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Telomerase Activator 65 (TA-65) Favorably Affects Parameters of Metabolic Syndrome - A Pilot Study

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**Telomerase Activator 65 (TA-65) Favorably Affects Parameters of
Metabolic Syndrome - A Pilot Study**

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MSc Medical Biochemistry, Amrita Vishwavidyapeetham, India, 2010

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Approval page

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Telomerase Activator 65 (TA-65) Favorably Affects Parameters of Metabolic Syndrome-

A Pilot Study

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List of Abbreviations

Alanine Transaminase	ALT
American Diabetic Association	ADA
Aspartate Amino Transaminase	AST
Blood pressure	BP
Body Mass Index	BMI
C Reactive Protein	CRP
Carbohydrate Restricted Diet	CRD
Coronary heart disease	CHD
DNA damage response	DDR
Enzyme Linked Immunosorbent Assay	ELISA
European Association on the Study of Diabetes	EASD
European Group for the Study of Insulin Resistance	EGIR
Glycemic Index	GI
Glycemic Load	GL
Glycosylated hemoglobin	HbA1C
High Density Lipoprotein cholesterol	HDL-c
International Diabetes Federation	IDF
Low Density Lipoprotein cholesterol	LDL-c

Metabolic syndrome	MetS
National Cholesterol Education Program/ Adult Treatment Panel III	NCEP/ATP III
Nutrition Data System for Research	NDSR
Polymerase Chain Reaction	PCR
Repressor/activator protein 1	RAP1
Standard deviation	SD
Telomerase Activator- 65	TA-65
Telomere length	TL
Telomeric Repeat Amplification protocol	TRAP
Telomeric repeat binding factors 1 and 2	TRF1, TRF2
Terminal restriction fragmentation	TRF
Type 2 Diabetes	T2D
Waist circumference	WC
World Health Organization	WHO

Abstract

Background: Telomerase Activator 65 (TA-65), a compound extracted from *Astragalus membranaceus*, was developed to increase or maintain telomere length. **Objectives:** To determine the effects of TA-65 on the parameters of metabolic syndrome (MetS). **Methods:** We recruited 40 patients aged 32-70 years with MetS to determine the effects of TA-65 on dyslipidemias and anthropometrics in this at-risk population. This was a double-blind, randomized crossover design in which patients were allocated to consume either 16 mg daily of a TA-65 supplement or placebo for 12 weeks. Following a 3-week washout, participants were allocated to the alternate treatment for an additional 12 weeks. Anthropometric and biological markers were measured at the end of each treatment. Plasma lipids, fasting blood glucose, C-reactive protein (CRP), liver enzymes, and glycosylated hemoglobin were measured using a Cobas c-111. Plasma insulin was measured by ELISA and telomere length was measured in lymphocytes and granulocytes using q-FISH assay. Diet records were analyzed by using the Nutrition Data System for Research (NDSR) software. **Results:** There were no changes in dietary intake between treatments and most of the parameters of MetS were not altered. Compared to the placebo period, HDL cholesterol (HDL-c) was higher while body mass index, waist circumference, and the LDL-c/HDL-c ratio were lower during TA-65 treatments ($p < 0.05$). Negative correlations of changes in HDL-c and CRP ($r = -0.511$, $p < 0.01$) and HDL-c and alanine aminotransferase ($r = -0.61$, $p < 0.001$) were observed during the treatments, suggesting that the favorable changes observed in HDL-c were associated with decreases in inflammation. Telomere length was not changed from baseline even after 3 months of TA-65 treatment. **Conclusion:** TA-65 slightly improved key markers of cardiovascular disease risk, while sustaining telomere length in patients with MetS

Chapter 1

Introduction

Telomere, a compound structure of unique DNA repeats (TTAGGG) present at the end of each strand of DNA, represents a mechanism that offers protection to chromosomes against degradation (1). During each cell division, DNA polymerase cannot fully replicate the 3' end of linear DNA, which results in progressive telomere shortening, leaving DNA vulnerable to damage (2). Shortening telomeres act as the aging clock in every cell. As they shorten, they signal change in gene expression, changing the cell's phenotype to that of an older cell. Furthermore, short telomeres lead to genetic mutations and can result in serious complications associated with age (3). In addition to aging, telomere length (TL) can also be shortened by oxidative stress, smoking, obesity, lack of exercise and a poor diet (3,4,5). Shortening of TL is observed in connection to several disease conditions such as cardiovascular diseases, stroke, diabetes, osteoporosis, liver disease and cancer. Normally, TL can be maintained or increased by addition of TTAGGG telomere sequences to the ends of chromosomes guided by the enzyme telomerase. However, the telomerase production declines with age, mortality and disease conditions. TA-65® (Telomerase Activator-65), a patented compound developed by TA Sciences claims to activate telomerase production and thus help maintain or rebuild telomeres without any side effects.

TA-65® is a patented, natural, small molecule that activates the telomerase enzyme and enhances telomere length. It rejuvenates the immune system and other critical cells compromised by age and daily stress. TA-65 is the only known commercially available telomerase activator on the market and is an expensive nutraceutical. TA-65 is a concentrated herbal extract from the root of

Astragalus membranaceus, which has been used safely for millennia in traditional Chinese medicine (6). Several studies have shown beneficial effects of TA-65 in increasing telomerase activity and telomere length (7). Additionally, it has been demonstrated to improve cardiac function and possibly decrease insulin resistance (9). These protective effects of TA-65 have been attributed to various fractions of the root extract, most notably the extract that is the proprietary molecule TA-65. TA-65 has shown a significant capacity to stimulate the rescue of short telomeres, both in vitro (in mouse embryonic fibroblasts) and in vivo, according to a study performed in mice by Blasco et al (7). There was a significant drop of very short telomeres (less than 2, 3, and 4 kb) in the groups of mice previously treated with TA-65, following 3 months of treatment (7). Essentially, the TA-65 molecule, increases protective cytokine release and enhances the immune system without increasing inflammatory responses. In a prospective, randomized, double-blind study conducted by Marshall-Blum et al in men aged 60 and 84, participants took TA-65 at a dose of between 2 and 4 capsules a day, depending on the treatment group assigned, for 24 weeks (8). Laboratory tests were performed 2 weeks before inclusion, at the baseline visit, and at 6, 12, and 24 weeks of treatment. Cardiac events frequency decreased on average in the subjects that took TA-65 extract in comparison with the baseline value (9). Subjects treated with TA-65 showed a statistically significant improvement ($P < 0.0001$) in quality of life parameters, measured both by self-evaluation and by health personnel.

Telomere length is maximum at birth and decreases progressively with age, thus it is considered as a biomarker of chronological aging. This age associated shortening of TL is linked to various age-related diseases like diabetes, hypertension, Alzheimer's disease, cancer etc. and their associated complications. Telomere length is a result of combined effect of oxidative stress,

inflammation and repeated replication of cells, thus forming an association between telomere length and chronological aging and age-related diseases. Shortening of telomere length in peripheral blood mononuclear cells is associated with mortality in patients with stable coronary artery disease, suggesting that telomere length can be used as a predictor of adverse outcomes in these patients. Shorter leukocyte telomere length has been linked to impaired glucose tolerance, Type 2 Diabetes, and coronary heart disease. Thus, telomere length may play an important role in predicting cardiovascular disease and diabetes. TA-65 has proven to be effective in supporting various disease conditions by activating the immune system, decreasing inflammation, boosting anti-oxidant profile in addition to telomerase activation which prevents telomere length shortening. Considering the problems of age related metabolic diseases, TA-65 can be considered as a possible supplement to correct the parameters affecting these conditions. It may not only ameliorate the symptoms associated with these disease condition but could be a preventive measure as well.

Although we have some information on the effects of TA-65 on clinical studies, the effects of TA-65 on Metabolic Syndrome (MetS) is scarce. MetS is a constellation of metabolic conditions which double the risk for heart disease and quintuple the risk for diabetes mellitus (10). MetS is characterized by central obesity, hypertension, hyperglycemia, and dyslipidemia in combination with oxidative stress and systemic inflammation (11). There are many proposed strategies to reduce the biomarkers of MetS including dietary interventions (12), increased physical activity (11), or the use of bioactive components that may target specific physiological pathways associated with the symptoms (12,13). A large population-based study with 6 years of follow-up by Revesz et al revealed that short telomere length could a predictor for prevalence and progression of MetS components (14). It was shown particularly that increasing abdominal obesity was accompanied

by accelerated telomere attrition. Since TA-65 has been found to ameliorate disease conditions involving MetS components, the possibility of TA-65 to correct or modify the metabolic abnormalities in patients suffering from MetS is high. This study fills that gap in being the first to evaluate the effectiveness of TA-65 in individuals with MetS.

This study is a life style intervention in 11 men and 37 women classified with MetS. The objective of the study was to evaluate the efficacy of TA-65 on the parameters of MetS. We hypothesized that when compared to placebo, TA-65 would improve glucose metabolism by lowering plasma glucose, plasma insulin and glycosylated hemoglobin, reduce dyslipidemias by decreasing plasma triglycerides, increase HDL-c and lower LDL-c, lower blood pressure and waist circumference and reduce systemic inflammation in patient with MetS thus, favorably modify the parameters of MetS.

