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Research Article

Safety Study of AAV hTert and Klotho Gene Transfer Therapy for Dementia

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Abstract

A sponsored, interventional, non-randomized study without a control group using a novel and proprietary central nervous system gene transfer method to deliver AAV hTert and Klotho genes to five patients with mild or moderate dementia was performed to primarily evaluate safety. Clinical response data was gathered as a secondary interest. The therapy demonstrated a very high safety profile with no serious adverse effects identified. Clinical evaluation of the patients over the course of the one year follow up yielded significant findings with all five patients demonstrating evident reversal of Dementia symptoms such as sustained cognitive improvement as measured by the Folstein exam. Telomere analysis was performed before and after the therapy. A measurable elongation of the participants telomeres was identified, and biological age was reduced as chronological age increased.

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Introduction

It is estimated that 5 million adults in the United States suffered dementia in 2014. This number is projected to reach nearly 14 million by 2060. There are an estimated 50 million dementia sufferers worldwide; one new case is diagnosed about every 3 seconds. Dementia is a medical term used to describe cognitive decline and a group of symptoms affecting memory, communication, critical thinking skills, and social abilities. Alzheimer's disease is one of the most common causes of progressive dementia in older adults, but there are several other less well-known types of dementia and include Frontotemporal Dementia (FTD) and Multi-Infarct Dementia (MID) that are just as devastating. Millions of

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people die with dementia every year. Dementia is consistently linked with higher mortality and poor quality of life.

Massive public and private resources have been devoted to dementia research yet currently there are no proven disease-modifying interventions. We have been unable to affect the progression and incidence of the disease. Given this situation, it is not surprising that the disease incidence is increasing.

Over the prior decades, researchers of the neurodegenerative dementias have listed several implicated pathologies for neuronal dysfunction. Leading and persistent offenders on that list include Amyloid Beta plaque congestion, Tau tangles, and mitochondrial dysfunction. In the last few years there has been a shift regarding the significance of these pathologies. Many consider these pathologies a consequence of a more fundamental upstream pathology suggesting that measures to address these known pathologies might affect a clinical improvement and perhaps a slowing of disease progression; however, this approach may have little impact on disease incidence.

Adeno-associated virus (AAV) vector-mediated gene delivery has been shown to be generally safe in both human and animal models although there are reports of hepatic toxicity and death. These complications have been reported in studies that administered the dose intravenously and at a dose equaling or exceeding 1E14 vg/kg. This high dose Intravenous AAV carries a significant risk of hepatic toxicity and death. The mechanisms include direct hepatic insult and stimulation of an aggressive innate immune response leading to hepatic insult and dysfunction [1,2].

AAV is believed to be safe regarding cancer induction owing to its extremely low nuclear integration rate. Interestingly, ectopic hTERT expression can restore telomerase activity in normal human cells and immortalize cells without inducing a malignant phenotype [3-6].

AAV vector mediated gene therapy has been approved for the treatment of inherited blindness and spinal muscular atrophy, and long-term therapeutic effects have been achieved for other rare diseases, including hemophilia and Duchenne muscular dystrophy. Over 46 trials with AAV have been completed and 37 are currently active.

Research has shown that the effects of aging are associated with a reduction in telomere length at the ends of chromosomes and telomere shortening was observed with progressive cell division in vitro and increased age in vivo [7-10]. Several studies have shown that the upregulation of telomerase reverse transcriptase (Tert) gene increases longevity and does not increase cancer. Recent experimental data indicate that expression of telomerase is sufficient for the escape of cells from the two barriers to proliferation (M1 and M2) and for the immortalization of many cell types [11]. The Human gene (hTert) has been shown to affect the tau pathologies in mice and human hippocampus tissue supporting the key assumption that TERT plays a significant role in maintaining neuronal health. There is an abundance of research

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suggesting that reduction in telomerase is associated with several forms of dementia. Oxidative stress and insufficient antioxidative defense are most probably an important pathogenetic factor for vascular as well as so-called mixed dementias [12]. The telomerase protein TERT seems to be a protective factor in the brain and may offer neuronal resistance against pathological tau by reducing production of oxidative species and improving mitochondrial function [13]. Conversely, a decline in TERT is implicated in the cognitive decline of aging and of dementia.

