ABSTRACT

Title of Document:	*TNF* PROMOTER POLYMORPHISMS ASSOCIATED WITH SKELETAL MUSCLE PHENOTYPES IN HUMANS.
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Skeletal muscle plays a central role in the overall health of individuals across all ages, and skeletal muscle phenotypes are influenced by both genetic and environmental factors. Tumor necrosis factor alpha (TNF-α), a key player in the innate and adaptive immune responses, has long been recognized as a potent catabolic factor mediating muscle wasting in various pathological conditions. Overproduction of TNF-α has been implicated in the etiology of age-associated muscle loss (sarcopenia). Individual capacities to produce TNF-α vary widely, which is partially attributable to gene sequence variations. The TNF-α coding gene, *TNF*, is highly polymorphic and single nucleotide polymorphisms (SNPs) in the promoter region of *TNF* have been implicated for the transcriptional regulation of TNF-α production, and associated with numerous inflammatory and infectious diseases. The purpose of the present study was to investigate the association of muscle phenotypes, including sarcopenia, with 5 *TNF* promoter SNPs, which are potentially of biological significance.

A total of 1050 volunteers participating in the Baltimore Longitudinal Study of Aging (352 and 407 white women and men, 127 and 107 black women and men, and 30 and 27 non-white and non-black women and men) were genotyped for 5 *TNF* SNPs, and their regional and total body soft tissue masses and muscle strengths of upper and lower limbs were measured. Results indicated that *TNF* promoter SNPs are associated with muscle phenotypes in the participants: putative high TNF-α-producing alleles at positions -1031 and -863, individually or in combination in haplotype '1031C-863A-857C-308G-238G', are associated with lower muscle mass in males. These results suggest that genetic variation in the *TNF* locus may contribute to the inter-individual variation in muscle phenotypes, and imply that TNF-α may have a potential role in regulating body composition even in healthy people.

TNF PROMOTER POLYMORPHISMS
ASSOCIATED WITH SKELETAL MUSCLE PHENOTYPES IN HUMANS

By

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This page is the most precious page in my dissertation and will be kept in record of my life for ever. From this page, people whom I care about and who care about me will know how much I appreciate what they have done for me; from this page, I am reminded of how much I am indebted to the people around me.

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LIST OF ABBREVIATIONS

3'UTR - 3' untranslated region

ANCOVA - Analysis of covariance

ASM - Appendicular skeletal muscle mass

ASMI - Appendicular skeletal muscle mass index

BLSA - Baltimore longitudinal study of aging

BMI - Body mass index

CRP – C-reactive protein

CT – Computed tomography

DXA - Dual energy X-ray absorptiometry

FFM - Fat free mass

FM - Fat mass

Grip - Hand grip strength

HRT -Hormone replacement therapy

H-W - Hardy Weinberg equilibrium

IGF-I - Insulin-like growth factor -I

IL-6 - Interleukin -6

Knee Isokinetic 30°/s - Knee extensor isokinetic peak torque at the speed of 30 deg/sec

Knee Isokinetic 180°/s - Knee extensor isokinetic peak torque at the speed of 180 deg/sec

Knee Isometric 120° - Knee extensor isometric peak torque at the angle of 120

Knee Isometric 140° - Knee extensor isometric peak torque at the angle of 140

LmArm - Lean soft tissue mass of the arms

LmLeg - Lean soft tissue mass of the legs

LmTrunk - Lean soft tissue mass of the trunk

MHC - Major histocompatibility complex

MI -Myocardial Infarction

mTNF - Membrane bound form of tumor necrosis factor alpha

PT - Peak torque

PMA - phorbol myristyl acetate

SE - standard error

SNP - Single nucleotide polymorphism

sTNF -Soluble form of tumor necrosis factor alpha

T -Testosterone

TNF – Gene encoding protein tumor necrosis factor alpha

TNF-α -Tumor necrosis factor alpha

TNFR -Tumor necrosis factor receptor

INTRODUCTION

The loss of muscle mass and strength during aging has been termed sarcopenia. Data from the Third National Health and Nutrition Examination Survey indicated that about 45% of the older population is sarcopenic with a healthcare cost of about 1.5% of the total healthcare expenditures for the year 2000 in the U.S. (70). Sarcopenia has significant implications for individuals' physical and social well-being as it has been associated with a decline in mobility (94), an impairment in balance and increased risk of falls (158), physical disability (68), frailty (172), morbidity (74), as well as mortality (112; 118).

Although the pathophysiological mechanisms underlying sarcopenia remain elusive, there is a growing consensus that sarcopenia is a multifactorial condition resulting from withdrawal of, or resistance to, several anabolic stimuli to muscle including loss of central nervous system input, declining growth hormone, estrogen and testosterone, inadequacy in dietary protein and caloric intake, decreasing physical activity and muscle resistance to insulin and certain amino acids (30; 113), and the development of several catabolic stimuli (113; 142; 159), such as inflammatory cytokines, IL-1β, TNF-α, and IL-6.

Aging is accompanied by a 2-4 fold increase in plasma levels of inflammatory mediators including tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 1 receptor antagonist (IL-1Ra), soluble TNF-α receptor (sTNFR), acute phase proteins such as CRP, and neutrophils (24; 83). Since increases in these inflammatory mediators are far lower than the increases observed during acute infections, age-related inflammation is often referred to as low-grade inflammation (24). Low-grade inflammation may play an important role in age-related diseases such as Alzheimer's disease, atherosclerosis, type-2 diabetes, osteoporosis, as well as sarcopenia (24). In the elderly, skeletal muscle mass and

strength have been inversely associated with circulating levels of IL-6 (145; 177) and TNF-α (177); the muscle atrophic response to strength training has been inversely related to baseline levels of sTNFR-I (23); and a lower muscle protein synthesis rate has also been associated with increased levels of sTNFR-II (166).

Among the variety of inflammatory markers, TNF-α may be the driving force behind many age-related problems, whereas other cytokines like IL-6, IL-1Ra, sTNFR, as well as acute phase proteins (APPs) like CRP, reflect responses to local on-going TNF-α activity (21; 139). TNF-α, along with IL-1β, is an initiator of the inflammatory cascade, able to induce the second wave of inflammatory cytokines and acute phase proteins including IL-6 and CRP (20). IL-6, IL-1Ra, sTNFR and APPs work as anti-inflammatory and immunosuppressive mediators to inhibit excessive synthesis and activity of TNF-α (164). TNF-α is mainly produced and functional at the local tissue level, and its short half-life makes it difficult to detect in the circulation unless large amounts are produced (20), which may explain why in some studies significant increases were found in plasma concentrations of IL-6, CRP and/or sTNFR, but not in TNF-α in aged people (166).

A strong catabolic effect of TNF-α on muscle has been well documented (134). TNF-α was originally designated as 'cachectin' in recognition of its catabolic property, and it has been implicated (134) in muscle wasting associated with diseases such as cancer, AIDS, congestive heart failure, chronic obstructive pulmonary disease (COPD), and rheumatoid arthritis. TNF-α can induce muscle wasting through direct and indirect pathways. Indirectly, TNF-α can induce anorexia and hypermetabolism (167), suppress expression of insulin-like growth factor-I (IGF-I) (49), and induce skeletal muscle insensitivity to insulin (18). Directly, TNF-α can affect protein transcription efficacy in

skeletal muscle (90), can induce skeletal muscle protein breakdown by the ubiquitin/proteasome system via activation of nuclear factor-kB (NF-κB) (134), and can induce loss of myonuclei by apoptosis via interaction with TNFR1 (36).

Several lines of research support the hypothesis that age-related up-regulation of TNF-α in skeletal muscle plays an important role in sarcopenia. Greiwe et al. (53) found elevated TNF-α mRNA and protein levels in skeletal muscle from frail elderly subjects, which was mitigated by resistance exercise training along with increases in muscle strength. They also reported that the muscle protein synthesis rate was inversely associated with muscle TNF-α protein levels (53). TNF-α basal expression has been shown to be fiber type specific with the expression predominantly found in type II fibers (128). Phillips and Leeuwenburgh (125) found that 1) the expression of TNF-α in type II fibers was greater in aged rats than in young rats; 2) TNF-α signaling leading to apoptosis was preferentially active in type II fibers from old rats; 3) the age-related changes were reduced by caloric restriction. The results of the above two studies are in line with the findings that sarcopenia disproportionately affects type II muscle fibers vs. type I fibers (34). As suggested by Ferrucci and Guralnik (46), even transient elevations in TNF-α can inhibit the degree of protein synthesis over a relatively long period of time, which could ultimately lead to muscle frailty in older people.

While it is prevalent in older people, sarcopenia does not develop at the same age and at the same rate in individuals, as reflected by the fact that the prevalence of sarcopenia is always far less than 100% in any population studied (12; 27; 37). The variation in susceptibility to sarcopenia may be genetically controlled to some extent, as skeletal muscle is highly heritable (6; 82; 147; 190). Many candidate genes for muscle

phenotypes have been studied and genetic polymorphisms in those genes have been found to contribute to the inter-individual variations in muscle phenotypes (156), but none of those associations can be considered conclusive at present.