Chapter 2

Literature Review

2.1 Metabolic syndrome

Metabolic syndrome (MetS) is a multi-faceted condition characterized by increased central adiposity, arterogenic dyslipidemias, insulin resistance, high blood pressure, low grade inflammation and oxidative stress (15). In line with national obesity trends in the United States, it has been estimated that ~34% of adults have MetS (16). The progression of these metabolic abnormalities amplifies the risk for cardiovascular disease by two-fold and the risk for type 2 diabetes mellitus by five-fold (17). The pathophysiology in the development of the disease is complex and not completely understood. Despite ethnicity, MetS is being identified globally as a growing epidemic due to the sedentary life style and poor diet (18). The clustering of metabolic syndrome risks with cardiovascular disease and DM has been recognized for more than 80 years, but the modern concept of metabolic syndrome began when Reaven proposed a conceptual framework linking the metabolic events in a single pathophysiological construct (19). In 1947 Vague, a physician from Marseilles, observed that upper body obesity was significantly associated with diabetes mellitus, atherosclerosis, gout and calculi, in which all these conditions were improved by eating low carbohydrate diet (20,21). Haller in 1977 described metabolic syndrome to be associated also with obesity, diabetic mellitus, hyperlipoproteinemia, hyperuricemia and hepatic stenosis (22). In the same year, Singer added that hypertension and gout could also be associated with the syndrome (23) In 1988, Reaven proposed the theory that insulin resistance provides common mechanisms underlying the associated abnormalities such as blood pressure, lipid

abnormalities and glucose intolerance in his landmark Banting lecture (24). Reaven also termed the disease as syndrome X. He did not include obesity as one of the factors in the syndrome as the previous authors did. However, obesity in particular, central obesity came later to be recognized as one of the important underlying factor of metabolic syndrome. The terms metabolic syndrome, insulin resistance syndrome or syndrome X were used to define the clustering of factors that increase the risk for cardiovascular disease and type 2 diabetes. The exact pathology of metabolic syndrome is still not yet fully known (25). The first clinical definition was released by the WHO in 1998. It included impaired glucose tolerance (IGT/IFG), diabetes, or insulin resistance, calculated according to the homeostasis model assessment (HOMA) or through oral glucose tolerance test (OGTT) as an essential component in addition to dyslipidemia (low HDL-C and high TG), hypertension and microalbuminuria. In 1999, the European group for the study of Insulin Resistance (EGIR) proposed the modification to the WHO definition.

In 2001 the NCEP ATP III released a recommendation which excluded insulin resistance and introduced five equal components based on routine clinical measurements of which three have to be present for the diagnosis of MetS (26). These criteria were: elevated waist circumference, high TG, low HDL-c, elevated BP and fasting glucose. This was again revised in 2003 based on the recommendations of the American Diabetic Association (ADA) by decreasing the fasting glucose cut off point to <100mg/dL. In 2004, the International Diabetes Federation (IDF) published new criteria that modified the ATP III definition. The IDF considered central adiposity as a key component to MetS and highlighted that the determination of central obesity requires the attention to gender and ethnic specificity. In 2005 The American Diabetes Association (ADA) and European Association on the Study of Diabetes (EASD) pointed out specific controversies in the diagnosis of metabolic syndrome (27) For instance the value of including diabetes in the definition was

questionable. Also, the use of insulin resistance as the unifying etiology was considered to be uncertain. The ADA/EASD committee also pointed out that there was no clear basis for including or excluding cardiovascular disease (CVD) risk factors in the diagnosis of the metabolic syndrome. There are a number of arguments regarding this topic and the debate on the definition and diagnosis of metabolic syndrome continues. There are several existing criteria in use for defining the metabolic syndrome like the definition used by the American Heart Association (AHA), European Group for the Study of Insulin Resistance (EGIR), National Cholesterol Education Program-Adult Treatment Panel III (NCEP/ATP III), World Health Organization (WHO) and International Diabetes Federation (IDF). The most commonly used definition of MetS was described by the WHO and NCEP ATP III criteria (27) According to the NCEP ATP III definition, metabolic syndrome is present if three or more of the following five criteria are met: waist circumference over 40 inches (men) or 35 inches (women), blood pressure over 130/85 mmHg, fasting triglyceride (TG) level over 150 mg/dL, fasting high-density lipoprotein (HDL-c) cholesterol level less than 40 mg/dL (men) or 50 mg/dL (women) and fasting blood sugar over 100 mg/dL.

The NCEP ATP III definition is one of the most widely used criteria of metabolic syndrome. It incorporates the key features of hyperglycemia/insulin resistance, visceral obesity, atherogenic dyslipidemia and hypertension. It uses measurements and laboratory results that are readily available to physicians, facilitating its clinical and epidemiological application. It is also simple and easy to remember. Importantly, it does not require that any specific criterion be met; only that at least three of five criteria are met. Thus, the definition does not build on any preconceived notion of the underlying cause of metabolic syndrome, whether it is insulin resistance or obesity.

Regardless of the existing controversies in diagnosis and definition, the metabolic syndrome is still considered to be a useful diagnostic tool in primary care prevention. It gives opportunity for early patient identification and education on proper and early health behavioral changes implicated in the development of the deadly cardiovascular diseases like hypertension and diabetes. Patients could be educated early about the connection between their lifestyle, health risks, and medical outcomes. For instance, NCEP/ATP III identifies metabolic syndrome as an indication for vigorous lifestyle intervention. Effective interventions include diet modification, exercise, and judicious use of pharmacologic agents to address specific risk factors. Weight loss will significantly improve all aspects of metabolic syndrome (28) . Increase in physical activity and decrease in caloric intake by reducing portion sizes have been found to improve metabolic syndrome abnormalities, even in the absence of weight loss. Specific dietary changes that are appropriate for addressing different aspects of the syndrome include reducing saturated fat intake to lower insulin resistance, reducing sodium intake to lower blood pressure, and reducing high-glycemic-index carbohydrate intake to lower triglyceride levels. These dietary changes include carbohydrate restricted diet (CRD) (28), the Mediterranean diet (29), low fat diet (30), eggs as a part of low fat diet (31) and several others (32).

2.2 Telomere

The important role of chromosome ends in ensuring chromosomal stability was first proposed from the classic studies of the 1930s by Hermann Muller working with fruit flies (Nobel Prize 1945) (33) and Barbara McClintock working with maize (Nobel Prize 1983) (34). These conclusions have stood the test of time, and since this work was published, an enormous amount of data on telomeres and their function has been produced. Ever since these conclusions were made,

enormous amount of research on telomeres, its functions and the details of the mechanism and molecules associated were focused. Muller coined the term, from the Greek for “end” (telos) and “part” (meros). McClintock pointed out that without these special end structures, chromosomes would fuse and often break upon mitosis, resulting in chromosome instability which was detrimental to cells. These pioneering studies established that functional “telomeres” are required to protect chromosome ends, to provide chromosome stability, and to ensure faithful segregation of genetic material into daughter cells upon cell division. This protective function of telomeres is known as ‘telomere capping’. The molecular mechanisms underlying telomere capping involve specialized protein complexes bound to telomeres (35-37) as well as telomere-associated noncoding RNAs known as TERRA (38-40).

If telomere capping is disrupted, telomere fusions generate dicentric chromosomes that are susceptible to breakage during mitosis, ultimately leading to aneuploidy and disease states including premature aging pathologies and cancer (41,42,43) Telomere science has raised to further glory and attention when the 100th Nobel Prize in Medicine and Physiology (2009) was awarded jointly to Elizabeth H. Blackburn, Carol W. Greider and Jack W. Szostak for the discovery of “how chromosomes are protected by telomeres and the enzyme telomerase”(44). Telomeres can be naively described as specific cap like segments of DNA at the end of chromosomes. They are composed of a DNA component characterized by noncoding repetitive sequences rich in Guanine (G) and multiple protein components. The DNA component of telomeres is characterized in all vertebrates by tandem repeats of (TTAGGG/CCCTAA) n and the repeat sequences can vary from one species to another (45). They protect the genome from nucleolytic degradation, unnecessary recombination, repair and inter-chromosomal fusion, thereby playing a pivotal role in preserving the information in the genome. As a normal cellular process in

all cells, a small portion of telomeric DNA is lost with each cell division. The diminishing of TL has been linked with progression of age, diabetes, cancer and an increased risk of death. A myriad of proteins is directly or indirectly associated with telomeric DNA. Some of these proteins are found in telomeres at any time, even though there is a highly dynamic exchange between proteins that are telomere-bound and unbound (46).

2.3 Structure and formation of telomere

Telomeres form a special heterochromatic structure at the end of linear chromosomes. They are cap like structures that protects chromosomes from degradation, preventing fusion of sticky arms and are essential for preventing genomic instability (47). Thus, telomeres are essential to ensure chromosome stability (48). Mammalian telomeres comprise several kilobases, between 10 and 15 kb in humans and 25 and 50 kb in mice, of tandem TTAGGG DNA repeats (47) Telomeres are characterized by the presence of a 30–400-nucleotide-long 3' overhang of a G-rich strand, known as the G-strand overhang. The G-strand overhang can fold back and invade the double-stranded telomeric region, forming the so-called T-loop and generating a displacement loop, or D-loop. The T-loop structure has been proposed to protect chromosome ends from degradation and DNA repair activities as well as from telomerase activity (49,50). Telomeres are bound by a specialized complex known as shelterin that has crucial functions in telomere length regulation and in the protection of telomeres from the DNA damage response (DDR) by masking the chromosome ends from the DNA repair machinery through repression of the ATM and ATR signaling pathways (48). The shelterin complex is composed of six proteins: telomeric repeat binding factors 1 and 2 (TRF1 and TRF2), TRF1-interacting protein 2 (TIN2), protection of telomeres protein 1 (POT1), TIN2, POT1-interacting protein (TPP1), and repressor/activator protein 1 (RAP1) (50-52). Telomeres

shorten with each cell division as a result of the incomplete replication of linear DNA molecules by conventional DNA polymerases, which is called the end-replication problem (53,54).

2.4 Telomere homeostasis

Due to repetitive DNA replication, the end telomere sequence is lost in each cycle. Shortening is restored by the cell using various mechanisms to maintain homeostasis. Telomerase adds bases to the ends of telomeres (37). In young cells, telomerase keeps telomeres from wearing down too much. But as cells divide repeatedly, there is not enough telomerase, so the telomeres grow shorter and the cells age. As shortening advances, this may lead to aging, cellular death or even cancerous transformation of somatic cells, thereby affecting the health and life span of the individual. Telomerase remains active in sperm and eggs, which are passed from one generation to the next. If reproductive cells did not have telomerase to maintain the length of their telomeres, any organism with such cells would soon go extinct (42).