Klotho is an enzyme that in humans is encoded by the KL gene [14]. klotho can suppress oxidative stress and inflammation, thereby reducing endothelial dysfunction and atherosclerosis [15]. Klotho is required for oligodendrocyte maturation, myelin integrity, and can protect neurons from toxic effects [16]. Mice, deficient in klotho, expressed a reduced number of synapses and cognitive deficits, whereas mice overexpressing klotho have enhanced learning and memory [17]. Klotho-deficient mice manifest a syndrome resembling accelerated human ageing and extensive and accelerated arteriosclerosis. Klotho deficient mice also exhibit impaired endothelial dependent vasodilatation and impaired angiogenesis, suggesting that the klotho protein may protect the cardiovascular system through endothelial-derived nitrous oxide (NO) production. Further, Klotho is postulated to play a protective role in Alzheimer's disease patients [18,19]. It was demonstrated that an over-expression of klotho in mice could extend their average life span between 19% and 31% compared to normal mice [10]. In addition, variations in the Klotho gene (SNP Rs9536314) are associated with both life extension and increased cognition in human populations [20].

Assessing the cognitive status in dementia patients is best done using a serial testing strategy. The Folstein test is an acceptable diagnostic tool used in the clinical setting to assess the degree of cognitive function at diagnosis and over time.

Methodology

Five patients participated in the study and were classified as having mild or moderate dementia. All patients performed witnessed informed consent and provided information from their primary doctor including diagnosis, brain images, and bloodwork. All accepted to participate in genetic testing and telomere testing. Pre-treatment Folstein cognitive testing was performed on all patients and repeated post treatment at intervals of approximately once a month following the initial treatment. Pre-treatment medical laboratory blood analysis was performed and repeated post treatment along with doctor office visits at 3, 6-, 9-, and 12-month periods. Pre-treatment brain Magnetic Resonance Imaging was performed and repeated 10 months post treatment. Participants were subjects with diagnosed dementia and were qualified as detailed in Table 1.

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Inclusion Criteria	• All subjects are diagnosed with dementia • Patients include adult females and males over 40 from any ethnic or racial group • Female patients >40 years of age must be post-menopausal • Could cooperate for cognitive testing • Brain imaging • High school graduate or higher education
Exclusion Criteria	• Female participant who is pregnant, lactating, or planning pregnancy during the study • Female participant who is premenopausal • Significant renal or hepatic impairment • Scheduled elective surgery or other procedures requiring general anesthesia during the study • Participant with life expectancy of less than 6 months • Any other significant disease or disorder which, in the opinion of the Investigator, may either put the participants at risk because of participation in the study, or may influence the result of the study, or the participant's ability to participate in the study • Participants who have been in another research study involving an investigational product in the past 12 weeks • Hypersensitivity to product constructs • Active viral infection based on clinical observations • Patients with increased bleeding risk (such as thrombocytopenia) should not be treated due to the large number of intramuscular injections required • Immunodeficiency • Serological evidence of HIV infection, or Hepatitis A, B or C infection • Ongoing immunosuppressive therapy or immunosuppressive therapy within 3 months of starting the study (e.g., corticosteroids, cyclosporine, tacrolimus, methotrexate, cyclophosphamide, intravenous immunoglobulin, rituximab) • Concomitant illness or requirement for chronic drug treatment that in the opinion of the PI creates unnecessary risks for gene transfer. Patients taking any of the following drugs will be excluded: drugs for treatment of myopathy or neuropathy or agents used to treat diabetes mellitus • Mental illness that would make progress testing difficult or impossible • Patients with history of angina and patients with a history of myocardial infarction in the past 6 months • Anti-platelet or other anticoagulant medicinal products must not be used concomitantly at the time of injection and for at least one week before or one day after the injection

Table 1: Inclusion and exclusion criteria.