The capacity to produce cytokines, including TNF-α, differs among individuals, which may be ascribed to polymorphisms within the regulatory regions or signal sequences of cytokine genes (81). The TNF-α gene (*TNF*) is located on chromosome 6p21.3, which is within a highly polymorphic region of the human genome, the major histocompatibility complex (MHC) region (56). Many single nucleotide polymorphisms (SNPs) and microsatellites have been identified in the *TNF* locus and the ones in the promoter region of *TNF* are thought to influence *TNF* transcription rate and likely to be of direct functional significance in regulating TNF-α production (144). In the promoter, 5 SNPs at positions (relative to the transcription start site) -1031T/C (rs1799964), -863C/A (rs1800630), -857C/T (rs1799724), -308G/A (rs1800629), -238G/A (rs361525) have been well characterized: their influence on gene expression and association with various inflammatory diseases have been frequently reported (56; 57). The transcriptional promoter activities of variant alleles -1031C, -863A and -857T were found to be 2 fold higher than wild-type alleles in peripheral blood mononuclear cells upon stimulation (64). The -308A-allele, with the presence of 3' UTR of *TNF* gene, has been associated with a 2-fold increase in transcription as compared with the -308G-allele in PMA-stimulated Jurkat cells (a cell line derived from human T-cell leukaemia) and U937 cells (a human leukemic monocyte lymphoma cell line) (85). The ubiquitous transcription factor OCT-1 has been found to bind specifically with variant alleles at positions -863 and -857, but not with wild-type alleles (65). Furthermore, the -863 (C/A) SNP was found to alter the relative binding

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affinity of different forms of the NF-κB complex (170). It is believed that the interaction between nuclear proteins and these *TNF* SNPs confers an important pathway for the allele-specific modulation of *TNF* expression (56). Association studies have shown that these 5 SNPs were differentially linked to various infectious and autoimmune diseases (16; 60; 66), as well as longevity (101) and age-related diseases (22; 87; 87; 100; 152; 173), but negative results have also been reported (66; 79; 136; 152; 189). Despite extensive research, to date no human *TNF* genetic variation has been consistently identified to result in substantial alterations in *TNF* expression, and no definitive disease-related *TNF* polymorphism has been characterized (15; 144).

Despite the evidence indicating the importance of inflammatory factors including TNF-α on sarcopenia, sarcopenia has not been studied in relation to genetic polymorphisms in the *TNF* promoter. Therefore, the objectives of this study were to 1) analyze the relationship between *TNF* promoter polymorphisms and muscle phenotypes in healthy people aged between 20 to 97 years, and 2) explore the association of *TNF* promoter SNPs with sarcopenia specifically in people 55 years and older. This study may provide insight into the possible mechanisms of sarcopenia, as well as potentially influence the development of effective interventions against sarcopenia, for example using anti-inflammatory treatment in individuals carrying specific *TNF* genotypes.

HYPOTHESES

Objective # 1: To determine the relationship between muscle phenotypes and 5 *TNF* promoter SNPs in the participating adult men and women.

Hypothesis #1: Putative high-expressing alleles in the promoter region of *TNF*, namely -1031C, -863A, -857T, -308A and -238A, will be associated with lower muscle mass and lower muscle strength in the participating adult men and women.

Objective # 2: To construct haplotypes for the *TNF* promoter and test the association between major haplotypes and muscle phenotypes in participating adult men and women.

Hypothesis #2:   *TNF* promoter haplotypes containing risk alleles will be associated with inferior muscle phenotypes in the participating adult men and women.

Objective # 3: To test the association of *TNF* risk alleles and risk haplotypes with the presence or absence of sarcopenia in the participating elderly sub-population.

Hypothesis #3: *TNF* risk alleles and risk haplotypes will have higher frequencies in the group of elderly people (≥55 yr) who are sarcopenic than in the group of elderly people who are not sarcopenic.

METHODS

Subjects

The subjects consisted of 1059 adult men and women (20 to 97 years of age) from the Baltimore Longitudinal Study of Aging (BLSA). Most of the participants of the BLSA are of European descent, healthy, and well-educated (146). Subjects visited the Gerontology Research Center at 1- to 2-year intervals for 2 days of medical, psychological, and physiological testing, which includes assessment of muscle mass and strength. Subjects with acute inflammatory diseases or conditions including cancer, HIV, autoimmune disease, or major surgical operations within the past year were excluded. All BLSA participants provide written informed consent to participate in both direct and ancillary studies related to their collected data. The experimental protocols were approved by the Institutional Review Boards (IRB) for Medstar Research Institute, Johns Hopkins Bayview Medical Center, and the University of Maryland.

Potential Confounders

Based on previous studies, several factors that are predictive of muscle mass and/or strength, and/or are associated with TNF-α levels (systemic or local) were identified and included in the analyses.

Body weight and height were measured to the nearest 0.1 kg and 0.5 cm, respectively with a Detecto medical beam scale. Age was also included as a covariate in the statistical analysis. Subjects were classified into three race groups: whites, blacks, and other, which included all non-white and non-black individuals. Tobacco smoking was evaluated by using a standardized questionnaire. Smoking habits were evaluated in 3

groups: current smoker, former smoker, and never smokers. Menopausal status and HRT status were collected from female subjects by inquiring if she was post-menopausal, and if she was using hormone replacement therapy. Medical history was assessed on each visit. Heart disease, diabetes mellitus, and cancer were recorded as has ever had or never. Body fatness was obtained from DXA (see below). The details regarding measurement of these factors and subject exclusion criteria have been reported elsewhere (99; 127; 161).

Measurements of Skeletal Muscle Mass

Total body fat (FM) and soft tissue fat free mass (FFM), and lean soft tissue mass for both legs (LmLeg), lean soft tissue mass for both arms (LmArm), and lean soft tissue mass of the trunk (LmTrunk) were assessed by dual-energy X-ray absorptiometry (DXA) (model DPX-L Lunar Radiation, Madison, WI) using previously described methods (99). FFM has been used previously as a valid indicator of muscle mass (51; 181). The scanner was calibrated daily before testing. Reliability has been assessed by performing two total body scans, 6 wk apart, on 12 older men (> 65 yr), and the difference between the two scans was ~ 0.01% for both FM and FFM. The appendicular skeletal muscle mass (ASM) was calculated as the sum of the FFM for both arms and legs (51). The limbs were isolated from the trunk by using DXA regional computer-generated lines with manual adjustment. With the use of specific anatomic landmarks, the legs were defined as the soft tissue extending from a line drawn through and perpendicular to the axis of the femoral neck and angled with the pelvic brim to the phalange tips and the arms as the soft tissue extending from the center of the arm socket to the phalange tips. The system software provides the total mass, ratio of soft tissue attenuations, and bone mineral mass for the total body and isolated regions. The ratio of soft tissue attenuation for each region was used to divide

bone mineral-free tissue of the extremities into fat and fat-free components. The fat and bone mineral-free portion of the extremities were assumed to represent appendicular skeletal muscle mass along with a small and relatively constant amount of skin and underlying connective tissues (51). The appendicular skeletal muscle mass index (ASMI) was calculated as ASM/height^2.

Measurements of Skeletal Muscle Strength

Five indicators of muscle strength were measured: isometric hand grip strength (grip), isokinetic knee extension peak torque at a speed of 30 deg/sec (knee isokinetic 30°/s), isokinetic knee extension peak torque at a speed of 180 deg/sec (knee isokinetic 180°/s), isometric knee extension peak torque at an angle of 120° (knee isometric 120°), and isometric knee extension peak torque at an angle of 140° (knee isometric 140°). Grip strength was measured using the Smedley hand-held dynamometer (Stoelting, Wood Dale, IL). The dynamometer was adjusted individually for hand size, and three trials were performed for each hand. For the present study, the maximum strength obtained for the right hand was used as the measure of grip strength. Knee extension strength represented by peak torque (PT) was measured using the Kinetic Communicator dynamometer (Kin-Com model 125E, Chattanooga Group, Chattanooga, TN). For the dominant knee extensors, shortening PTs were measured at angular velocities of 0.52 rad/s (30°/s) and 3.14 rad/s (180°/s), and static PTs were measured at the angle of 120° and 140°. The terms "shortening" and "lengthening" were substituted throughout the present study for the more commonly used terms "concentric" and "eccentric," respectively, based on the recommendations of Faulkner (42). For each test, subjects performed three maximal efforts, separated by 30-sec rest intervals, from which the highest value of the three trials

was accepted as the PT. PT was assessed by using the Kin-Com computer software

(version 3.2). Reliability of strength testing when using the Kin-Com dynamometer has

been reported elsewhere (63). Detailed procedures regarding subject positioning and

stabilization, gravity correction, and Kin-Com calibration are described elsewhere (99;

104). Briefly, subjects were positioned sitting with the backrest at an angle of 105°, with

the hip angle between 80-85°, and were stabilized by using chest, waist, and thigh straps.

The rotational axis of the dynamometer was aligned with the lateral femoral epicondyle

and the resistance pad positioned just proximal to the lateral malleolus of the ankle joint.

The Kin-Com angle reading was calibrated to the anatomic joint angle measured by a

goniometer. Gravity corrections to torque were based on leg weight at 170° and calculated

by the gravity correction program in the Kin-Com software package (Version 3.2). The

acceleration/deceleration rate was set at low, the activation force (i.e. force threshold

required for movement of the dynamometer arm) was set at 50 N, and the minimum force

for lever arm movement was set at 20 N.

Genotyping

In this study, a total of five SNPs were genotyped in the *TNF* gene including

rs1799964/T-1031C, rs1800630/C-863A, rs1799724/C-857T, rs1800629/G-308A and

rs361525/G-238A. Blood samples (10 ml) were obtained from all individuals by using

standard procedures, and genomic DNA was prepared from the EDTA-anticoagulated

whole blood samples by standard salting-out procedures (Puregene DNA Extraction,

Gentra Systems Inc.). Genotyping was done using TaqMan allele discrimination assays.

All experiments were performed using the Applied Biosystems 7300 Real-Time

PCR system. Each well of a 96-well optical reaction plate contained: 6.25 μl 2X TaqMan

Universal PCR Master Mix (Perkin-Elmer, Applied Biosystems Division), 0.625 μl of 20X

TaqMan SNP mix, 1.5 μl (10-20 ng) of genomic DNA, and 4.125 μl DNase free water. In

addition to experimental samples, each plate contained 2 no-template controls and 6

positive controls (2 for each genotype). The genotypes of the positive control samples were

validated by direct sequencing. Two different fluorescent dyes (FAM and VIC) were

utilized to identify the alleles of interest. The PCR was done using 10 min at 95°C

(AmpliTaq Gold Enzyme activation) and 40 cycles of 15 s at 92°C (denaturation) and 1

min at 60°C (annealing and extension). Analysis of raw data to determine genotypes was

performed by the ABI 7300 Sequence Detection System software.