Several studies have derived evidence on the inverse relationship between telomere length and increased incidence of disease resulting in higher mortality rates. Life style modifications have been proven to reverse these effects (74).

2.5 Quantification of Telomere Length

Multiple methods have been developed for the study of telomere length. These techniques include quantification of telomere length by southern blots of terminal restriction fragmentation (TRF)- which was one of the earliest tools for length assessment- making it the gold standard in telomere biology. TL has also been successfully measured using real-time quantitative polymerase chain reaction (qPCR), which provides the advantage of requiring small samples of DNA for analysis.

Another method, Quantitative Fluorescence in situ hybridization (q-FISH) of telomeric repeats is performed by assessing metaphase chromosome or interphase nuclei using a fluorescent (CCCTAA)₃ probe in cells, rather than DNA. Alternate and the recently used mechanism is by Flow FISH assay. telomere length measurement of leukocytes is very accurate using flow-FISH and provides cell population specific measure, but its utility in population-based studies is limited by its need for viable cells. The telomere length in both granulocytes and lymphocytes are measured using the assay. The correlation of telomere length between these measurements is generally modest. In an epidemiological study of 681 elderly individuals, a weak correlation of ($r = 0.52$) was generated between Southern blot and qPCR. A similar result ($r = 0.47$) was seen when comparing telomere length measured by qPCR and flow FISH in 52 normal individuals. No data are available for the correlation between flow FISH and Southern blot telomere length beyond the strong correlation reported in the initial flow FISH study ($r = 0.9$) (71,72)

One of the major drawbacks in using telomere length as a clinical measure is its high variability between different individuals, which is determined at birth. Furthermore, shortening with age is more rapid in males than females, and the rates can also differ between various ethnic groups. All these factors limit the usefulness of telomere measurement in cross-sectional studies; thus, longitudinal studies measuring actual telomere erosion rates in individuals over times represent more powerful study designs for demonstrating causal effects. (73)

2.6 Shortening of Telomere Length

In 1961, Leonard Hayflick discovered that human cells could undergo only a limited number of cell divisions when cultured in vitro (55), a phenomenon known as replicative senescence or the Hayflick limit (56). This is an important feature of telomeres; their length determines the number

of cell divisions that a cell can undertake. Alexei Olovnikov linked the Hayflick limit to the replication of telomeric DNA. The existence of a compensatory mechanism for telomere shortening was first found in 1973 by Alexy Olovnikov who also suggested the telomere hypothesis of aging and the telomere's connections to cancer (57). Replication of the 5'-3' strand requires RNA primers that are removed afterward, leaving gaps. These gaps are filled-in using the adjacent Okazaki fragments as primers. The very terminal gap at the 5' end of telomeres cannot be filled because of the lack of such a primer. Olovnikov proposed that the DNA replication machinery could not copy chromosomal ends completely and, therefore, cells could not compensate for the chromosomal shortening associated with cell division, suggesting that progressive telomere shortening may be a key factor to limit the number of cell divisions. James D Watson (Nobel Prize 1962) also recognized that the unidirectional nature of DNA replication was a problem for the complete copy of chromosomal ends (58). This was called the 'end-replication problem'. In this manner, during each cycle of cell division, a small fragment of telomeric DNA is lost from the end. After several rounds of division, telomeres eventually reach a critically short length, which activates the pathways for senescence and cell death (59,60) A progressive decline in telomere length may also occur due to DNA damage. There is a consensus that their length is reduced with age, smoking and stress thereby impairing health. (61)

The properties of eukaryotic telomeres are usually identified as the "capping function", with a principal mission to protect chromosome ends from DNA degradation, DNA repair mechanism and fusion with other chromosomal ends. Their length serves as an intrinsic biological clock that regulates the life span of the cell, *i.e.* they provide limits on the number of replications a cell can go through. Uncapped telomeres are able to activate the DNA damage response and cause end-to-end fusions, resulting in chromosomal instability, cellular senescence and apoptosis (programmed

cell death). Telomere repeats are lost with each round of cell replication by a plethora of different mechanisms, and most somatic cells express insufficient telomerase to compensate for the loss of telomere repeats.

Human cells lose telomeric DNA at a modest rate of about 15-60 bp per year, likely reflecting the small number of stem cells that are actively dividing in proliferative tissues compared to the total stem cell reserve and the quiescent state of cells in other tissues. Telomere shortening has been investigated in human cells in culture, in human genetic diseases with mutated telomerase and in animal models of telomere deficiency (62-70)

2.7 Factors affecting telomere length

Telomere length as generally assessed in leukocytes, is a novel marker of cellular aging and is associated with increased risks of morbidity and mortality. Telomere length reduces with age and may predict life span. Telomere length can be affected by combination of factors including age, lifestyle, genetic make-up, social and economic status, body weight, smoking, exercise, which may affect overall health, lifespan and rate of aging. Also, exposure to harmful agents, pollution, lack of physical activity, certain work atmospheres, stress and related hormones, high fat diet and low fiber diet increase telomere length shortening. This can be modified by life style and diet modification such as regular exercise, yoga or other physical activities that can reduce stress and obesity in addition to diets rich in anti-oxidants such Vitamin E, Vitamin C and beta carotene or carbohydrate restricted diet may reduce the pace of preserving telomere length by reducing oxidative stress. (74)

Rate of telomere shortening in human liver cells have been reported to lose 55 base pairs of telomeric DNA per year (75). Similar shortening has been reported in rapidly renewing gastric mucosal cells. Telomere length decreases by the increased action of proteins responsible for aging and thus negatively correlates with age (76) Individuals with shorter telomeres have significantly poor survival due to higher mortality rates caused by heart and infectious diseases (77). Progressive shortening leads to senescence, apoptosis or formation to cancerous cells (78,79) Certain individuals may also be born with shorter telomeres or may have a genetic disorder leading to shorter telomeres. Such individuals are at a greater risk to develop premature coronary heart disease (80, 81) and premature aging. Deficiency of telomerase gene in a genetic disorder dyskeratosis congenita leads to shorter telomeres and is associated with premature graying, predisposition to cancer, vulnerability to infections, progressive bone marrow failure, and premature death in adults (82).

2.8 Telomere length and age

Although telomerase is expressed in adult stem cell compartments, this is not sufficient to counteract telomere attrition associated with cell division throughout life, and therefore telomeres shorten with age in vitro and in vivo (83-89). This progressive telomere shortening eventually leads to critically short telomeres that can impair the regenerative capacity of tissues and has been proposed as one of the molecular hallmarks of aging (90) In mice, it has been shown that the rate of increase in the percentage of short telomeres, rather than the rate of telomere shortening throughout life, is a significant predictor of life span (91). Shortened telomeres induce a DDR that leads to a growth arrest, during which cells attempt to repair the damage and, if DNA damage is irreparable, triggers replicative senescence (92,93). Senescent cells progressively accumulate

during life and secrete factors that influence age-associated diseases (94). Indeed, senescence has been proposed as a mechanism that evolved to protect from cancer, with the drawback of promoting age-associated diseases (95) Therapeutic interventions based on either chemical activators of telomerase or telomerase-based gene therapy are currently being investigated in mouse models for their potential to improve health and extend life span, and as a treatment for short telomere syndromes (96-9).

2.9 Telomere length and MetS

The Netherlands Study of Depression and Anxiety reported that cellular aging is associated with abdominal obesity and dyslipidemia (low HDL cholesterol and high triglycerides (100) Shorter leukocyte telomere length is cross-sectionally associated with abdominal obesity, dyslipidemia, hyperglycemia, and the presence and severity of metabolic syndrome. Thus, short telomere length is associated with metabolic risk profile and is a known cellular marker which may predict a person's deteriorating metabolic condition (100,101). In patients with MetS compared to healthy volunteers, significant telomerase activity was detected in the circulating PBMC, along with elevated markers of inflammation and endothelial dysfunction. This suggest a prolonged activity of inflammatory cells in the studied state of this metabolic disorder that could represent a contributory pathway in the pathogenesis of atherosclerosis (102). However, it is still largely unknown how telomere maintenance might influence disease processes.

2.10 Telomerase

Telomerase is composed of a reverse transcriptase subunit (TERT) as well as an associated RNA component (*Terc*), which is used as a template for the de novo addition of telomeric repeats (12).

Telomerase is an RNA containing ribonucleoprotein enzyme that catalyzes the extension of telomeric DNA in eukaryotes. Although there is practically no activity of the telomerase in somatic cells, a low level is present in mitotically active cells. It has reverse transcriptase activity and is composed of two main parts – a telomere RNA component and a telomere reverse transcriptase. Once telomerase has been recruited to the telomere, it appears to undergo a separate activation step, which may include an increase in its repeat addition process. Telomerase reverses telomere shortening.

