was less consistent across groups, however, so we currently consider the LDL increase to be more noteworthy. We do not, however, view the LDL increase, as large enough to warrant strong consideration as an exclusion criterion, except possibly in extreme cases. Improved diet, exercise, and/or prophylactic statin use is potentially sufficient to abrogate such LDL increase.

Dramatic reversal of epigenetic age estimates

Epigenetic analyses of aging were first developed 10 years ago [69], and they are currently widely considered premier biomarkers to assess individual aging [70]. Loss of epigenetic information is a major cause of mammalian aging [71]. IEA and EEA methylation-based measures, the latter of which is considered more meaningful because it takes immune cell counts into consideration [72], both decreased dramatically after FST treatment (Fig. 8(A), Fig. 8(B)). This was unexpected and will require further investigation to propose a model of relevant mechanisms. Our current hypothesis is that FST-mediated inhibition of Activin might reverse epigenetic estimates of age by decreasing inflammation.

Telomeres lengthened slightly, and DunedinPACE rate of aging trended towards a very small increase, but both changes were heavily outweighed by the IEA and EEA decreases. It is counterintuitive that rate of aging would increase or even remain constant while IEA and EEA dropped so precipitously. Again, further investigation will be required to generate mechanistic understanding.

FST and longevity-optimized mTOR signaling

Additionally, FST might be an mTOR sensitizing/potentiating agent. FST inactivates ActRIIB, and ActRIIB inactivation can induce muscle hypertrophy to a similar degree in control and rapamycin-treated animals [73]. FST might uniquely potentiate mTOR signaling by preventing SMAD3 phosphorylation [74]. mTOR has mostly prolongevity effects [75], but it can negatively impact aging-induced sarcopenia and resulting frailty [76, 77] If FST renders mTOR more sensitive to anabolic signals, it could hypothetically allow more effective acute bursts of anabolism in the context of general mTOR suppression. Such a paradigm is consistent with our results demonstrating fat-free mass gain with simultaneous, dramatic reversal of epigenetic estimates of age.

Future studies

Subsequent FST trials should focus on multiple dosages, a longer study window of 6-12 months, strength testing in addition to body composition measurement, potential benefits in patients with inflammatory conditions like rheumatoid arthritis, careful examination of glucose and lipid metabolic changes. Beyond FST, our results illuminate 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.

ABBREVIATIONS	
AAV	Adeno-associated virus
AV	Adenovirus
ActRIIB	Activin receptor type II B
A/G	Android/Gynoid fat ratio
BF%	Body fat %
BMD	Bone mineral density
Cas9	CRISPR-associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
DEXA	Dual energy x-ray absorptiometry
DP	Dunedin PACE of aging calculated from the epigenome
EEA	Extrinsic epigenetic age
EILSA	Enzyme linked immunosorbent assay
FFM	Fat-free mass
FST	Follistatin
HbA1C	Hemoglobin A1C
Hcy	Homocysteine
HDL	High density lipoprotein
IEA	Intrinsic epigenetic age
LDL	Low density lipoprotein
LNP	Lipid nanoparticle
mTOR	Mechanistic target of rapamycin
PEI	Polyethyleneimine
SMAD	Small (worm) + Mothers against Decapentaplegic (fly)
TG	Triglycerides
TGFβ	Transforming growth factor beta

Tables

Participant Statistics

GROUP	n	AGE		
		MEDIAN	MIN.	MAX
All	43	46	23	88
Male	29	42	23	88
Female	14	57	27	86

Table 1: Number of subjects and sex and age statistics.

Results

Value	Mean change (Δ) from baseline	Min Δ	Max Δ	Unit	SE	*p*-value
FST	**+15.18**	**+1.67**	**+59.51**	**μg/ml**	**2.13**	**0.000*** **
FFM	**+1.69**	**-4.65**	**+12.15**	**lbs.**	**0.64**	**0.014***
BF%	**-0.80**	**-3.80**	**+1.50**	**%**	**0.30**	**0.015***
A:G	-0.015	-0.155	+0.150	-	0.015	0.320
BMD	+0.0015	-0.06	+0.074	g/cm^2	0.0055	0.787
hsCRP	-0.2	-6.6	+2.9	mg/L	0.3	0.530
Hcy	-2.5	-71.7	+5.7	μmol/L	3.3	0.460
Glucose	+1	-17	+27	mg/dL	2	0.739
HbA1c	+0.1	-0.3	+0.4	%	0.0	0.228
Insulin	+1.2	-9.0	+20.1	μIL/ml	1.1	0.228
Leptin	+0.3	-2.4	+3.3	ng/ml	0.3	0.252
TC	+10	-28	+60	mg/dL	6	0.118
LDL	+10	-21	+65	mg/dL	5	0.055
HDL	+1	-11	+32	mg/dL	2	0.508
TGFβ	-5	-100	+36	mg/dL	7	0.497
IEA	**-6.03**	**-21.08**	**+18.35**	**years**	**1.86**	**0.036***
EEA	**-8.18**	**-27.91**	**+10.19**	**years**	**2.06**	**0.001****
Tel	+0.06	-0.27	+0.36	kb	0.03	0.071
DP	+0.007	-0.390	+0.260	years per year	0.026	0.785

Table 2: Results.

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