Haplotype Construction

Haplotypes were constructed based on the population genotypes for 5 *TNF* SNPs

using PHASE software (v2.1) (108). PHASE is a program for constructing haplotypes

from population genotype data. It has been widely used in genetic studies and has been

suggested to be the best available method used to infer haplotype from population

genotype data (106). An input dataset was prepared according to the instructions in the

documentation for PHASE, version 2.1 (108), and the program was run according to the

documentation. The haplotypes with frequencies of 5% and above were extracted from the

output files and used in the association studies of haplotype and muscle phenotype.

Sarcopenia Identification

Cutoff values for sarcopenia were used to divide the subjects aged 55 years and

older into sarcopenic and non-sarcopenic groups as follows: relative appendicular skeletal

muscle mass (ASMI) (appendicular skeletal muscle mass divided by height squared) less

than 7.26 kg/m^2 in men, and less than 5.45 kg/m^2 in women were considered sarcopenic based on the work of Baumgartner et al. (12). Sarcopenia defined using this approach has been associated with self-reported physical disability (12) and instrumental activities of daily living disability in the elderly (14).

Statistical Analysis

A total of 1059 subjects were genotyped for *TNF* promoter SNPs and were all included in inferring haplotypes and in computing distributions in the study population of alleles, genotypes and haplotypes. Nine subjects didn't have measures on either muscle mass or muscle strength and were excluded from the genotype-phenotype association analyses. The characteristics of the remaining 1050 subjects are presented in Table 6.

Hardy-Weinberg (H-W) equilibrium was determined for the 5 *TNF* SNPs by using a χ^2 test to compare the observed genotype frequencies to those expected under H-W equilibrium. Pair-wise linkage disequilibrium of 5 *TNF* SNPs, and race and sex differences for genotype and allele frequencies were assessed by χ^2 test, and the Fisher's Exact test was used when the sample size for any cell was 5 or fewer.

Subjects' characteristics including muscle mass and muscle strength were compared between females and males using independent T tests. Pair-wise correlations between continuous variables were assessed by Pearson's correlation coefficients.

Since men and women differed significantly for almost all the variables studied, genotype-phenotype association studies were performed separately for females and males. Eleven muscle phenotypic traits were included as outcome variables in the study: four muscle mass measurements: lean soft tissue mass of both arms (LmArm), lean soft tissue mass of both legs (LmLeg), and lean soft tissue mass of trunk (LmTrunk), total body lean

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soft tissue mass (FFM); five muscle strength measurements: right hand isometric grip strength (Grip), knee extensor isokinetic peak torque at the speed of 30 deg/sec (Knee Isokinetic 30°/s), knee extensor isokinetic peak torque at the speed of 180 deg/sec (Knee Isokinetic 180°/s), knee extensor isometric peak torque at the angle of 120 deg (Knee Isometric 120°), knee extensor isometric peak torque at the angle of 140 deg (Knee Isometric 140°); and two derived muscle mass indicators: appendicular skeletal muscle mass (ASM), and appendicular skeletal muscle mass index (ASMI, ASM divided by height squared). Muscle traits were analyzed in all statistical analyses as continuous variables. Normality of each quantitative trait was confirmed by the Shapiro-Wilk test.

Because of small sample sizes for homozygotes of variant alleles and variant haplotypes, homozygotes and heterozygotes of variant alleles/haplotypes were combined as variant allele/haplotype carriers.

The differences in muscle phenotypes between two genotype groups of each SNP within each sex were tested using analyses of covariance (ANCOVA) to adjust for multiple confounding variables. ANCOVA was also used to assess the association between *TNF* promoter haplotypes and muscle phenotypes. Considering the existence of the subjects carrying two different variant haplotypes, carriers of each variant haplotype were compared separately with wild-type haplotype homozygotes within each sex in ANCOVA. Comparisons were also made between carriers and non-carries of each haplotype. In ANCOVA, muscle mass and strength measures were the response variables modeled one at a time; and the categorical explanatory variables were genotypes/haplotypes and race. The model was developed to make adjustment for the effects of age, body height, and total body fat, as well as regional muscle mass in the analyses of muscle strength; additional

control was also considered for smoking status and chronic diseases, as well as menopause status and HRT conditions in females. Both the first-order and the second-order effects of age on muscle mass/strength have been shown in previous studies (112). The quadratic term of age (age*age) was included in the model only when significant. Genotype/haplotype-by-race interaction was included in the model, and if significant, pre-planned comparisons with Bonferroni adjustment were then made between genotype groups for race stratified subgroups.

Characteristics between men/women with sarcopenia (sarcopenic group) and those with normal ASMI values (normal group) were compared using a t-test. χ^2 tests were used to compare the frequencies of genotypes and haplotypes between sarcopenic and normal groups for each sex. Binary logistic regression was used to examine whether genotype or haplotype is a significant predictor for sarcopenic status (presence or absence of sarcopenia) in the elderly people after controlling for multiple confounding variables consisting of race, sex, age, height and FM.

The Statistical Analysis Software System (SAS version 9.1, SAS institute Inc, Cary, NC) was used for statistical analysis. The α level to declare an effect significant was set at 0.05.

RESULTS

Allele and Genotype Frequencies

A total of 1059 BLSA subjects were genotyped for *TNF* promoter polymorphisms. The genotype and allele frequencies for the 5 SNPs in the *TNF* promoter, rs1799964/T-1031C, rs1800630/C-863A, rs1799724/C-857T, rs1800629/G-308A and rs361525/G-238A, are shown in Table 1. No significant deviation from Hardy Weinberg (H-W) equilibrium was detected for any of the 5 *TNF* SNPs (p=0.2~0.9) (Table 1). The genotype frequencies for variant allele homozygotes were low (n=1~25), so they were combined with the heterozygotes into one group as variant allele carriers for all 5 SNPs in the subsequent data analyses.

Table 1. Observed genotype and allele frequencies for *TNF* promoter polymorphisms and H-W test.

SNP	Genotype	Genotype Frequency (N & %)	Allele Frequency %	p
-1031 (1047)	CC	8 (3.6)	20.2 (C)	0.7
	CT	347 (33.1)	79.8 (T)	
	TT	662 (63.2)		
-863 (1045)	AA	47 (4.5)	18.9 (A)	0.2
	AC	301 (28.8)	81.1 (C)	
	CC	697 (66.7)		
-857 (1053)	TT	16 (1.5)	10.2 (T)	0.2
	TC	182 (17.3)	89.8 (C)	
	CC	855 (81.2)		
-308 (1053)	AA	27 (2.6)	16.9 (A)	0.8
	AG	301 (28.6)	83.1 (G)	
	GG	725 (68.9)		
-238A (1056)	AA	4 (0.4)	5.5 (A)	0.9
	AG	108 (10.2)	94.5 (G)	
	GG	944 (89.4)		

p values were generated by χ^2 test for Hardy Weinberg equilibrium.

The pairwise linkage disequilibrium of the 5 *TNF* promoter SNPs was assessed by χ^2 test (or Fisher's exact test when appropriate) (Table 2). Strong allelic association was detected between the -863C/A and the -1031T/C polymorphisms (R^2=0.48, p<0.001).

Race-specific genotype frequencies are displayed in Table 3. χ^2 test (or Fisher's exact test when appropriate) was conducted to compare genotype frequencies in whites compared to blacks (other races, n = 57, were not included in this analysis). Genotype distributions were the same in the two race groups for the -1031T/C, -863C/A, -308G/A and -238G/A (p=0.28~0.40) SNPs, while the genotype distribution of the -857C/T SNP differed between whites and blacks (p<0.01). The variant allele 'T' at site -857 was less represented in blacks than in whites (5.2% vs. 11.8%) (Table 3). No significant sex difference in genotype distribution was detected for any SNP (p>0.05) (Table 4).

Table 2. Linkage disequilibrium between *TNF* promoter polymorphisms.

	-863C/A	-857C/T	-308G/A	-238G/A
-1031T/C	0.48 (p<0.001)	0.02 (p=0.0002)	0.04 (p<0.0001)	0.19 (p<0.0001)
-863C/A		0 (p<0.0001)	0.04 (p<0.0001)	0.01 (p=0.0005)
-857C/T			0.01 (p=0.1)	0 (p=0.6)
-308G/A				0.01 (p=0.03)

Values are R^2 and p values for χ^2 tests.

Table 3. Genotype and allele frequencies for *TNF* promoter SNPs in whites and blacks.

Genotype		Whites	Blacks	p
-1031T/C	CC	26 (3.44)	8 (3.46)	0.28
	CT	256 (33.91)	69 (29.87)	
	TT	473 (62.65)	154 (66.67)	
	C (%)	20.39%	18.39%	
-863C/A	AA	40 (5.35)	4 (1.71)	0.31
	AC	214 (28.61)	67 (28.63)	
	CC	494 (66.04)	163 (69.66)	
	A (%)	19.77%	16.02%	
-857C/T	TT	14 (1.84)	1 (0.43)	<0.001
	TC	151 (19.89)	22 (9.44)	
	CC	594 (78.26)	210 (90.13)	
	T (%)	11.79%	5.15%	
-308G/A	AA	22 (2.89)	5 (2.16)	0.29
	AG	229 (30.13)	63 (27.16)	
	GG	509 (66.97)	164 (70.69)	
	A (%)	17.96%	15.73%	
-238G/A	AA	3 (0.39)	1 (0.43)	0.40
	AG	80 (10.5)	20 (8.58)	
	GG	679 (89.11)	212 (90.99)	
	A (%)	5.64%	4.72%	

Values are frequencies and percentages (in parenthesis).

p values were generated by χ^2 or Fisher's Exact Test

Table 4. Genotype frequencies for female and male subjects.