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An identical intranasal dose of an enzymatically augmented neurotropic AAV subtype was administered to each patient. Surveillance for minor and serious adverse effects was performed. All patients completed the SAE forms as requested.

Statistical Analysis

Initially a linear model was developed considering the follow up months as independent variable and Folstein testing score as the dependent variable. Age and gender were included in the models to evaluate interactions with follow up time and to obtain adjusted effect sizes. Non-linear associations were assessed with generalized additive models. The follow up time showed a non-linear association with Folstein testing score and a piece-wise linear function was adopted. The know position was determined using the loglikelihood ratio test. The linear model showed a significant overdispersion (i.e., residual deviance=666.81, df=39, P<0.001).

Each followed up person had a different Folstein testing score to start with making it justifiable to include random intercepts to the model. Therefore, a mixed effect model was fitted incorporating individual level random effects. The variance of the individual level random effect was significantly different from zero (delta deviance =37.5, df =1, P <0.001). Therefore, mixed effect model with individual level random effects were considered as the final model.

Pre and post comparison were done with Wilcoxon Rank Sum test. A P value of 0.05 was considered as significant. R programming language version 3.6.3 were used in the analysis.

Results

We studied five patients with mild or moderate dementia for a period of 12 to 15 months (Median=13.4 months, interquartile range=12.6 to 14.6 months). Three were males (68, 83 and 86 years) and two were females (83 and 84 years).

Initial Folstein testing scores of the patients ranged from 13 to 26 (median=22, interquartile range=16–22). The changes in the Folstein testing scores for the follow up is shown in figure 1.

The Folstein testing showed an increase from the baseline after the intervention. There was a sharp increase in cognitive test scores during the first 3.5 months after treatment with the average per month test score increase being 1.7 points. After 3.5 months, the scoring showed a slight decrease with an average drop of 0.07 points per month. The age or gender did not show significant effect on the scoring nor interaction with the follow up time.