2.11 TA-65®

TA-65® is a registered trademark of Telomerase Activation Sciences, Inc. formulated to help maintain or rebuild telomeres. It is a plant based dietary supplement, extracted from the dried root of a Chinese herb, *Astragalus membranaceus* and was It is considered as a nutraceutical and also the only one of its kind commercially available claiming to safely activate telomerase enzyme. The active ingredient in TA-65 activates telomerase enzyme thereby possibly reversing the telomere shortening or rebuilding the shortened cap. Harley et al at Geron Corporation and the Hong Kong University of Science and Technology conducted a screening program for telomerase activators, which led to the discovery that certain small molecule components of Huang Xi, a traditional Chinese medicine reputed to maintain health, were activators of telomerase (103-105,109) Thus, in 2000, TA-65 was discovered as a chemically defined small molecule activator of telomerase. Since then, there has been research and observational studies on TA-65 in humans and animal models supporting improvements in biomarkers of aging, including immune, cardiovascular, metabolic, bone, and inflammatory markers, without significant signs of toxicity. TA-65® showed striking in vivo effects declined senescent and natural killer cells together with a significant

reduction in the percentage of cells with short telomeres without any adverse events in cytomegalovirus (CMV) seropositive subjects (103). Telomerase activation and functional studies on a related molecule (TAT2) from the same plant have been previously reported for human skin keratinocytes and immune cells in culture leading to a decline of senescent and natural killer cells together with a significant reduction in the percentage of cells with short telomeres (106). Murine studies have reported that TA-65 increased mouse telomerase reverse transcriptase (mTERT) expression, leading to telomerase dependent elongation of short telomeres thereby rescuing the DNA damage (107). Patton Protocol-1 (TA-65 in combination with other supplements and physician counseling) launched in January, 2007, by TA Sciences (New York, NY) as a commercial age-management product produced reduction in fasting blood sugar, insulin, cholesterol, blood pressure, and homocysteine, and increases in bone mineral density, all considered positive health changes in humans (108)

2.12 *Astragalus membranaceus*

Astragalus membranaceus (Chinese: Huang Qi; milk-vetch root) is an important herb in traditional Chinese medicine. Astragalus roots are harvested from 4-year-old plants and are the only part that are used medicinally. It has been used widely as an herbal blend in natural remedies because of the anti-inflammatory, anti-oxidant, cardio-protective and longevity effects. (110) However, it is still largely unknown how telomere maintenance might influence disease processes. Astragalus contains saponins, flavonoids and polysaccharides which gives the positive impact on human health and may be related to its mechanism of action. These bioactive compounds are known to give anti-inflammatory effects, boost immune system and prevent oxidative stress while giving cardio protective and anti-diabetic effects (111). Also used generally for asthma, colds, flu, wound

healing and prevention of scarring. Astragalus membranaceus contains an active ingredient such as astragaloside IV (AST IV), which has been demonstrated to have cardio protective effects mainly against hypertension. RevGenetics in 2014, demonstrated that CAG (cycloastragenol), which increases telomere length (112). However, the active ingredient extracted from Astragalus for TA-65 preparation remains as a proprietary secret of TA Sciences.

2.13 TA-65 and Telomere length

TA-65 claims to be the only scientifically proven molecule that can dramatically rejuvenate aging human cells. A study to prove the effect of TA-65 in telomere length was conducted in mice. Experiments to demonstrate the effect of Telomerase activator by increasing the telomere length in a dose dependent manner in ex vivo mouse embryonic fibroblasts (MEF) incubated with TA-65. However, TA-65 administration for 4 months did not change the maximum life span of female mice (107)

It has been demonstrated that TA-65 is an effective telomerase activator in human immune cells, neonatal keratinocytes and fibroblasts. The impact of telomerase-dependent telomere extension was investigated in a telomerase-haplosufficient model by an ex vivo experiment. Investigators crossed $Terc^{+/-}$ ($Terc$ gene mutation/deficient could result in decreased. Telomerase activity and accelerated telomere shortening) female mice with G2 $Terc^{+/-}$ male mice to generate littermate populations of MEF which is either G3 $Terc^{+/-}$ or G3 $Terc^{-/-}$. Using TRAP mechanism, TA-65 was capable of activating Telomerase by approximately 2-fold telomerase-haplosufficient model and showed G3 $Terc^{+/-}$ treated with $10\mu\text{m}$ of TA-65, showed a raise in average telomere length (103).

The effects of TA-65 in alleviating telomere attrition in CMV⁻ (cytomegalo-virus) was studied in 117 subjects divided into 3 groups: placebo, low dose TA 65 (250 IU) and high-dose TA 65 (1000

IU).The study period consisted of 90 days with 14 days abstinence and telomere length was measured by qFISH. For the median TL, the placebo group showed a decrease at 9 and 12 months, while the low dose TA-65 group showed a significant increase at 3 months followed by stability. The high dose TA-65 group showed a trend of improvement in median telomere length when compared to placebo group, although the values were not significant (113).

2.14 TA-65 on glucose tolerance and Insulin resistance

Administration of AT-65 for 4 months significantly improved the capacity for glucose uptake in 1year old mice while post treatment, no significant changes were seen in the control group at 6 and 12 months. Additionally, treated mice showed a tendency of lowering insulin levels and HOMA-IR scores at 6 months post treatment, with no statistical significance (107).

2.15 TA-65 on cancer, immunity and age related macular degeneration.

The role of telomeres and telomerase in cell aging and cancer was established by scientists at biotechnology company Geron with the cloning of the RNA and catalytic components of human telomerase (107). They did this by developing a PCR based assay for telomerase activity called the TRAP assay, which surveys telomerase activity in multiple types of cancer. While assessing the long-term effects of TA-65 supplementation in mice with tumors, a decreased incidence of sarcomas, slightly decreased lymphomas, as well as a decreased incidence of hepatic cancer in the TA-65 treated group was observed (107). Another study showed improved immune function with TA-65 treatment on CD8T in individuals infected with HIV (103). An increase in the ability of T cells to reduce HIV production was noted by a 1.5 - 2.5-fold increase of telomerase enhancement in the treatment group compared with the control. In patients with AMD (Age related macular

degeneration), TA-65 treatment improved macular functions when compared to the placebo treatment.

2.16 Impact of TA-65 on parameters of MetS

Short telomeres are strongly linked to increased risk of cardiovascular disease and diabetes, indications where tissue aging and senescence play significant roles. Shorter leukocyte telomere length has been linked to impaired glucose tolerance, Type 2 Diabetes, and CHD (1). Telomere length and telomerase activity have been shown to be significantly lower in CAD patients (2). Telomere length may play an important role in predicting cardiovascular disease and diabetes. Over a 5-year period, and with an estimated 7000 person-years of use, TA-65 claims positive results in immune remodeling while improving or maintaining markers of metabolic, bone and cardiovascular health (103,108). With the millennia old reference of cardio-protective and anti-diabetic effects of Astragalus, the impact of TA-65 in improving the parameters of MetS is a possibility, however further studies are required in the area for stronger evidence.

Chapter 3

Materials and methods

3.1 Study design

Data from this study is derived from a double blind, randomized, cross over clinical trial on 40 subjects aged 32-72 and classified with MetS, defined as having 3 or more of the following criteria: blood pressure (BP) \geq 130/85 mm of Hg (either number, or use of antihypertensive medications); plasma glucose \geq 100 mg/dL; triglycerides (TG) \geq 150 mg/dL, waist circumference (WC) \geq 88 cm for women or \geq 102 cm for men, and HDL cholesterol (HDL-c) $<$ 40 mg/dL for men or $<$ 50 mg/dL for women. The criteria for exclusion were: participants with a BMI \geq 40 kg/m², current or past diagnosis of liver or renal disease, diabetes, cancer, stroke, heart disease, severe infectious or autoimmune diseases, and pregnant or lactating women. Other exclusion criteria were use of any glucose-lowering medications or supplements, use of immune-suppressants, anticoagulants, methadone, suboxone, MAO inhibitors, or lithium. Participants with fasting plasma triglycerides (TG) \geq 500 mg/dL, glucose \geq 126 mg/dL, or BP \geq 145/100 mm Hg were also excluded.

Subjects were recruited at the University of Connecticut, Storrs by advertising flyers, e-mails, social media posts as well as advertising booths in various health fairs. The study was approved by the University of Connecticut Institutional Review Board (IRB) under protocol H14-278 and all subjects signed a consent form prior to screening. The study was registered at Clinicaltrials.gov, protocol # NCT02531334. The study design is schematically represented in **Figure 1**.

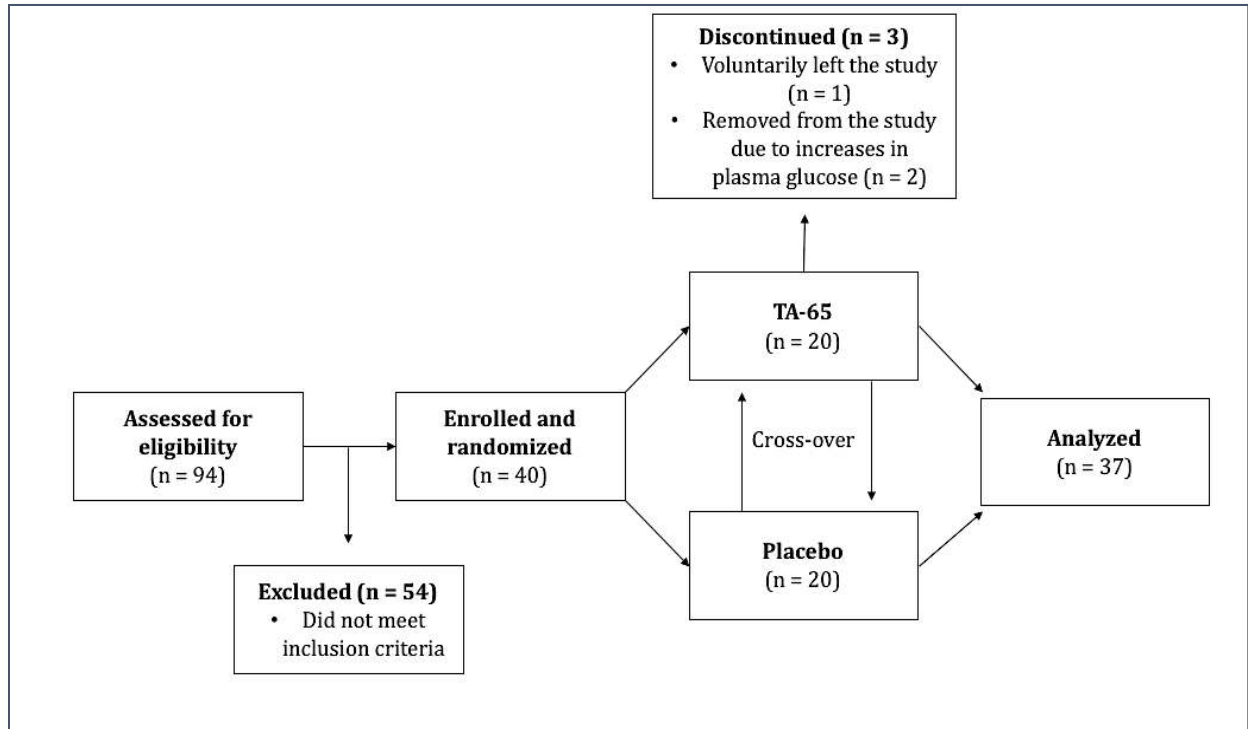


Figure 1. Schematic representation of the intervention

3.2 Screening of subjects

A total of ninety-four participants underwent a screening to determine eligibility of MetS criteria by measuring blood pressure, waist circumference, and calculating plasma lipids and glucose by use of the Cobas c 111 Analyzer. Subjects fasted 12 hours prior to screening, which took place at the University of Connecticut Department of Nutritional Sciences. Screening procedures included signing an informed consent form, followed by filling a comprehensive medical history form detailing any allergies, supplements and medications participants were taking, a personal health history and a medical history of family members. After collection of non-invasive anthropometric measurements and blood pressure, blood was drawn for analysis. All medical history data were examined by a physician (Jeffrey Anderson, MD). Individuals meeting 3 out of 5 inclusion criteria, with no exclusion criteria were enrolled in the study as per their consent.