Genotype		Female (510)	Male (543)	p
-1031T/C	CC (38)	19 (3.78)	19 (3.54)	0.88
	CT (343)	169 (33.67)	174 (32.40)	
	TT (658)	314 (62.55)	344 (64.06)	
-863C/A	AA (46)	21 (4.17)	25 (4.68)	0.69
	AC (297)	150 (29.82)	147 (27.53)	
	CC (694)	332 (66.00)	362 (67.79)	
-857C/T	TT (16)	7 (1.39)	9 (1.67)	0.92
	CT (180)	86 (17.03)	94 (17.44)	
	CC (848)	412 (81.58)	436 (80.89)	
-308G/A	AA (27)	10 (1.97)	17 (3.16)	0.07
	AG (299)	132 (25.98)	167 (31.04)	
	GG (720)	366 (72.05)	354 (65.80)	
-238G/A	AA (4)	1 (0.20)	3 (0.56)	0.40
	AG (108)	57 (11.22)	51 (9.44)	
	GG (936)	450 (88.58)	486 (90.00)	

Values are frequencies and percentages (in parenthesis).

p values were generated by χ^2 or Fisher's exact tests for the test of sex difference in genotype frequencies.

Muscle Mass, Strength and *TNF* Promoter Polymorphisms

A total of 1050 BLSA participants (509 female and 541 male subjects) with both genotype and muscle phenotype data were included for the mass and strength analyses. Subjects with incomplete data were also included and the exact sample sizes for the study variables are reported in appropriate tables. Disease frequencies and smoking status by sex, and menopause and HRT status for females are shown in Table 5. Compared with females, males had higher incidence of myocardial infarction (6.1% vs. 2.2%, $p<0.01$), cancer (19.2% vs. 7.9%, $p<0.01$), diabetes (15.2% vs. 3.3%), and hypertension (43.4% vs. 30.3%, $p<0.01$), and more males were indicated to be smokers than females (current smoking: 5.9% vs. 2.2%, former smoking: 55.8% vs. 37.1%; $p<0.01$) (Table 5).

Table 5. Race, diseases and smoking status for female and male subjects, menopause and HRT status for females.

Variable		Female (509)	Male (541)	p
Race	White (759)	352 (69.16)	407 (75.23)	0.09
	Black (234)	127 (24.95)	107 (19.78)	
	Other (57)	30 (5.89)	27 (4.99)	
MI	No (1006)	498 (97.84)	508 (93.90)	<0.01
	Yes (44)	11 (2.16)	33 (6.10)	
Cancer	No (906)	469 (92.14)	437 (80.78)	<0.01
	Yes (144)	40 (7.86)	104 (19.22)	
Diabetes	No (951)	492 (96.66)	459 (84.84)	<0.01
	Yes (99)	17 (3.34)	82 (15.16)	
Hypertension	No (661)	355 (69.74)	306 (56.56)	<0.01
	Yes (389)	154 (30.26)	235 (43.44)	
Smoking	Current (43)	11 (2.16)	32 (5.91)	<0.01
	Former (491)	189 (37.13)	302 (55.82)	
	Never (516)	309 (60.71)	207 (38.26)	
Menopause	No	164 (32.22)		
	Yes	345 (67.78)		
HRT	No	437 (85.85)		
	Yes	72 (14.15)		

Values are frequencies and percentages (in parenthesis).
MI: myocardial infarction event
HRT: Hormone replacement therapy
p values were generated by $\chi 2$ test for the test of sex difference for each variable

The genetic associations of the *TNF* promoter polymorphisms with muscle mass and muscle strength were assessed for males and females separately. Subject characteristics are displayed in Table 6 for females and males. T tests demonstrated that all the variables were significantly different between females and males: males are older, have higher weight, height, BMI, FFM, arm lean mass, leg lean mass, trunk lean mass, ASM, and ASMI than females ($p<0.01$); females have higher FM than males ($p<0.01$) (Table 6).

Table 6. Characteristics of subjects by gender.

Variables	Females	Males	p
Age (Yrs)	60.5±0.7(509)	66.1±0.7(541)	<0.01
Weight (Kg)	70.3±0.7(504)	85.1±0.7(536)	<0.01
Height (m)	1.6±0.0(504)	1.8±0.0(536)	<0.01
BMI (Kg/m^2)	26.4±0.2(504)	27.5±0.2(536)	<0.01
FM (Kg)	28.2±0.5(447)	24.8±0.5(437)	<0.01
Arm lean mass (Kg)	3.8±0.0(411)	6.5±0.1(407)	<0.01
Leg lean mass (Kg)	12.3±0.1(412)	18.0±0.2(407)	<0.01
Trunk lean mass (Kg)	19.6±0.2(412)	26.9±0.2(407)	<0.01
FFM (Kg)	39.8±0.2(447)	56.4±0.3(437)	<0.01
ASM (Kg)	16.2±0.1(411)	24.6±0.2(407)	<0.01
ASMI (Kg/m^2)	6.1±0.0(408)	8.0±0.1(406)	<0.01
Grip strength (Kg)	25.2±0.4(355)	38.6±0.5(374)	<0.01
Knee Isokinetic 30°/s (Nm)	97.3±1.6(441)	142.6±2.4(469)	<0.01
Knee Isokinetic 180°/s (Nm)	65.8±1.1(436)	102.3±1.8(466)	<0.01
Knee Isometric 120° (Nm)	372.1±5.9(400)	486.6±8.1(414)	<0.01
Knee Isometric 140° (Nm)	279.5±5.1(397)	350.7±6.7(411)	<0.01

Values are mean ± standard error (sample size)
p: p values for mean comparisons between males and females using two independent T tests
BMI: body mass index
FM: total body fat mass in Kg
FFM: total body lean soft tissue mass in Kg
ASM: appendicular skeletal muscle mass in Kg
ASMI: appendicular skeletal muscle mass index in Kg/m^2

T tests were used to compare subject characteristics including muscle mass and muscle strength between the two genotype groups for each of the 5 *TNF* SNPs in females and males, respectively. In females, no statistically significant differences were found between genotype groups of each SNP for all the confounding variables. The -238A

carriers tended to be younger than -238G homozygotes with the difference being marginally significant (A carriers vs. GG homozygotes: 57.0±2.2 vs. 60.9±0.8 years; p=0.08) (Table 7). In males, no statistically significant differences were found for any confounding variables within any genotype group pairs. Marginal differences between genotype groups were found for height for -863C/A (A carriers vs. G homozygotes: 1.75±0.01 vs. 1.76±0.00 m; p=0.06) and -857C/T (T carriers vs. CC homozygotes: 1.75±0.01 vs. 1.76±0.00 m; p=0.06), and for BMI for -238G/A (A carriers vs. G homozygotes: 28.4±0.6 vs. 27.4±0.2; p=0.07) (Table 8).

Table 7. Age, weight, height, BMI and FM by genotype in the female BLSA subjects.

	-1031T/C		-863C/A		-857C/T		-308G/A		-238G/A	
	CC+CT	TT	AA+AC	CC	TT+CT	CC	AA+AG	GG	AA+AG	GG
Age (Yrs)	60.3±1.2 (189)	60.8±1.0 (314)	59.6±1.2 (172)	61.3±0.9 (332)	61.6±1.7 (93)	60.1±0.8 (413)	59.7±1.4 (142)	60.8±0.9 (367)	57.0 ±2.2 (58)	60.9±0.8 (451)
	p=0.74		p=0.26		p=0.43		p=0.52		p=0.08	
Weight (Kg)	70.4±1.2 (188)	70.4±0.8 (311)	69.9±1.1 (169)	70.6± 0.8 (330)	69.3±1.4 (93)	70.6±0.7 (409)	71.5±1.1 (142)	69.9±0.8 (362)	70.7±2.2 (58)	70.3±0.7 (446)
	p=0.93		p=0.52		p=0.48		p=0.23		p=0.83	
Height (m)	1.63±0.01 (188)	1.63±0.00 (311)	1.64±0.01 (169)	1.63±0.00 (330)	1.62±0.01 (93)	1.63±0.00 (409)	1.64±0.01 (142)	1.63±0.00 (362)	1.63±0.01 (58)	1.63±0.00 (446)
	p=0.43		p=0.50		p=0.20		p=0.43		p=0.74	
BMI (Kg/m$^{2)}$)	26.5±0.4 (188)	26.4±0.3 (311)	26.1±0.4 (169)	26.6±0.3 (330)	26.2±0.5 (93)	26.4±0.3 (409)	26.8±0.4 (142)	26.3±0.3 (362)	26.6±0.8 (58)	26.9±0.3 (446)
	p=0.82		p=0.31		p=0.79		p=0.30		p=0.71	
FM (Kg)	28.5±0.9 (165)	28.3±0.6 (276)	27.8±0.9 (149)	28.7±0.6 (295)	28.9±1.2 (82)	28.2±0.6 (362)	29.0±0.9 (127)	28.0±0.6 (320)	27.9±1.7 (50)	28.3±0.5 (398)
	p=0.89		p=0.35		p=0.55		p=0.30		p=0.84	

Values are mean ± standard error (sample size)

p: p values are for mean comparisons between genotype groups of each SNP using two independent T test.

BMI: Body mass index

FM: total body fat mass in Kg

Table 8. Age, weight, height, BMI and FM by genotype in the male BLSA subjects.