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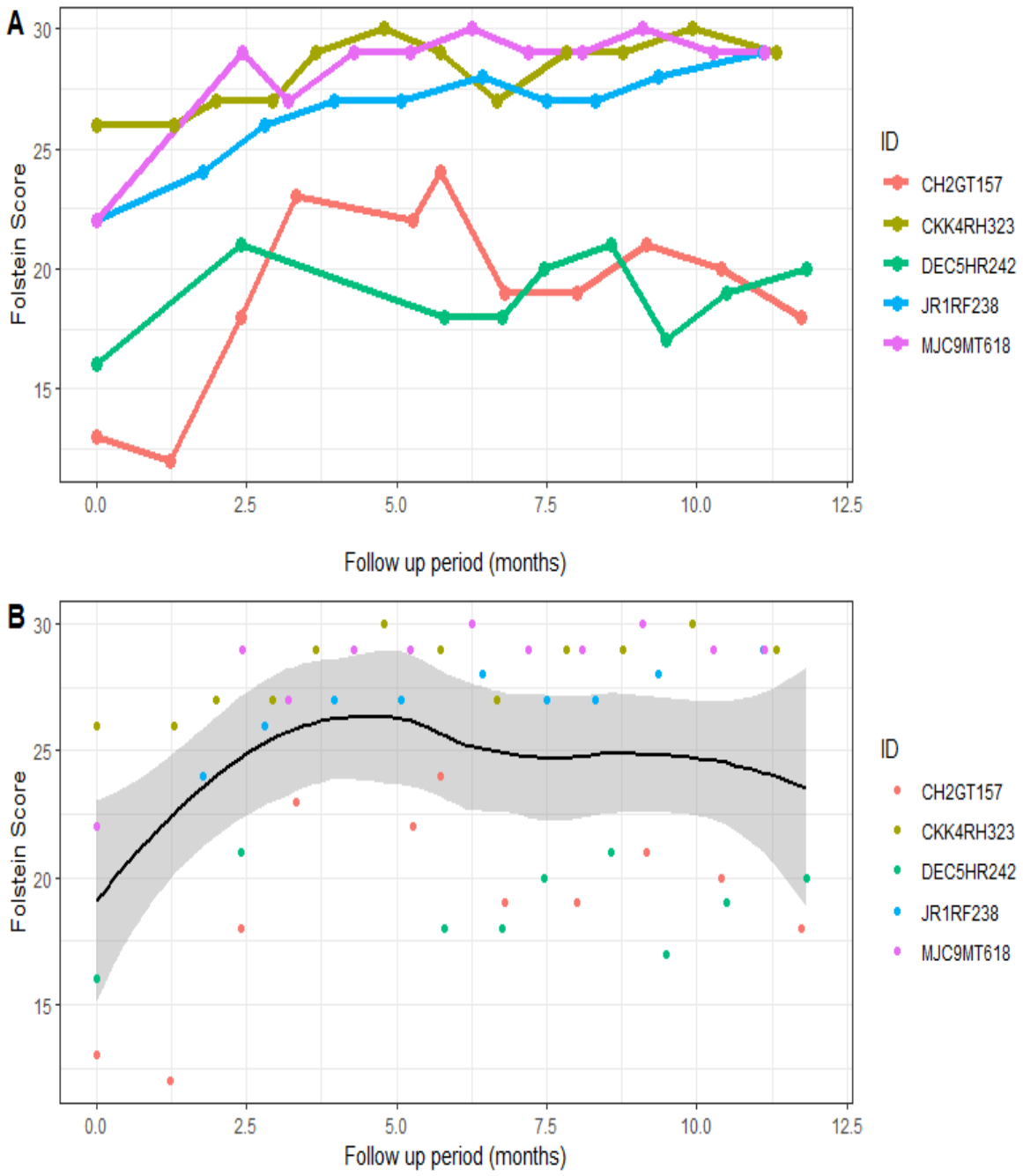

Figure 1: Graphical demonstration of the individual Folstein testing results beginning with the pre-treatment baseline scores and continuing with the 12-month post treatment results. A) line graphs showing individual patient scores. B) individual scores with locally estimated scatterplot smoothing (LOESS).

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Parameter	Estimate	Std. error	T value	P value
Intercept	7.81	32.04	0.24	0.808
Age (years)	0.176	0.38	0.46	0.69
Gender: male	-4.377	5.05	-0.87	0.477
Follow up months	1.68	0.21	7.94	<0.001
Follow up months >3.5	-1.73	0.25	-6.87	<0.001
SD (Random intercept)	5.08	-	-	-

Table 2: Parameter estimates of the mixed effect model.

Telomere analysis

Telomere length shortens with cell division. Short telomeres have been associated with increased incidence of diseases and decreased survival. Progressive shortening of telomeres leads to cell senescence (inability to replicate) and ultimately apoptosis (programmed cell death). Cells with shortened telomeres have increased inflammation and oxidative stress, and exposed chromosomal DNA which can result in aborted apoptosis thru oncogenic transformation of somatic cells [22]. For this reason, we looked specifically at the cells with critically short telomeres (20th percentile and under). This group holds special significance; a measurable increase in the groups cell telomere length is consistent with some degree of rescue of that cell population from impending senescence, apoptosis, or oncogenic transformation.

Figure 2 demonstrates the test subjects various ages and telomere lengths [D1]. The Pre-treatment mean age was 80.6 (SD=7.0) years and the post-treatment mean age was 81.5 (SD=7.0) (Figure 2A). The biological age decreased in four of the five patients with the pretreatment mean biological age measuring 82.8 years (SD=6.6) and the post treatment mean biological age measuring 79.4 years (SD=9.1). There was an increase in the Median telomere length in Four of the five patients with the median pre-treatment telomere length being 8.88 (SD=0.89) and the median post treatment telomere length being 9.54 (SD=1.11). There was an increased telomere length in the 20th percentile group of telomers in all 5 patients with the mean pretreatment measuring 3.86 (SD=0.59) and the mean post treatment measuring 5.70 (SD=1.27). This increase was statistically significant (Wilcoxon Rank Sum test, P=0.027).