3.2.1 Pregnancy Test

Women of child-bearing age were tested four times for pregnancy using a qualitative immunoassay that measures Human Chorionic Gonadotropin (hCG) in urine. The pregnancy screening is performed at baseline (week 0) and repeated at the beginning of the washout (week 12), at the beginning of the second supplement period (week 15), and at the end of the intervention (week 27). Subjects with positive result had to discontinue the study.

3.3 Experimental period

The period between recruiting and finishing the last intervention lasted 24 months, from August 2015-August 2017. One participant discontinued due to health problem unrelated to the study and two others were removed due to sudden increase in plasma glucose which were above the required criteria. Thirty-seven subjects comprising 11 men and 26 women, completed the intervention. The experimental period involved a 27-week intervention, where the subjects were randomly allocated to consume either a daily serving of TA-65 (two capsules per day of 8 mg each, n=20) or a placebo (n=20) for 12 weeks. Randomization was by TA Sciences; they sent labeled supplement bottles for each participant and retained the key in their possession. After a 3-week washout, participants were allocated to the alternate treatment for an additional 12 weeks.

Participants were advised not to change their diet or exercise protocols during the 27-week intervention. They recorded a 3-day dietary record and exercise questionnaire before and after the two treatment periods to ensure that there were no changes in diet or physical activity. Participants were also asked to report to the department every 4 weeks to check compliance on supplement intake, to assess weight, and to monitor blood pressure on those subjects who were classified with blood pressure $\geq 130/85$ and $\leq 145/100$ mm Hg at baseline.

3.6 Anthropometrics and blood pressure

Weight was measured to the closest 0.1 kg and height to the closest 0.5 centimeter on a portable stadiometer/scale. Height was converted into metric units to calculate BMI (kg/m^2). Waist circumference was measured at the top of the iliac crest to the adjacent iliac crest to the nearest 0.5 cm, using a flexible measuring tape placed against the skin. Blood pressure was measured on the right arm using an Omron automated blood pressure cuff. Subjects were seated quietly for 5 min in a chair, bladder empty, and upper arm supported at heart level as described by Pickering et al (114). Blood pressure and waist circumference were measured 3 times and the mean was recorded to account for variability.

3.7 Dietary analysis

Detailed diet records composed of all the food and beverages consumed over a 3-day period (two non-consecutive weekdays and a weekend day) were collected four times i.e., before starting the study (Week 0), at the end of each supplement period (TA-65 or placebo) (Week 12), at the end of the washout period or beginning of the alternate treatment (Week15) and the end of the second phase (Week 27). The dietary intake data were analyzed using the Nutritional Data Systems for Research (NDSR, 2013) software, developed by the Nutrition Coordinating Centre, University of Minnesota. The mean values were obtained for nutrient intake at each data collection point. Values for total energy as well as absolute and percent contribution from the macronutrients, types of dietary fat, dietary cholesterol, and dietary fiber were calculated as well as dietary carotenoids.

3.8 Blood sample collection

After a 12 hour fast, blood was collected from an antecubital vein into EDTA tubes, which were immediately centrifuged at 2,000 x g for 20 min. The plasma was aliquoted and preserved in labelled plastic vials and were stored at -80 °C. This procedure was carried out on 5 occasions; during the screening visit, the baseline and end of each intervention i.e., week 0, week 12, week 15, week 27.

3.9 Plasma lipids, fasting blood glucose, glycosylated hemoglobin, CRP, liver enzymes and insulin

Plasma lipids such as total cholesterol (TC), triglycerides (TG) and HDL-c, as well as glucose, C-reactive protein (CRP), liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using the Cobas c-111 Analyzer (Roche Diagnostics, Indianapolis, IN). Glycosylated hemoglobin (HbA1c) was also measured in whole blood in the Cobas c-111 analyzer. LDL cholesterol (LDL-c) was calculated by the Friedewald equation as previously reported (115), Insulin was measured by ELISA using a kit, according to manufacturer instructions (Crystal Chem, Elk Grove Village, IL).

3.10 Telomere length analysis

Telomere length was analyzed in whole blood at baseline and after each supplement period by Repeat Diagnostics Inc. in Vancouver, Canada in both granulocytes and lymphocytes using flow FISH assay. Flow-FISH (fluorescent in-situ hybridization) is a cytogenetic technique to quantify the copy number of specific repetitive elements in genomic DNA of whole cell populations via the combination of flow cytometry with cytogenetic fluorescent in situ hybridization staining

protocols. To assess the durable effects of TA-65, the telomere length was measured 6 months after the end of the intervention.

3.11 Statistical analysis

Differences in anthropometric and parameters of MetS were compared between the TA-65 and the placebo periods by Student's paired t-test using SPSS software. Data are presented as mean \pm SD. Pearson correlations were calculated between changes between supplement and placebo on plasma HDL-c and changes in CRP and liver enzymes. Level of significance was set at $p < 0.05$.

Chapter 4

Results

4.1 Baseline characteristics

The baseline characteristics of 40 participants consisting of 11 men and 29 women are presented in **Table 1**. Participants age ranged from 32 to 71 with a mean of 52.4 ± 9.5 years. Their mean weight was 90.7 kg and the mean BMI was 32.3 ± 2.7 kg/m², placing them in the category of obese. In terms of the NCEP ATP III definition of the MetS, was used in this study, all subjects met the criteria for waist circumference with a mean of 113.9 cm in men and 105.1cm in women. When compared to the MetS criteria, 100% of the participants met WC as the common criteria, 70% were hyperglycemic, 63% either had high systolic BP, high diastolic BP or both, 48% had elevated plasma TG, and 43% had low HDL-c (**Figure 2**). Total cholesterol was 183.6 mg/dL and LDL-c were elevated at 104.5 mg/dL. Interestingly 50% of subjects had LDL-c values higher than 100 mg/dL although this parameter is not a component of the MetS. Mean plasma insulin and HbA1c were within normal ranges. To provide information about inflammation, CRP was determined with an average plasma concentration of 0.45mg/dL.

Table 1. Baseline characteristics of subjects (n = 40) with Metabolic Syndrome

Parameter	Values¹
Age (years)	52.4 ± 9.5
BMI (kg/m²)	32.3 ± 3.7
Waist Circumference (cm)	113.9 ± 13.2 for men and 105.1 ± 8.9 for women
Systolic BP (mm Hg)	126.3 ± 12.5
Diastolic BP (mm Hg)	84.1 ± 7.9
Total Cholesterol (mg/dL)	183.6 ± 33.9
LDL-c (mg/dL)	104.5 ± 30.2
Triglycerides (mg/dL)	136.0 ± 71.5
HDL-c (mg/dL)	40.1 ± 9.2 for men and 56.1 ± 19.0 for women
Glucose (mg/dL)	105.7 ± 9.9
HbA1c (%)	5.6 ± 0.5
Insulin (pmol/L)	52.4 ± 30.0

¹ Values are expressed as mean ± SD.