	-1031T/C		-863C/A		-857C/T		-308G/A		-238G/A	
	CC+CT	TT	AA+AC	CC	CC+CT	TT	AA+AG	GG	AA+AG	GG
Age (Yrs)	67.6±1.1 (194)	65.4±0.9 (345)	67.4±1.2 (173)	65.5±0.9 (363)	66.8±1.6 (105)	66.1±0.8 (436)	65.8±1.2 (185)	66.4±0.9 (355)	67.3±2.2 (54)	66.1±0.7 (488)
		p=0.15		p=0.24		p=0.69		p=0.77		p=0.62
Weight (Kg)	85.9±1.2 (192)	84.8±0.8 (342)	84.5±1.3 (170)	85.7±0.8 (361)	84.2±1.6 (105)	85.4±0.7 (431)	85.2±1.0 (183)	85.1±0.9 (352)	88.6±2.4 (54)	84.8±0.7 (483)
		p=0.40		p=0.44		p=0.42		p=0.96		p=0.13
Height (m)	1.76±0.01 (192)	1.76±0.00 (342)	1.75±0.01 (170)	1.76±0.00 (361)	1.75±0.01 (105)	1.76±0.00 (431)	1.77±0.01 (183)	1.76±0.00 (352)	1.76±0.01 (54)	1.76±0.00 (483)
		p=0.54		p=0.06		p=0.06		p=0.14		p=0.84
BMI (Kg/m^2)	27.8±0.3 (192)	27.3±0.2 (342)	27.5±0.4 (170)	27.5±0.2 (361)	27.6±0.5 (105)	27.5±0.2 (431)	27.3±0.3 (183)	27.5±0.2 (352)	28.4±0.6 (54)	27.4±0.2 (483)
		p=0.22		p=0.93		p=0.98		p=0.46		p=0.07
FM (Kg)	25.1±0.8 (157)	24.8±0.6 (280)	24.4±0.8 (134)	25.2±0.6 (298)	24.4±1.1 (92)	26.0±0.5 (345)	24.8±0.8 (144)	24.9±0.6 (292)	27.1±1.3 (44)	24.6±0.5 (394)
		p=0.78		p=0.42		p=0.46		p=0.90		p=0.10

Values are mean ± standard error (sample size)

p: p values are for mean comparisons between genotype groups of each SNP using two independent T test.

BMI: Body mass index

FM: total body fat mass in Kg

In females, no statistically significant difference was found in muscle mass and strength measurements between genotype groups for any SNP, the only tendency for a difference being for isometric knee extension peak torque at 140° between -863A carriers and -863C homozygotes (A carriers vs. C homozygotes: 291.44±8.95 vs. 271.18±6.11 Nm; p=0.06) (Table 9). In males, statistically significant differences in muscle mass, but not in muscle strength, were observed between genotype groups of -1031T/C, -863C/A, and -857C/T (Table 10). For -1031T/C, 'C' carriers were lower in arm lean mass, leg lean mass, and ASM than 'T' homozygotes (p=0.01-0.04; Table 10). For -863C/A, compared with 'C' homozygotes, 'A' carriers were lower in arm lean mass, trunk lean mass, FFM, and ASM (p=0.002-0.05; Table 10). For -857C/T, 'T' carriers were lower than 'C' homozygotes in leg lean mass and ASM (p=0.01-02; Table 10). No statistically significant difference in muscle mass and strength was found between genotype groups of -308G/A and -238G/A in men, though a tendency was observed for -238A carriers to have higher trunk lean mass than 'G' homozygotes (p=0.06; Table 10).

Table 9. Muscle mass and strength by genotype in the female BLSA subjects.

Variables	-1031T/C		-863C/A		-857C/T		-308G/A		-238G/A	
	CC+CT	TT	AA+AC	CC	CC+CT	TT	AA+AG	GG	AA+AG	GG
LmArm (Kg)	4.2±0.3 (146)	3.9±0.1 (259)	4.2±0.4 (136)	3.9±0.1 (273)	3.8±0.1 (76)	4.0±0.2 (332)	4.0±0.1 (114)	4.0±0.2 (297)	3.8±0.1 (41)	4.0±0.1 (371)
	p=0.38		p=0.37		p=0.25		p=0.99		p=0.3	
LmLeg (Kg)	12.2±0.2 (146)	12.4±0.1 (259)	12.2±0.2 (135)	12.4±0.1 (274)	12.0±0.2 (76)	12.4±0.1 (32)	12.5±0.2 (115)	12.30±0.13 (296)	12.2±0.4 (42)	12.4±0.1 (370)
	p=0.47		p=0.37		p=0.16		p=0.5		p=0.73	
LmTrunk (Kg)	19.4±0.3 (147)	19.7±0.2 (259)	19.4±0.3 (135)	19.7±0.2 (274)	19.6±0.4 (76)	19.6±0.2 (333)	19.7±0.4 (115)	19.5±0.2 (297)	19.4±0.7 (42)	19.6±0.2 (371)
	p=0.51		p=0.44		p=0.93		p=0.76		p=0.74	
FFM (Kg)	39.4±0.4 (164)	40.1±0.3 (276)	39.7±0.4 (148)	40.0±0.3 (295)	39.6±0.6 (82)	40.0±0.3 (361)	40.3±0.4 (127)	39.7±0.3 (319)	40.0±0.8 (50)	39.8±0.2 (397)
	p=0.13		p=0.55		p=0.54		p=0.19		p=0.85	
ASM (Kg)	16.1±0.2 (145)	16.3±0.2 (259)	16.0±0.2 (135)	16.3±0.2 (273)	15.8±0.3 (76)	16.3±0.2 (331)	16.4±0.2 (114)	16.1±0.2 (296)	16.0±0.5 (41)	16.2±0.1 (370)
	p=0.48		p=0.45		p=0.22		p=0.34		p=0.58	
ASMI	6.1±0.1 (144)	6.1±0.1 (258)	6.0±0.9 (133)	6.1±0.7 (272)	6±0.09 (76)	6.1±0.05 (329)	6.2±0.1 (114)	6.1±0.1 (293)	6.1±0.2 (41)	6.1±0.1 (367)
	p=0.83		p=0.34		p=0.37		p=0.36		p=0.82	
Grip (Kg)	24.9±0.6 (132)	25.4±0.5 (220)	25.2±0.6 (122)	25.3±0.5 (233)	24.7±0.9 (61)	25.4±0.4 (292)	25.6±0.6 (95)	25.1±0.5 (261)	24.9±1.2 (30)	25.2±0.4 (326)
	p=0.48		p=0.91		p=0.47		p=0.49		p=0.8	
Knee Isokinetic 30°/s (Nm)	98.1±2.7 (167)	97.0±2.0 (268)	99.2±2.7 (155)	95.7±2.0 (282)	96.5±4.0 (80)	97.7±1.8 (358)	99.5±2.9 (125)	96.5±1.9 (317)	102.6±5.5 (52)	96.6±1.7 (389)
	p=0.73		p=0.31		p=0.78		p=0.41		p=0.23	
Knee Isokinetic 180°/s (Nm)	64.3±1.7 (165)	66.8±1.5 (265)	66.0±1.7 (153)	65.3±1.4 (279)	64.8±2.7 (79)	66.2±1.2 (354)	66.0±1.9 (124)	65.8 ±1.3 (313)	65.0±3.8 (50)	66.0±1.2 (386)
	p=0.3		p=0.76		p=0.64		p=0.95		p=0.78	
Knee Isometric 120° (Nm)	369.6±9.4 (150)	373.5±7.6 (245)	381.3±10.3 (140)	364.6±7.0 (257)	373.8±14.7 (74)	372.7±6.5 (323)	368.4±10.1 (115)	374.3±7.2 (286)	373.6±18.2 (43)	372.1±6.2 (357)
	p=0.75		p=0.17		p=0.94		p=0.65		p=0.93	
Knee Isometric 140° (Nm)	281.2±9.0 (149)	277.7±6.3 (243)	291.4±9.0 (138)	271.2±6.1 (256)	278.3±11.9 (72)	280.2±5.8 (322)	284.0±8.6 (114)	278.4±6.3 (284)	296.4±18.5 (43)	277.5±5.3 (354)
	p=0.74		p=0.06		p=0.89		p=0.63		p=0.25	

Values are mean ± standard error (sample size)

Comparisons are for variant allele carriers vs. wild-type allele homozygotes for each *TNF* promoter SNP

Table 10. Muscle mass and strength by genotype in the male BLSA subjects.