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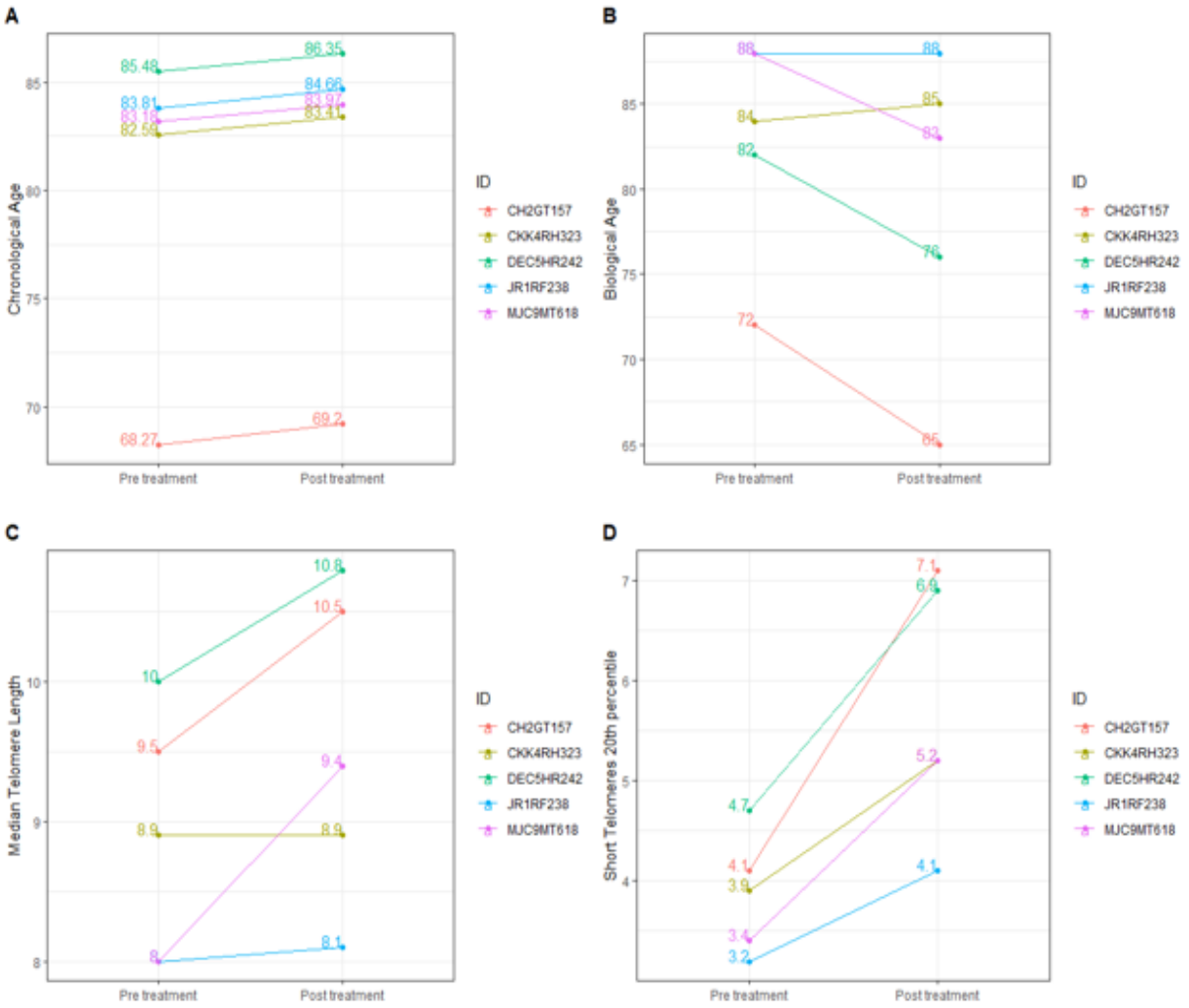

Figure 2: Subjects chronological age (A), biological age (B), median telomere length (C) and 20th percentile group of telomers (D) at pre and post treatment.