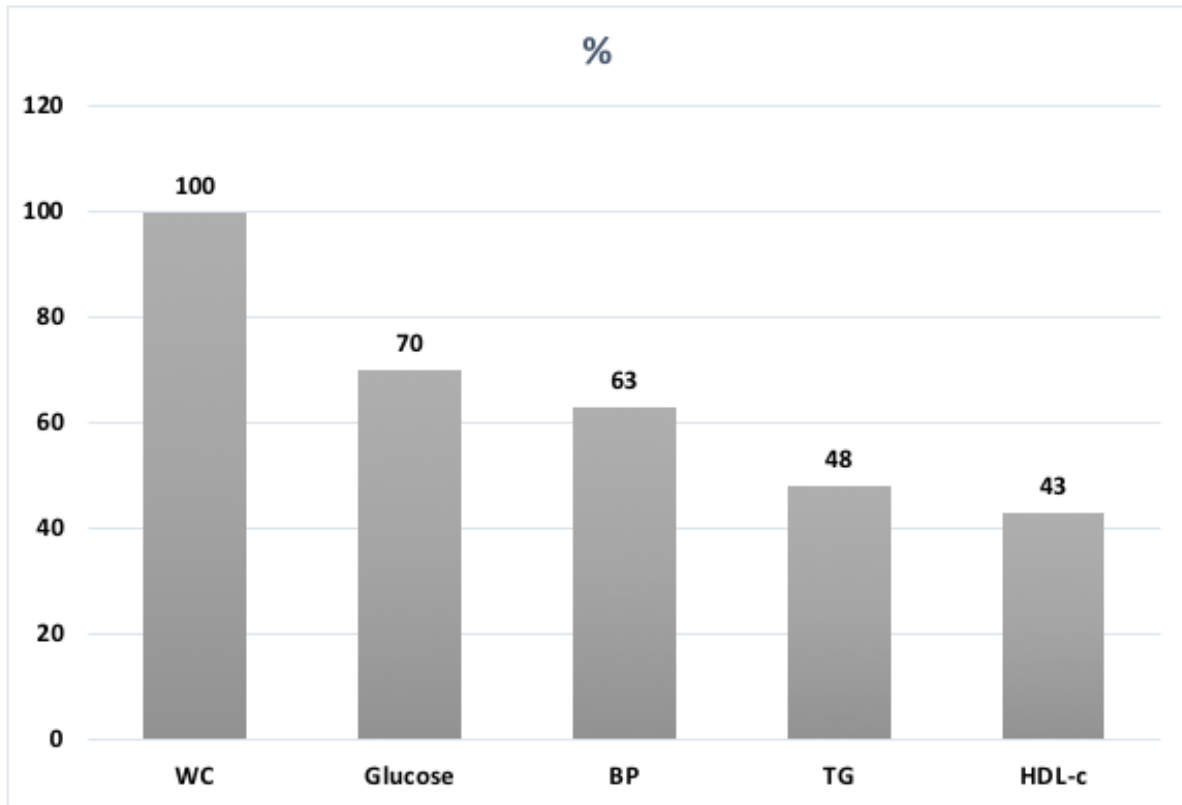


Figure 2. Percent of subjects who met each criterion for MetS

4.2 Dietary Intake

Results from daily dietary intake are presented in **Table 2**. There were no differences in macronutrient intake, type of fatty acids, dietary fiber, added sugar, and carotenoids throughout the intervention. Even the values for glycemic load and glycemic index were not statistically significant when the two treatments were compared. Overall, participants consumed a high fat and low fiber diet, when compared to dietary recommendations. There was a high degree of inter-individual variation in carotenoid consumption, however participants remained consistent between dietary periods.

Table 2. Dietary daily intake of participants with Metabolic Syndrome (n = 37) during the TA-65 and the placebo periods

Dietary Component	TA-65	Placebo
Total Energy (kcal)	1713 ± 463	1754 ± 501
Fat Energy (%)	40.1 ± 6.4	38.9 ± 6.9
Carbohydrate Energy (%)	38.4 ± 8.3	40.2 ± 7.5
Protein Energy (%)	18.3 ± 4.4	17.5 ± 3.6
Cholesterol (mg)	287.2 ± 124.7	286.9 ± 145.9
SFA (g)	27.5 ± 10.6	25.9 ± 9.9
MUFA (g)	27.0 ± 10.5	27.7 ± 10.1
PUFA (g)	16.2 ± 5.9	16.9 ± 10.1
Omega-3 Fatty Acids (g)	1.85 ± 0.84	1.78 ± 1.65
Trans Fatty Acids (g)	2.47 ± 1.17	2.63 ± 1.47
Added Sugar (g)	32.9 ± 22.9	34.2 ± 29.3
Total Fiber (g)	16.3 ± 5.9	17.5 ± 7.8
Soluble Fiber (g)	5.7 ± 2.3	6.2 ± 2.3
Insoluble Fiber (g)	10.2 ± 4.6	11.7 ± 6.1
Glycemic Index	58.8 ± 5.4	59.2 ± 5.3
Glycemic Load	92.5 ± 43.1	98.8 ± 38.6
β-Carotene (μg)	2641 ± 2837	3321 ± 2575
α-Carotene (μg)	348 ± 442	564 ± 559
Cryptoxanthin (μg)	120 ± 362	142 ± 287
Lycopene (μg)	3317 ± 3073	3679 ± 3770
Lutein + Zeaxanthin (μg)	2416 ± 4393	2385 ± 2773

¹ Values are expressed as mean ± SD.

4.3 Anthropometric measurements

As indicated in **Table 3**, there was no significant change in weight during the treatment periods. However, participants under TA-65 treatment had a BMI of 32.6 kg/m² which was significantly lower when compared to the BMI of 32.9 kg/m² during the Placebo treatment. ($p < 0.05$). The waist circumference was also lowered from 109.8 to 108.9 cm during the TA-65 treatment period when compared to the supplement period ($p < 0.05$) (**Figure 3**).

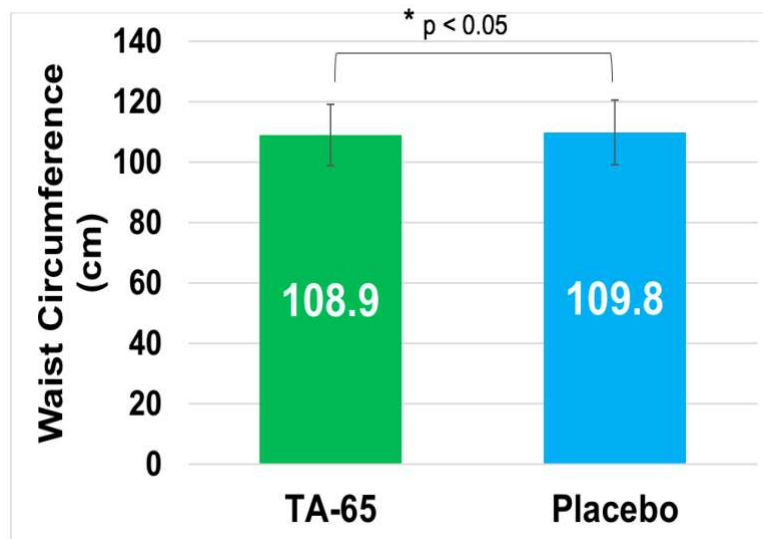


Figure 3. Change in WC in MetS participants (n=37) after consuming TA-65 or placebo for 12 weeks

Table 3. Anthropometric and plasma biomarkers of participants with Metabolic Syndrome (n = 37) after consuming TA-65 or placebo for 12 weeks

Parameter	TA-65	Placebo
Weight (kg)	92.1 ± 16.8 ^a	92.7 ± 17.1 ^a
BMI (kg/m²)	32.6 ± 3.8 ^a	32.9 ± 3.9 ^b
Waist Circumference (cm)	108.9 ± 10.1 ^a	109.8 ± 10.8 ^b
Systolic BP (mm Hg)	123.8 ± 10.5 ^a	124.9 ± 13.5 ^a
Diastolic BP (mm Hg)	83.6 ± 8.0 ^a	83.8 ± 8.9 ^a
Total Cholesterol (mg/dL)	178.4 ± 32.8 ^a	182.7 ± 40.1 ^a
LDL-c (mg/dL)	100.5 ± 30.1 ^a	104.1 ± 36.3 ^a
HDL-c (mg/dL)	52.9 ± 21.5 ^a	49.3 ± 17.8 ^b
LDL-c/HDL-c Ratio	2.15 ± 0.90 ^a	2.43 ± 1.14 ^b
Triglycerides (mg/dL)	124.8 ± 67.3 ^a	131.2 ± 62.7 ^a
Glucose (mg/dL)	104.5 ± 13.3 ^a	103.6 ± 11.7 ^a
Insulin (pmol/L)	58.3 ± 37.7 ^a	57.9 ± 43.4 ^a
HbA1c (%)	5.54 ± 0.45 ^a	5.54 ± 0.48 ^a
ALT (U/L)	31.2 ± 11.1 ^a	34.5 ± 18.7 ^a
AST (U/L)	26.2 ± 7.1 ^a	28.9 ± 21.8 ^a
CRP (mg/dL)	0.39 ± 0.41 ^a	0.41 ± 0.44 ^a

^{a,b} Values are expressed as mean ± SD. Values in the same row with different superscripts are significantly different at p < 0.05

4.4 Plasma lipids, fasting blood glucose, glycosylated hemoglobin, insulin, liver enzymes and CRP

Results from **Table 3** shows no differences in systolic and diastolic BP, total cholesterol, plasma TG, or in parameters of glucose metabolism (plasma glucose, HbA1c, or insulin).

However, HDL-c was higher ($p < 0.05$) (**Figure 4**) and the LDL-c/HDL-c ratio ($p < 0.05$), a key marker of cardiovascular disease risk, was lower in participants following the TA-65 period (**Figure 5**). In addition, there were no changes in ALT, AST or CRP between treatments.

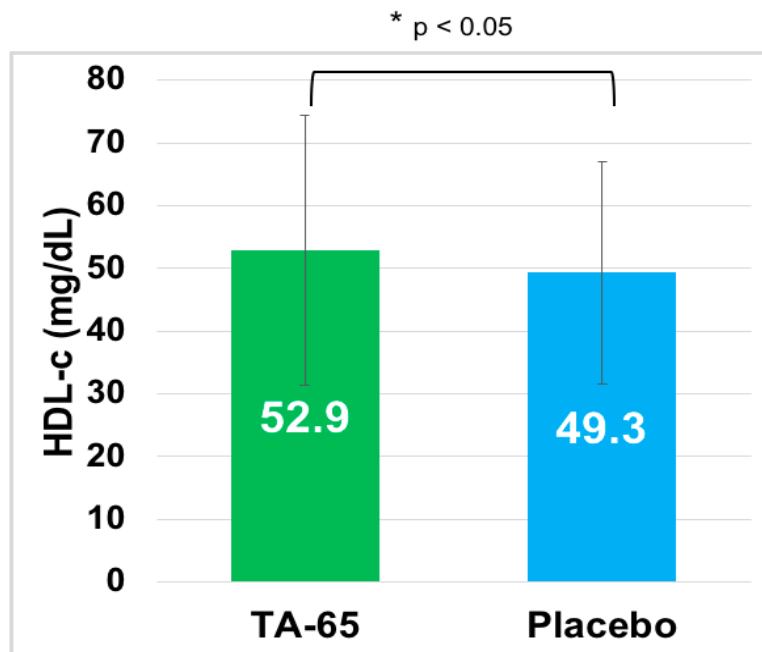


Figure 4. Change in HDL-c in MetS participants (n=37) after consuming TA-65 or placebo for 12 weeks