	-1031T/C		-863C/A		-857C/T		-308G/A		-238G/A	
	CC+CT	TT	AA+AC	CC	CC+CT	TT	AA+AG	GG	AA+AG	GG
LmArm (Kg)	6.3±0.1 (142)	6.6±0.1 (256)	6.2±0.1 (122)	6.7±0.1 (282)	6.3±0.2 (86)	6.6±0.1 (321)	6.6±0.1 (135)	6.5±0.1 (271)	6.8±0.2 (41)	6.5±0.1 (367)
		p= 0.01		p=0.002		p=0.14		p=0.2		p=0.22
LmLeg (Kg)	17.6±0.2 (142)	18.3±0.2 (256)	17.5±0.3 (122)	18.3±0.2 (282)	17.3±0.4 (86)	18.3±0.2 (321)	18.3±0.3 (135)	17.9±0.2 (271)	18.3±0,4 (41)	28.0±0.2 (367)
		p=0.04		p=0.02		p=0.01		p=0.29		p=0.64
LmTrunk (Kg)	26.7±0.4 (142)	27.0±0.3 (256)	26.2±0.4 (122)	27.2±0.3 (282)	26.5±0.6 (86)	27.0±0.2 (321)	271.0±0.4 (135)	26.8±0.3 (271)	28.1±0.6 (41)	26.8±0.2 (367)
		p=0.51		p=0.04		p=0.4		p=0.57		p=0.06
FFM (Kg)	55.8±0.6 (156)	56.7±0.5 (279)	55.5±0.6 (133)	57.0±0.4 (297)	55.5±0.8 (90)	56.7±0.4 (345)	57.0±0.6 (143)	56.1±0.4 (291)	57.8±1.1 (44)	56.3±0.4 (392)
		p=0.20		p=0.05		p=0.18		p=0.22		p=0.19
ASM(Kg)	23.9±0.3 (141)	24.9±0.3 (264)	23.8±0.4 (121)	25.0±0.3 (281)	23.6±0.5 (84)	24.8±0.2 (321)	24.9±0.4 (134)	24.4±0.3 (270)	25.0±0.6 (41)	24.5±0.2 (365)
		p=0.03		p=0.01		p=0.02		p=0.29		p=0.46
ASMI	7.8±0.1 (141)	8.0±0.1 (263)	7.8±0.1 (121)	8.0±0.1 (280)	7.8±0.1 (84)	8.0±0.1 (320)	8.0±0.1 (134)	7.9±0.1 (269)	8.1±0.2 (41)	8.0±0.1 (364)
		p=0.10		p=0.16		p=0.09		p=0.54		p=0.34
Grip (Kg)	37.6±0.9 (131)	39.1±0.7 (243)	37.5±1.0 (113)	39.2±0.7 (258)	37.5±1.4 (74)	38.9±0.6 (300)	39.3±0.9 (130)	38.1±0.7 (243)	39.1±1.7 (36)	38.5±0.6 (339)
		p=0.21		p=0.17		p=0.32		p=0.27		p=0.77
Knee Isokinetic 30°/s (Nm)	141.2±3.8 (173)	144.3±3.5 (294)	141.9±4.0 (146)	144.7±3.3 (318)	144.1±7.7 (88)	142.9±2.6 (381)	143.7±4.7 (163)	142.5±3.0 (306)	146.3±7.8 (50)	142.8±2.7 (420)
		p=0.54		p=0.59		p=0.88		p=0.83		p=0.68
Knee Isokinetic 180°/s (Nm)	101.4±2.8 (174)	103.5±2.5 (290)	102.4±3.1 (146)	103.6±2.4 (315)	102.8±5.5 (88)	102.6±1.9 (378)	104.4±3.5 (160)	101.8±2.2 (306)	100.6±5.5 (51)	102.9±2.0 (416)
		p=0.57		p=0.77		p=0.96		p=0.54		p=0.7
Knee Isometric 120° (Nm)	495.1±16.9 (160)	511.1±26.3 (254)	495.5±13.6 (131)	510.8±24.9 (280)	579.9±83.6 (78)	486.0±8.9 (337)	531.9±44.8 142)	489.8±12.2 (273)	528.6±47.1 (46)	500.9±18.5 (370)
		p=0.61		p=0.59		p=0.27		p=0.37		p=0.62
Knee Isometric 140° (Nm)	341.9±10.8 (158)	372.9±20.3 (252)	353.5±12.1 (130)	366.3±18.6 (277)	407.4±62.1 (78)	348.9±7.0 (333)	386.7±35.1 (140)	347.2±8.1 (271)	340.5±20.7 (45)	362.7±14.4 (367)
		p=0.18		p=0.56		p=0.35		p=0.28		p=0.38

Values are mean ± standard error (sample size)
Comparisons are for variant allele carriers vs. wild-type allele homozygotes for each *TNF* promoter SNP

We next examined the association between muscle phenotypes and *TNF* promoter genotypes using ANCOVA in whites and blacks. Adjustment was made for age, body height and total body fat in the first model. In a second model, additional adjustment was considered for smoking status and chronic diseases, as well as menopause status and HRT conditions for females. Interaction of race with genotype was assessed by adding a product term of race*genotype in the first model and, if significant, pre-planned comparisons were employed for race stratified subgroups (i.e. whites and blacks). To correct type I error inflation in multiple comparisons, the Bonferroni method was used: since two comparisons were made, $\alpha <= 0.025$ was accepted as the significance level. The other race group was exclude from this part of the analyses since this group has only a small number of subjects (n=57) and its ethnic composition is complex.

In females, no significant differences for muscle mass measures were found between genotype groups of any SNP, though a trend was found for -863A carriers to have less leg lean mass than non-carriers (12.3±0.2 vs. 12.7±0.1 kg; p=0.07; Table 11). Knee extensor isokinetic peak torque at 30°/s was found to be marginally associated with -1031T/C (p=0.07). Knee extensor isokinetic peak torque at 30°/s and isometric peak torques at 120° and 140° were significantly associated with -863C/A (0.01-0.02, Table 11), but not with -857C/T, -308G/A and -238G/A. For -863C/A, compared with wild-type allele homozygotes, variant allele 'A' carriers had higher leg muscle strength (Table 11). Further controlling for leg muscle mass mitigated the association between -1031T/C and knee extensor isokinetic peak torque at 30°/s (p=0.04 increased to 0.07), but augmented the association between -863C/A and leg muscle strength (p=0.01-0.08

decreased to 0.01-0.03, Table 12). These observations suggested that the association between leg muscle strength and -863C/A in females was independent of muscle mass.

A significant race interaction with -238G/A was found for leg lean mass (p=0.005), ASM (p=0.011), and ASMI (p=0.009) in females. Post hoc comparisons showed that black females carrying the -238A allele tended to have lower muscle mass (p=0.037-0.053, Table 13); whereas white females carrying the allele 'A' tended to have higher muscle mass (p=0.029-0.061, Table 13). However, all these relationships did not reach Bonferroni significance level (i.e., p=0.025).

In males, no significant relationship was found between muscle phenotypes and *TNF* promoter SNPs at positions -857, -308 and -238. Significant genotypic associations of -1031T/C and -863C/A were found with muscle mass, but not with muscle strength. -1031C was significantly associated with lower arm lean mass and ASM (p=0.005, 0.020, Table 14), and was marginally associated with lower leg lean mass and ASMI (p=0.06, 0.07; Table 14). -863A was significantly associated with lower arm lean mass (p=0.041), and was marginally associated with lower ASM (p=0.081; Table 14). Interaction of race with -1031T/C was found to be statistically significant for grip strength (p=0.04) and this interactive effect was stronger after controlling for arm lean mass (p=0.01). Post hoc comparisons revealed that the association between -1031T/C and grip strength was accounted for by the association present in black males, with black males carrying variant allele 'C' being significantly higher in grip strength than 'T' allele homozygotic black males (p=42.2±1.7 vs. 36.8±1.0Kg; p=0.007; Table 13).

Table 11. Adjusted means for muscle mass and strength by genotype in female BLSA subjects.

Variables	-1031T/C			-863C/A			-857C/T			-308G/A			-238G/A		
	CC+CT	TT	p	AA+AC	CC	p	CC+CT	TT	p	AA+AG	GG	p	AA+AG	GG	p
LmArm (Kg)	4.0±0.1 (132)	4.0±0.1 (246)	0.8	3.9±0.1 (124)	4.0±0.1 (257)	0.48	4.0±0.1 (73)	4.0±0.1 (308)	0.88	4.0±0.1 (111)	3.9±0.1 (272)	0.22	3.9±0.1 (37)	4.0±0 (347)	0.67
LmLeg (Kg)	12.4±0.2 (133)	12.6±0.1 (246)	0.35	12.3±0.2 (124)	12.7±0.1 (258)	0.07	12.4±0.3 (73)	12.6±0.1 (309)	0.65	12.5±0.2 (112)	12.6±0.1 (272)	0.93	Race*genotype interaction		0.01
LmTrunk (Kg)	19.7±0.3 (133)	19.8±0.2 (246)	0.89	19.7±0.3 (124)	19.8±02 (258)	0.76	19.5±0.5 (73)	19.8±0.2 (309)	0.63	19.6±0.3 (112)	19.8±0.2 (272)	0.59	19.7±0.6 (38)	19.7±0.2 (347)	0.97
FFM (Kg)	39.9±0.3 (151)	40.5±0.2 (263)	0.15	40.0±0.3 (137)	40.4±0.2 (279)	0.33	40.0±0.6 (79)	40.3±0.2 (338)	0.69	40.4±0.4 (124)	40.2±0.2 (295)	0.59	40.4±0.7 (46)	40.2±0.2 (374)	0.84
ASM (Kg)	16.4±0.2 (132)	16.6±0.1 (246)	0.4	16.2±0.2 (124)	16.6±0.1 (257)	0.11	16.4±0.4 (73)	16.5±0.1 (308)	0.77	16.6±0.2 (111)	16.5±0.1 (272)	0.79	Race*genotype interaction		0.01
ASMI (Kg/m^2)	6.1±0.1 (131)	6.2±0.1 (245)	0.45	6.1±0.1 (123)	6.2±0.1 (256)	0.11	6.2±0.1 (73)	6.2±0.1 (306)	0.8	6.2±0.1 (111)	6.2±0.1 (270)	0.56	Race*genotype interaction		0.01
Grip (Kg)	25.1±0.5 (118)	26.1±0.4 (207)	0.11	25.0±0.6 (110)	25.9±0.4 (218)	0.2	26.6±0.9 (58)	25.5±0.3 (268)	0.27	25.4±0.6 (092)	25.9±0.4 (237)	0.46	24.5±1.3 (27)	25.8±0.3 (302)	0.33
Isokinetic 30°/s Nm	103.3±2.5 (153)	96.7±1.9 (254)	0.07	103.1±2.6 (142)	96.5±1.9 (266)	0.02	99.3±4.7 (77)	98.4±1.6 (333)	0.87	97.7±2.8 (122)	99.7±1.8 (291)	0.56	105.9±5.4 (49)	98.4±1.6 (363)	0.18
Isokinetic 180°/s Nm	68.1±1.8 (151)	67.5±1.4 (251)	0.65	68.8±1.8 (140)	66.8±1.3 (263)	0.37	62.8±3.3 (76)	67.7±1.2 (329)	0.16	65.1±2.0 (121)	68.6±1.3 (287)	0.14	68.1±3.9 (47)	67.6±1.1 (360)	0.91
Isometric 120° Nm	379.0±10.3 (137)	368.7±7.7 (232)	0.42	388.1±10.3 (128)	363.6±7.5 (242)	0.01	376.9±18.3 (72)	371.0±6.6 (299)	0.76	359.5±11.2 (112)	380.0±7.2 (262)	0.14	394.2±24.6 (40)	371.9±6.3 (333)	0.38
Isometric 140° Nm	282.0±8.9 (136)	270.3±6.7 (230)	0.3	290.6±8.9 (126)	267.5±6.5 (241)	0.02	279.1±16.0 (70)	275.4±5.8 (298)	0.83	272.1±9.0 (111)	269.2±6.1 (260)	0.78	304.8±21.4 (40)	274.0±5.5 (330)	0.16

Comparisons are for variant allele carriers vs. wild-type allele homozygotes for each *TNF* promoter SNP

LmArm, LmLeg, LmTrunk, FFM, ASM were all adjusted for age, age*age (if significant), height, FM.