Radiological and Medical Laboratory Analysis

Diagnostic Radiology physicians interpreting the participants brain MRI exams reported no appreciable differences between the pretreatment and post treatment exams. No appreciable morphological changes were seen. There were no new findings on the post treatment imaging studies such as stroke, new or increased atrophy, intracranial bleeding, mass or mass effect, hydrocephalus, or evidence of trauma.

Complications and Safety

No short term or long term clinical or laboratory complications were observed or identified. Monitored serum chemistry, electrolyte, and hematological values

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remained stable throughout the follow-up time. No serious adverse effects such as a visible or laboratory detected innate or humoral immune response was observed or reported. Hepatic function as monitored by clinical laboratory values remained within normal parameters without evidence of hepatic insult. The treatment was well tolerated with only brief minimal physical discomfort at the injection site.

Discussion

Adeno-associated virus (AAV), first discovered in 1965 as a contaminant of adenovirus samples, is a very popular gene therapy vector due to its safety profile. It is currently a particular favorite vector for investigating gene therapies targeting the central nervous system. It is a member of the Parvoviridae family and can be described as a dependovirus due to the need of a helper virus or some other cellular stressor to replicate. Its size is around 20 nm which limits the gene payload size. There are hundreds of different strains or serotypes but only a couple of handfuls have been developed into therapeutic vectors. [23-30]

Recombinant AAV (rAAV) is particularly desirable as a vector since most of the viral DNA has been removed leaving 4% or less of the original viral DNA to invoke an immune response. This results in a much lower incidence of antigen specific immunity. [31] This reduced antigen specific immunity is presumed to be from a lack of antigen presenting cell (APC) transduction [32]. After rAAV traverses the cell membrane, thru a series of cellular transport mechanisms it ultimately ends up as an episome in the cell's nucleus adjacent to but not integrated into the nuclear DNA [33].

AAV may be poorly immunogenic compared with other viruses however the capsid proteins and the nucleic acid sequence delivered can trigger the various components of our immune system. Prior exposure to AAV, particularly the capsid proteins, can result in activation of neutralizing antibodies (NABs) as well as T cells. This immune response further primes the immune system against those capsid proteins and other AAV components. Future AAV administration then carries a high likelihood of invoking an immune response capable of reducing the efficacy of future therapies transfection rate as well as priming the system to attack previously transduced cells [34]. Innovations designed to reduce the incidence of an immune response in future AAV administrations include alteration of capsid proteins, utilization of new serotypes, and alteration of the promotor and enhancer technologies [35].

AAV mediated gene therapy can stimulate very specific innate immune system mechanisms that are particularly harmful to cells having a very large endoplasmic reticulum, such as hepatocytes. Historically, the risk of such an adverse innate immune system reaction has been addressed with a regimen of immunosuppressant medications. The degree of protection afforded by these immunosuppressant medications is dependent not only on the dose of the immunosuppressant drug administered, but

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also on the systemic dose of AAV administered as a therapeutic agent.

Recent reports of fatalities in the ASPIRO (AT132) trial highlighted the potential dangers of AAV therapy regarding dose limitations and immunological issues. In the AT132 trial, large doses (1E14 vg/kg or higher) of AAV administered via an intravenous route in patients with preexisting hepatic disease resulted in the death of 4 participants. Mechanisms felt to be causal in these deaths are direct hepatic toxicity from the large viral load as well as stimulation of an aggressive immune response [36].

With IV administration, hepatic toxicity is the greatest threat to the safety of the dose recipient because the liver is the critical organ-the organ receiving the largest dose. IV delivery also carries a higher risk of stimulating an aggressive innate immune response. Prior nonhuman primate studies have suggested that an intravenous dose of 2E14 vg/kg can cause significant hepatic dysfunction leading to death [37].