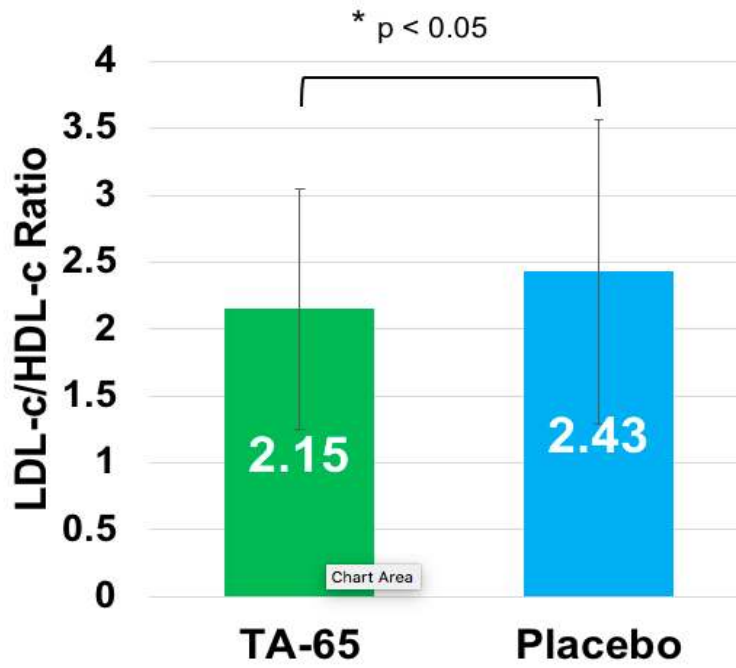


Figure 5. Change in LDL-c/HDL-c in MetS participants (n=37) after consuming TA-65 or placebo for 12 weeks

4.5 Lymphocyte telomere length

Telomere length did not change following TA-65 treatment across the whole intervention. However, **Figure 5** depicts a pattern observed in the 2 randomly assigned panels of treatment during the intervention. In panel A (consisting of 17 participants, who started with TA-65) had a 0.29% reduction in telomere length which reduced further to a 0.72% when they switched to placebo treatment. Whereas in Panel B consisting of 20 participants who started with placebo, there was a 2% reduction in telomere length followed by a 0.15% increase in telomere length when they switched to TA-65 treatment. These results suggest that if they had continued to take TA-65, telomere length would have been maintained.

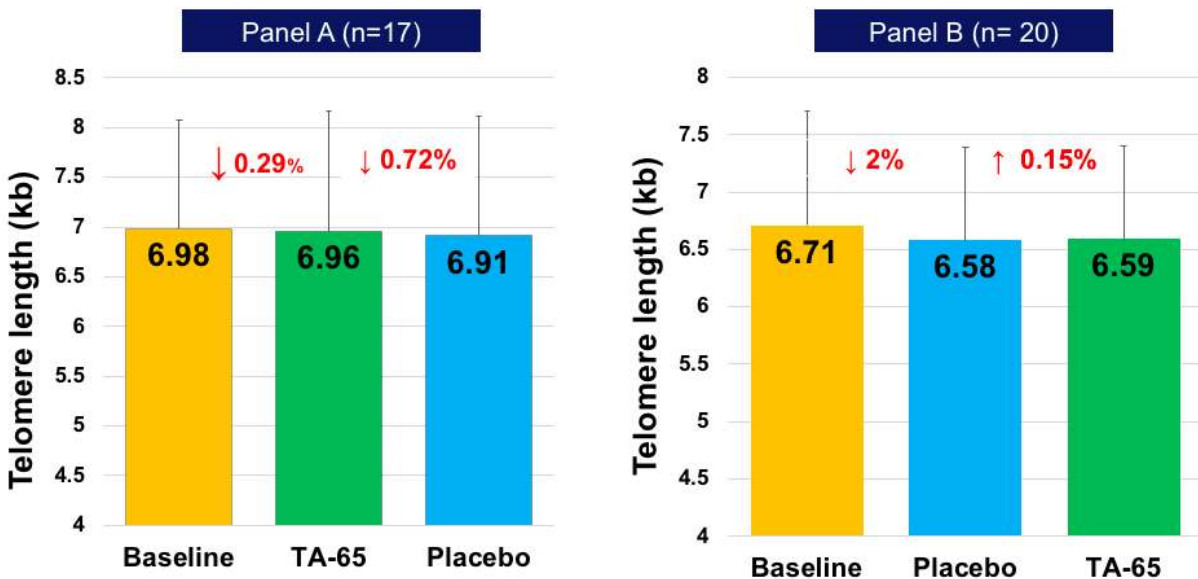


Figure 6. Comparisons of telomere length at baseline, followed by either the TA-65 or placebo supplementation in the 2 randomly assigned groups: Panel A (n=17) and Panel B (n=20). There were no significant differences in telomere length.

4.6 Correlation of granulocyte and lymphocyte telomere length with age

As depicted in **Figure 6**, an inverse relation was observed between the granulocyte and lymphocyte telomere length to the age of the MetS participants at baseline. In participants ranging from ages 32 to 71, the length of telomere decreased with increase in age.

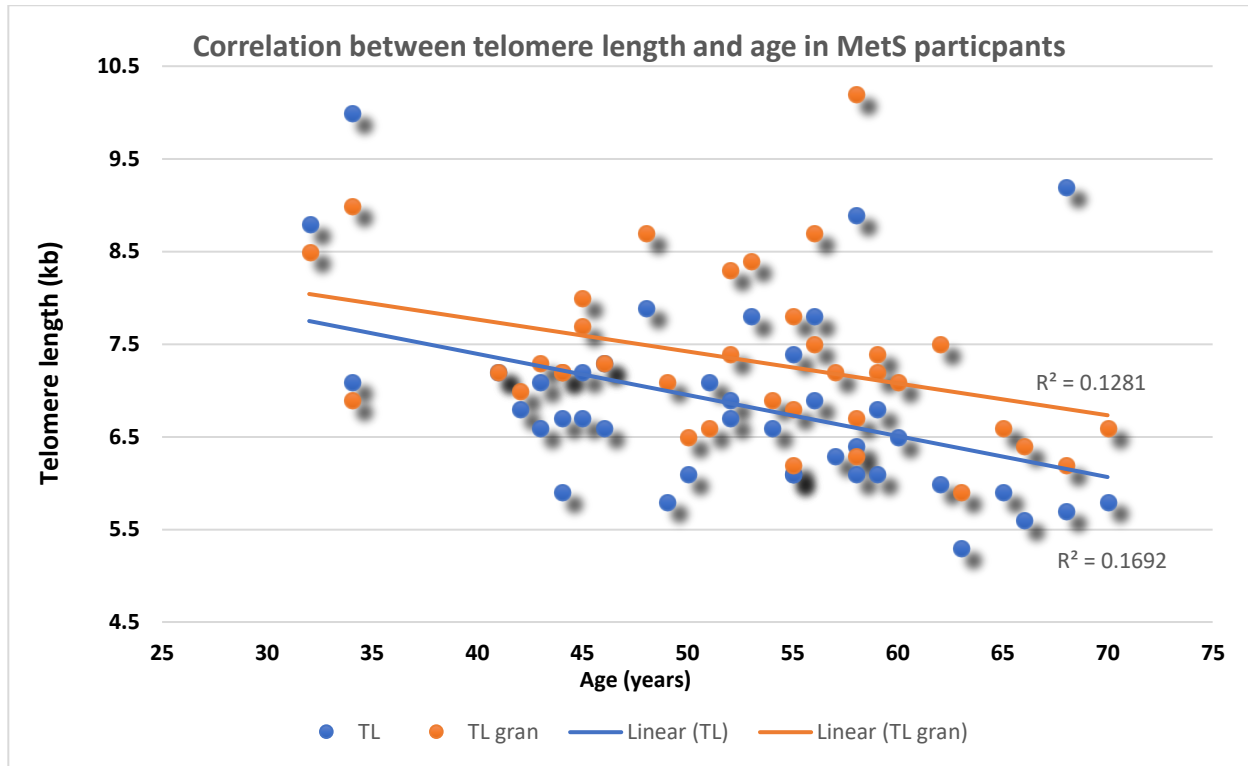


Figure 7. Correlation between telomere length and age in MetS participants at baseline (n=40)

4.7 Correlation between changes in HDL-c with changes in CRP

A negative correlation was observed between the changes in HDL-c with the liver enzymes (AST and ALT) and CRP. (Table 4). The correlation of changes in HDL-c with these biomarkers may suggest a protective role of HDL-c against inflammation.

Table 4. Correlations between changes in HDL-c with liver enzymes and CRP between the placebo and the TA-65 periods

Parameter	Correlation	P value
ALT	-0.610	0.0001
AST	-0.445	0.001
CRP	-0.511	0.001

Chapter 5

Discussion

By studying 37 participants who were treated with TA-65 for 12 weeks (August 2015 - August 2017), we were able to examine protective effects of this supplement and its association with the parameters of MetS, dietary intake and telomere length. In this pilot study, we have inferred that TA-65 exerts some protective effects in individuals with MetS as demonstrated by the lower BMI, WC and LDL-c/HDL-c ratio, and the higher HDL-c following a 12-week treatment as compared with placebo. The results show no significant changes in telomere length during both the treatment periods, while sustaining the telomere length after 3 months of not consuming TA-65 indicating possible extended effect of TA-65 in maintaining telomere length. However, this has to be investigated further.