ASMI was adjusted for age, age*age (if significant), FM.

Grip was adjusted for age, age*age (if significant), height, FM and lmArm

Knee Isokinetic 30°/s, Knee Isokinetic 180°/s, Knee Isometric 120°, Knee Isometric 140° were all adjusted for age, age*age (if significant), height and LmLeg.

Interaction: statistically significant race by genotype interaction

Table 12. Leg muscle strength by genotype before and after controlling for muscle mass in female BLSA subjects

		-1031		p	-863		p
		CC+CT	TT		AA+AC	CC	
Knee Isokinetic 30°/s (Nm)	Before	102.9±2.4	97.0±1.8	0.041	102.2±2.5	97.1±1.8	0.08
	After	99.2±2.5	93.7±1.9	0.069	100.0±2.5	93.2±1.9	0.024
Knee Isometric 120° (Nm)	Before				389.3±9.8	362.9±7.3	0.026
	After				385.4±9.9	355.1±7.3	0.011
Knee Isometric 140° (Nm)	Before				292.5±8.5	266.4±6.3	0.012
	After				285.0±8.5	260.5±6.2	0.016

Knee Isokinetic 30°/s, Knee Isometric 120°, Knee Isometric 140° were all adjusted for age, age*age (if significant), FM, and height

Before and After: before and after additional adjustment for LmLeg.

Table 13. Adjusted means of muscle phenotypes for genotype groups of *TNF* -238G/A and -1031T/C in Blacks and Whites.

		-238G/A		
Black females		AA+AG (N=11)	GG (N=115)	p
	LmLeg (Kg)	11.7±0.6	13.0±0.2	0.037
	ASM (Kg)	15.6±0.8	17.2±0.2	0.053
	ASMI (Kg/m^2)	5.9±0.3	6.5±0.1	0.050
White females		AA+AG (N=43)	GG (N=309)	
	LmLeg (Kg)	12.8±0.3	12.1±0.1	0.029
	ASM (Kg)	16.6±0.4	15.8±0.1	0.061
	ASMI (Kg/m^2)	6.2±0.1	5.9±0.1	0.050
		-1031T/C		
Black males		CC+CT (N=31)	TT (N=75)	
	Grip (Kg)	42.2±1.7	36.8±1.0	0.007
White males		CC+CT (N=152)	TT (N=252)	
	Grip (Kg)	38.1±0.8	38.3±0.6	0.87

Comparisons are for variant allele carriers vs. wild-type allele homozygotes for each *TNF* promoter SNP

LmArm, LmLeg, LmTrunk, FFM, ASM were all adjusted for age, age*age (if significant), height, FM.

ASMI was adjusted for age, age*age (if significant), FM.

Grip was adjusted for age, age*age (if significant), height, FM and LmArm

Table 14. Adjusted means for muscle mass and strength by genotype in the male BLSA subjects.

	-1031T/C			-863C/A			-857C/T			-308G/A			-238G/A		
	CC+CT	TT	p	AA+AC	CC	p	CC+CT	TT	p	AA+AG	GG	p	AA+AG	GG	p
LmArm (Kg)	6.4±0.13 (131)	6.8±0.1 (248)	0.01	6.4±0.1 (115)	6.8±0.1 (263)	0.04	6.8±0.2 (77)	6.7±0.1 (303)	0.48	6.8±0.1 (130)	6.6±0.1 (248)	0.3	6.8±0.2 (37)	6.7±0.1 (344)	0.68
LmLeg (Kg)	17.9±0.3 (131)	18.5±0.2 (248)	0.06	18.0±0.3 (115)	18.4±0.2 (263)	0.15	18.1±0.4 (77)	18.4±0.2 (303)	0.42	18.6±0.3 (130)	18.2±0.2 (248)	0.23	18.4±0.5 (37)	18.4±0.2 (344)	0.96
LmTrunk (Kg)	26.2±0.4 (131)	26.8±0.3 (248)	0.25	26.0±0.5 (115)	26.9±0.3 (263)	0.13	26.9±0.6 (77)	26.6±0.3 (303)	0.72	26.7±0.4 (130)	26.6±0.3 (248)	0.87	27.2±0.8 (37)	26.6±0.2 (344)	0.48
FFM (Kg)	56.4±0.6 (146)	57.1±0.4 (263)	0.3	56.6±0.7 (127)	57.0±0.4 (279)	0.58	56.8±0.8 (83)	56.9±0.3 (327)	0.91	57.2±0.6 (139)	56.7±0.4 (269)	0.41	56.6±1.0 (40)	56.9±0.3 (371)	0.75
ASM (Kg)	24.3±0.4 (131)	25.4±0.2 (248)	0.02	24.4±0.4 (115)	25.3±0.2 (263)	0.08	24.9±0.5 (77)	25.1±0.2 (303)	0.72	25.4±0.4 (130)	24.9±0.3 (248)	0.22	25.2±0.7 (37)	25.0±0.2 (344)	0.86
ASMI (Kg/m^2)	7.9±0.1 (131)	8.2±0.1 (247)	0.07	8.0±0.1 (115)	8.2±0.1 (262)	0.27	8.1±0.2 (77)	8.1±0.1 (302)	0.81	8.2±0.1 (130)	8.1±0.1 (247)	0.29	8.2±0.2 (37)	8.1±0.1 (343)	0.8
Grip (Kg)	Race*genotype interaction		0.01	40.1±1.2 (107)	38.5±0.6 (241)	0.22	38.0±1.3 (67)	38.6±0.5 (283)	0.67	38.7±0.9 (126)	38.4±0.6 (223)	0.78	40.6±1.7 (33)	38.4±0.5 (318)	0.21
Isokinetic 30°/s Nm	140.9±4.7 (163)	136.4±3.1 (278)	0.42	142.2±5.5 (140)	136.9±3.0 (300)	0.4	135.3±7.0 (79)	138.0±2.8 (365)	0.72	134.7±4.4 (157)	138.6±3.2 (286)	0.48	148.1±8.2 (46)	136.5±2.7 (399)	0.18
Isokinetic 180°/s Nm	104.1±3.1 (164)	98.5±2.0 (274)	0.13	104.6±3.6 (140)	99.1±2.0 (297)	0.18	97.6±4.6 (79)	100.4±1.8 (362)	0.57	98.5±2.9 (154)	100.7±2.1 (286)	0.54	106.2±5.4 (47)	99.4±1.8 (395)	0.23
Isometric 120° Nm	479.2±15.4 (149)	464.0±10.5 (238)	0.41	481.7±17.6 (125)	465.7±9.9 (261)	0.43	469.3±23.0 (68)	469.1±9.3 (321)	0.99	467.1±14.6 (136)	469.0±10.7 (252)	0.92	500.2±26.8 (41)	465.1±9.1 (349)	0.22
Isometric 140° Nm	337.8±12.6 (148)	340.3±7.6 (236)	0.53	346.4±14.5 (124)	338.3±8.1 (259)	0.63	336.9±18.8 (69)	339.0±7.1 (317)	0.92	339.5±12.1 (134)	338.1±8.8 (251)	0.93	335.1±22.0 (41)	339.5±7.4 (346)	0.85

Comparisons are for variant allele carriers vs. wild-type allele homozygotes for each *TNF* promoter SNP

LmArm, LmLeg, LmTrunk, FFM, ASM were all adjusted for age, age*age (if significant), height, FM.

ASMI was adjusted for age, age*age (if significant), FM.

Grip was adjusted for age, age*age (if significant), height, FM and lmArm

Knee Isokinetic 30°/s, Knee Isokinetic 180°/s, Knee Isometric 120°, Knee Isometric 140° were all adjusted for age, age*age (if significant), height, FM and LmLeg

Interaction: statistically significant race by genotype interaction with p values before and after additional adjustment for regional muslc mass being reported.

Haplotypes

TNF promoter haplotypes were constructed using PHASE software (v. 2.1) based on the genotype data for the 5 *TNF* promoter SNPs (rs361525/G-238A, rs1800629/G-308A, rs1799724/C-857T, rs1800630/C-863A, rs1799964/T-1031C). In total, 17 haplotypes were constructed by PHASE and their structure and frequencies are shown in Table 15.

The 5 haplotypes with frequencies of 5% and over, including CCCGA (4.8%), TCTGG (7.9%), CACGG (13.8%), TCCAG (16.2%) and TCCGG (50.3%), were assessed for genotypic association with muscle mass and muscle strength. For purposes of clarity, the GGCCT haplotype is referred to as the "wild-type haplotype" and the other 4 haplotypes are referred to as "variant haplotypes." Few subjects were homozygous for the four variant haplotypes (Table 16), so in the analyses of muscle mass and muscle strength, they were combined with subjects heterozygous for the corresponding variant haplotypes into variant haplotype carriers.

Table 15. Haplotypes for *TNF* promoter polymorphisms and their frequencies.

Haplotype	Frequency	Percent
TCCGG	1065	50.28
TCCAG	343	16.19
CACGG	292	13.79
TCTGG	168	7.93
CCCGA	101	4.77
TACGG	61	2.88
TATGG	34	1.61
CCCGG	24	1.13
CACGA	6	0.28
TCCGA	6	0.28
TCTAG	5	0.24
CCTGG	4	0.19
CCCAG	3	0.14
CCTGA	2	0.09
CACAG	2	0.09
TCCAA	1	0.05
CCTAG	1	0.05

Table 16. Haplotype frequency in BLSA subjects by sex.