Regional AAV gene transfer therapy is felt to be less risky due to the lower does utilized or required, minimal viral load to the liver, and that the regional therapy is often delivered to immune privileged areas like the ocular globes or the central nervous system. The patients in this study received the equivalent of 1/10000 of the dose of the patients in the AT132 trial. Additionally, in this case the dosing was regional rather than intravenous. This extremely low dose essentially minimized the risk of hepatoxicity to almost zero. Additionally, the regional delivery to the CNS minimized the risk of a significant humoral response thereby maximizing the potential for cell transduction.

AAV vector mediated gene transfer therapy reliably inserts the gene into the cytoplasm of the cell and subsequently the nucleus existing as an episome unintegrated into the nuclear DNA. The intended result of our therapy is the establishment of a nuclear episomal inclusion that will contain an extra copy of the hTERT gene and an extra copy of the KLOTHO gene. These nuclear episomal inclusions will produce extra telomerase and alpha-Klotho.

The cellular biological effects from the extra telomerase production in the central nervous system should be elongation of telomeres, improvement of mitochondrial health and function, reduced oxidative stress imposed by pathological Tau, improved genomic stability, reduced senescence, and reversal of cellular biological aging [38].

The cellular biological effects from the extra Alpha Klotho production in the central nervous system should be lowered intracellular oxidative stress and cellular inflammation with reduced endothelial dysfunction and atherosclerosis [15], clearing of neuronal damage by a restored microglial system, increased oligodendrocyte maturation, myelin integrity, and reduction or removal of Amyloid beta plaque [16,39,40]

Our telomere analysis before and after treatment demonstrated evidence of increased telomerase function. The median and the short telomeres were measurably

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elongated. Reduced biological age was seen in 4 of 5 participants.

Cognitive assessment before and serial cognitive testing after treatment demonstrated a rapid improvement in cognitive function during the first 3.5 months after which the improvement slowed until it leveled off at five months. Thereafter there is a slight decrease slope in the average test score with an average drop of 0.07 points per month. Given that Patients with Alzheimer's dementia typically show an annual decline of 3 points on the on the Folstein test [21], our results indicate that the AAV hTert and Klotho gene transfer therapy was successful, and the effects of that gene therapy reversed some of the dementia pathology.

Pretreatment brain MRI compared to post treatment Brain MRI demonstrated no significant changes. This doesn't seem surprising considering the imaging was separated by only 10 months. With longer follow-up and continued or sustained cognitive gains, one would expect to see additional morphologic changes in the brain which might include a decreased rate of atrophy or stabilization of anatomical structures, possibly a cessation of further atrophy, and even possibly, a measurable reversal of structure atrophy. Diminishing Amyloid Beta plaque presence and reduction of Tau tangles may become evident, and Functional MRI might show some slowing or reversal of the regional hypometabolism seen on Functional MRI as dementia advances.

Given the regional delivery of the therapy and given that the CNS is relatively immune privileged, it's not surprising that there were no significant immune responses.

Conclusion

Using a novel and proprietary CNS gene delivery method, rAAV hTERT and Klotho gene transfer therapy was administered to participants with mild or moderate dementia and was evaluated primarily for safety and secondarily for clinical benefit. Results demonstrated a very high safety profile with no significant adverse treatment events. Clinical findings were significant with all participants demonstrating improved cognitive function based on Folstein testing indication a successful gene transfer therapy and a reversal of underlying dementia pathology Telomere analysis detailed elongation of the participants telomeres, and a reduction in biological age.

While the results of this study are exciting and significant, we recognize it is a small study that should prompt further investigation with a larger cohort and more sophisticated analysis.

Limitations of Study

This interim report is limited by the number of patients and the duration of the follow-up period. The project is ongoing, and more patients will be enrolled; the five patients described here will continue to be followed per the study protocol, and future measures of safety and efficacy will be recorded at subsequent study visits.

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