5.1 Baseline characteristics:

5.1.1 Central obesity as the chief predictor of MetS

The high BMI ($32.3 \pm 2.7 \text{ kg/m}^2$) at baseline assessment is clear evidence that obesity plays a major role in the metabolic complications seen in this population. Central obesity is the most common feature of MetS, which generally increases the risk for developing T2D and CVD (127). However, not every obese subject is resistant to metabolic actions of insulin or at increased risk of developing T2D and CVD. This seems to be true for the population under study since although the fasting plasma glucose was elevated, their insulin as well as CRP were within the normal range. Therefore, obesity has been a well-defined modifiable risk factor for CVD on the same level with smoking, hypertension and dyslipidemia. However, aside from total body fat, subjects with

increased amounts of visceral fat (intra-abdominal fat) are more prone to be classified with insulin resistance and MetS than individuals without or with less abdominal obesity.

The main concerns of obesity are fat stores/deposition and fat distribution. Kissebah et al outlined that body fat distribution and adipocyte size are important markers of metabolic complications in women (128). BMI, however, does not have an ability to indicate body fat distribution, thus we also assessed WC as an anthropometric marker of central obesity. The mean value of WC in this population was 113.9 ± 13.2 for men and 105.1 ± 8.9 for women, therefore almost 12 cm greater than the cut-off point in the NCEP ATP III definition (102 cm) and even greater (17cm) than that for women (88 cm) (17).

5.1.2 Telomere length as a marker of aging

The MetS participants ranging in age from 32-70, showed a correlation of progressive shortening of telomere length as they aged. Leukocyte telomere length has been identified as a biomarker of aging (90). The baseline data depicted the same pattern reviewed by previous studies on telomere length shortening with age. This inverse relation in telomere length might be accelerated by metabolic defects of MetS such as dyslipidemias and insulin resistance (39) in addition to the production of inflammatory mediators and reduced anti-oxidant production, increases in dysregulation of the insulin receptor leading to insulin resistance and dyslipidemias. The shortening of telomere length with age could thus be a marker to predict an individual's deteriorating metabolism and health condition (100,101). In vitro research and controlled animal studies in human cells, have shown that long term TA-65 use supports enhanced telomerase-activation thereby resulting in enhanced lifespan (103). Telomerase enhances the number and

quality of healthful years, though it does not necessarily lengthen lifespan. There have been no negative side effects, as the molecule acts in a transient, non-pathogenic fashion (103)

5.2 Dietary intake

The 3-day dietary assessment and the analysis of daily dietary intake during the treatment showed no significant changes in eating pattern. This eliminated the chances of the interference of dietary components into the results. Life style changes including successful modifications by energy restriction of dietary macronutrients, incorporation of functional foods and bioactive nutrients, adherence to the Mediterranean diet in addition to regular exercise serves as a proved therapeutic treatment for MetS (12). Dietary intake modification is a key strategy to reduce MetS. The participants of this study who were blinded to the treatments maintained the same life style and did not modify their diet or exercise pattern throughout the intervention. Also, the diet intake pattern of the participants when compared to healthy adults seems to show a greater consumption of saturated fats with lower intake of dietary fiber. The prevalence of central obesity in this population might be a result of that dietary pattern. A deliberate change in the dietary pattern during the TA-65 treatment could prove to be more effective than the sole consumption of TA-65 following an unhealthy dietary pattern.

5.3 Improvement in anthropometric markers when comparing TA-65 and placebo treatments by lowering waist circumference and BMI.

Abdominal obesity is the most common feature of MetS and is associated with increased release of free fatty acids into the circulation, which target specific organs leading to dyslipidemia, insulin resistance, and inflammation (127). As seen in the results, a small but significant lowering in waist

circumference and BMI levels were observed after the TA-65 treatment when compared to the placebo. The mechanism for this improvement is unclear. However, the diet records of participants reveal a non-significant decrease in total calories and in carbohydrate intake, where high intake of both is highly associated with increased WC (129). Therefore, we speculate that TA-65 may have affected the behavior of our participants and that they followed a healthier eating pattern during the TA-65 period, which resulted in the positive effects on WC. Daubenmire, *et al.* (130) have reported previously that being enrolled in a mindfulness intervention pilot study affected telomerase activity in a positive manner and they found a positive correlation between restrained eating and telomerase activity.

5.4 Cardio-protective effects of TA-65

Previous studies have recorded that intake of TA-65 results in improvement of plasma lipids. In the current study, although no differences in TC or TG were observed between TA-65 and placebo, HDL-c was higher at the end of TA-65 and, consequently, there was a significant decrease in the LDL-c/HDL-c ratio, a very well-known biomarker of cardiovascular disease risk (133).

The Framingham study as well as others that followed have shown that HDL-c is an independent cardiovascular risk factor and an increase in HDL-c of only 10mg/dL leads to a reduction of 2-3% in CVD (116,117)). It was found that low HDL-c was even associated with increased mortality (118). Thus, raising HDL-c reduces the risk of CHD (119). HDL-c promotes reverse cholesterol transport from the periphery (mainly macrophages) to the liver but also exerts pleiotropic effects on inflammation, hemostasis and apoptosis (119). A retrospective cohort study in Taiwanese adults with MetS has shown that the individual components of MetS, especially low HDL-c and

hypertension were better predictors of CVD mortality than MetS as a whole (120). The Quebec cardiovascular study which followed 2103 middle aged men for 5 years showed that plasma HDL-c was an independent marker of CHD and recommended that raising plasma HDL-c is a therapeutic target for optimal prevention of CHD (121). Hence the slight increase in HDL-c that we observed during the TA-65 treatment is of utmost importance as it reduces the risk of CHD as well as CVD associated mortality.

While it is established that HDL-c is an independent protective risk factor for atherosclerotic CVD and LDL-c increases CVD risk, the LDL-c/HDL-c ratio reflects the two-way traffic of cholesterol entering and leaving the arterial intima in a way that the individual levels of LDL-c and HDL-c do not (132). It has also been suggested that LDL-c/HDL-c is a more robust risk indicator of CVD than the individual parameters (122-125,133). In a middle aged male population assessed at baseline in the Finnish Kuopio Ischemic Heart Disease prospective cohort study, it was found that a high serum LDL-c/HDL-c ratio was independently associated with an increased risk of SCD (Sudden cardiac death) (126). The current NCEP guidelines recommend levels of LDL and HDL that represent a ratio of 2.5 or lower (134). Coronary deaths spiked when LDL-c/HDL-c ratio was raised between 3.7 and 4.3 (135). In the Physician's health study involving around 15,000 men of ages 40 to 80, a 1 -unit increase in the ratio was associated with a 53% increase in risk of MI (130). In the Boston Area Health Study, which analyzed a group of men and women less than 76 years of age with no prior history of CVD but who had experienced a first MI, a 1-unit increase in the LDL-c/HDL-c ratio was associated with a 75% increase in risk of MI (131). In addition, comparison of individual LDL-c/HDL-c ratios from subjects in the Framingham Study clearly indicates that the ratios are significantly more durable predictors of CVD than the individual levels of LDL-c or HDL-c (132)

Thus, the raising of HDL-c and lowering of LDL-c/HDL-c during the TA-65 treatment exerts minor but powerful cardio protective effects.

5.5 Pattern of telomere length

The characterized function of TA-65 is its ability to decrease the shortening of telomeres during DNA transcription. In mouse embryonic fibroblasts, TA-65 has been shown to ameliorate the number of short telomeres and decrease the percentage of critically short telomeres as well as DNA damage that harbors critically short telomeres (7). In humans, it has also been shown that the small molecule telomerase activator (TAT2) isolated from *Astragalus membranaceus* induced telomerase activity in peripheral blood mononuclear cells and T lymphocytes and increased their antiviral functions (106). Furthermore, human supplementation with TA-65 as well as other dietary supplements have been shown to reduce the percentage of cells with short telomeres (9).

In the present study, no significant change in telomere length was observed during the two arms of treatment. However, a non-significant pattern was seen in the 2 panels of treatment. TA-65 does its job in protecting the end region of chromosomes by preventing loss of telomere length. Telomerase activator stimulates the production of Telomerase enzymes which prevents further attrition of the TL. The pattern showed no decrease in telomere length thereby showing a tendency to maintenance or prevent of shortening of telomere length which is the classic function of TA-65. Life style modifications with increased physical activity paired with carbohydrate restricted diet and/or Mediterranean diet has previously been shown to offer reduction in MetS risk and further complications of MetS (12). Boccardi, *et al.* on the other hand, reported that a healthy diet such as Mediterranean diet promoted health-span by maintaining rather than increasing telomere length

suggesting that both maintenance and increases of telomere length are important determinants of aging (131). Other studies have also shown that the main functions of telomerase activity are slowing telomere attrition by preserving the proliferative potential of stem cell (44,45). Hence diet restrictions with long term supplementation of TA-65 might pose better effects than the present study where diet restrictions were not present.

5.6 Conclusion

TA-65 supplementation in MetS participants for 12 weeks when compared to a placebo exerted some protective effects against dyslipidemia and waist circumference, which are metabolic abnormalities associated with MetS. While no significant change in telomere length was observed during the treatments, a pattern of maintenance of telomere length was observed after TA-65 treatment suggesting a protective effect in life expectancy. Since MetS is a precursor to the development of Type-2 diabetes or heart disease, dietary strategies for reversal of MetS biomarkers may be a preventative treatment for this at-risk population. Therefore, the effect of TA-65 supplementation along with strict diet and exercise modifications may improve symptoms of MetS or reduce future health complications.

Strengths of the study

Since scarce information exists on the effects of TA-65 on parameters of MetS, this was a unique pilot study involving a population at risk for heart disease and diabetes.

Limitations and future directions

1. The long-lasting effect of TA-65 could not be clearly depicted in the study as the intervention lasted only 27 weeks with participants consuming TA-65 only for 12 weeks. A similar study with larger sample size and longer duration would give more information on the protective effects of TA-65.

2. Another future direction would be to assess ethnicity and to adjust for ethnic differences in statistical analyses. It is well established that parameters of MetS vary in different populations, thus our results may have been biased by ethnicity.

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