	Female		Male	
	Homozygous Haplotype	Heterozygous Haplotype	Homozygous Haplotype	Heterozygous Haplotype
CCCGA	1	48	2	47
TCTGG	6	69	6	75
CACGG	9	124	11	126
TCCAG	10	127	15	165
TCCGG	133	257	141	253

Numbers are sample sizes

TNF Pomoter Haplotype and Muscle Mass and Strength

The association between *TNF* promoter haplotypes and muscle phenotypes was assessed in two steps. First, we compared carriers of each variant haplotype with wild-type haplotype homozygotes for muscle mass and strength; second, muscle pheonotypes were compared between carriers and non-carriers of each haplotype. ANCOVA was used in both steps with haplotype and race as explanatory variables, and age, height and total body fat mass as covariates. Further controlling for disease and smoking status, as well as menopause and HRT status (for females only) was also considered. Females and males were analyzed separately. No subject of other races was included. Interaction between

race and haplotype (race*haplotype) was tested and, if significant, pre-planned comparison was employed within whites and blacks. The Bonferroni corrected p, 0.025, was accepted as the significance level.

In females, compared with wild-type haplotype homozygotes, no statistically significant difference was found for any variant haplotype group for any muscle phenotype, though a tendency was shown for CACGG carriers to be higher in knee extensor isokinetic peak torque at 30°/s (p=0.071, Table 17). Consistently, in comparisons between carriers and non-carriers, no haplotype was associated with muscle phenotypes in the entire female population (Table 18). However, significant race interactions with haplotypes were observed for muscle mass or muscle strength, except CACGG, which did not show any significant interaction with race (Table 17, 18). Comparisons within race groups revealed that (Table 19, 20) compared with TCCGG homozygotic black females, black females carrying TCTGG were lower in knee extensor isokinetic peak torque at 180°/s (49.8±6.1 vs. 68.8±3.1, p=0.006; Table 19), and that black

females carrying TCCAG were lower in grip strength (25.3±1.1 vs. 29.0±1.0 kg, p=0.011, Table 19). Compared with non-carriers, TCTGG carriers had lower knee extensor isokinetic peak torque at 180°/s (50.1±6.6 vs. 68.6±1.9, p=0.007, Table 20) in black females; carriers of TCCGG, the wild-type haplotype, had higher leg lean mass, ASM, ASMI, grip strength and knee extensor isokinetic peak torque at 180°/s (p=0.0007-0.0048). The differences in white females were not significant, though a trend was shown for higher muscle mass in CCCGA carriers than in non-carriers (p=0.020-0.045, Table 20). As a whole, these post hoc comparisons appeared to suggest that in females the associations between *TNF* promoter haplotypes and muscle phenotypes were stronger in blacks than in whites and variant haplotypes were associated with inferior muscle phenotypes.

In males, compared with wild-type haplotype homozygotes, carriers of any variant haplotype showed no significant difference in any muscle phenotypic trait, though a trend was shown for CACGG carriers being lower in arm lean mass (p=0.07, Table 21). In comparisons between carriers and non-carriers of each haplotype, no significant difference in muscle phenotypes was found for CCCGA, TCTGG; CACGG was significantly associated with lower arm lean mass, trunk lean mass, ASM (p=0.008-0.048, Table 22), and marginally associated with lower leg lean mass, ASMI (p=0.07, 0.082, Table 22); TCCGG carriers was significantly lower in knee extensor isokinetic peak torque at 180°/s than non-carriers (96.6±1.9 vs.106.3±4.4; p=0.043; Table 22). TCCAG showed a trend to be associated with higher leg lean mass and ASM (p=0.067, 0.05; Table 22).

Significant race interactions with haplotypes were also found in males for various muscle phenotypes (Table 21, 22). Post hoc comparisons showed that the only significant difference was

between CACGG carriers and non-carriers in black males for grip strength (CACGG carriers vs.

non-carriers: 43.4±2.3 vs. 37.4±0.9 Kg, p=0.015; Table 23).

Table 17. Adjusted means of muscle mass and strength for variant haplotype carriers and wild-type haplotype homozygotes in female BLSA subjects.

	CCCGA Carrier	p	TCTGG Carrier	p	TCCAG Carrier	p	CACGG Carrier	p	TCCGG Homozygote
LmArm (Kg)	4.0±0.2 (29)	0.73	4.1±0.1 (58)	0.28	4.1±0.1 (108)	0.19	4.0±0.1 (95)	0.49	3.9±0.1 (101)
LmLeg (Kg)	Race*haplotype Interaction	0.006	12.6±0.3 (58)	0.97	12.6±0.2 (109)	0.78	12.4±0.2 (95)	0.52	12.6±0.2 (101)
LmTrunk (Kg)	20.0±0.7 (30)	0.98	19.4±0.6 (58)	0.48	19.8±0.4 (109)	0.71	19.9±0.3 (95)	0.96	19.9±0.3 (101)
FFM (Kg)	40.3±0.7 (38)	0.87	40.1±0.6 (63)	0.58	40.6±0.4 (120)	0.85	39.9±0.4 (105)	0.32	40.5±0.4 (107)
ASM(Kg)	Race*haplotype Interaction	0.015	16.6±0.4 (58)	0.74	16.7±0.2 (108)	0.72	16.4±0.2 (95)	0.78	16.5±0.2 (101)
ASMI (Kg/m^2)	Race*haplotype Interaction	0.014	6.2±0.2 (58)	0.65	6.3±0.1 (108)	0.5	6.1±0.1 (94)	0.88	6.2±0.1 (100)
Grip (Kg)	24.5±1.4 (23)	0.3	26.6±1.1 (48)	0.55	Race*haplotype Interaction	0.013 & 0.006	25.4±0.6 (90)	0.29	26.2±0.6 (88)
Isokinetic 30°/s (Nm)	106.7±5.7 (40)	0.12	98.1±4.5 (62)	0.64	99.9±2.8 (117)	0.51	103.3±2.9 (106)	0.07	96.5±3.0 (100)
Isokinetic 180°/s (Nm)	69.2±4.0 (39)	0.82	Race*haplotype Interaction	0.011 & 0.006	66.5±2.1 (116)	0.11	68.9±2.1 (104)	0.59	70.3±2.1 (100)
Isometric 120° (Nm)	398.8±23.5 (32)	0.19	372.8±16.9 (58)	0.5	364.0±10.3 (108)	0.91	374.5±11.8 (96)	0.45	364.3±11.0 (91)
Isometric 140° (Nm)	290.1±18.7 (32)	0.12	274.6±15.2 (58)	0.3	277.5±9.2 (107)	0.22	275.4±10.6 (95)	0.24	255.7±9.7 (91)

Comparisons are for variant allele carriers vs. wild-type allele homozygotes for each *TNF* promoter SNP

LmArm, LmLeg, LmTrunk, FFM, ASM were all adjusted for age, age*age (if significant), height, FM.

ASMI was adjusted for age, age*age (if significant), FM.

Grip was adjusted for age, age*age (if significant), height, FM and lmArm

Isokinetic 30°/s, Isokinetic 180°/s, Isometric 120°, Isometric 140° were all adjusted for age, age*age (if significant), height, FM and LmLeg.

Interaction: statistically significant race by genotype interaction

Table 18. Adjusted means of muscle mass and strength for carriers and non-carriers of *TNF* promoter haplotype in female BLSA subjects.

	CCCGA		TCTGG		TCCAG		CACGG		TCCGG	
	Carrier	Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier
LmArm(Kg)	3.9±0.1	4.0±0	4.0±0.1	3.9±0	4.1±0.1	3.9±0.1	3.9±0.1	4.0±0.1	4.0±0	3.9±0.1
	(29)	(355)	(58)	(326)	(108)	(276)	(95)	(289)	(299)	(85)
	p=0.79		p=0.47		p=0.15		p=0.78		p=0.61	
LmLeg(Kg)	Race*haplotype Interaction		12.6±0.3	12.5±0.1	12.6±0.2	12.5±0.1	12.4±0.2	12.6±0.1	Race*haplotype Interaction	
			(58)	(327)	(109)	(276)	(95)	(290)		
	p=0.003		p=0.96		p=0.9		p=0.3		p=0.001	
LmTrunk(Kg)	19.9±0.7	19.7±0.2	19.4±0.6	19.7±0.2	19.7±0.3	19.8±0.2	19.8±0.3	19.7±0.2	19.7±0.2	19.8±0.4
	(30)	(355)	(58)	(327)	(109)	(276)	(95)	(290)	(299)	(86)
	p=0.75		p=0.58		p=0.83		p=0.84		p=0.94	
FFM(Kg)	40.3±0.7	40.2±0.2	40.2±0.6	40.2±0.2	40.5±0.4	40.2±0.2	39.8±0.4	40.4±0.2	40.3±0.2	40.0±0.5
	(38)	(382)	(63)	(357)	(120)	(300)	(105)	(315)	(322)	(98)
	p=0.98		p=0.9		p=0.43		p=0.17		p=0.53	
ASM(Kg)	Race*haplotype Interaction		16.6±0.4	16.5±0.1	16.6±0.2	16.5±0.1	16.3±0.2	16.6±0.1	Race*haplotype Interaction	
			(58)	(326)	(108)	(276)	(95)	(289)		
	p=0.008		p=0.78		p=0.62		p=0.39		p=0.003	
ASMI (Kg/m^2)	Race*haplotype Interaction		6.2±0.2	6.2±0.1	6.2±0.1	6.2±0.1	6.1±0.1	6.2±0.1	Race*haplotype Interaction	
			(58)	(324)	(108)	(274)	(94)	(288)		
	p=0.006		p=0.74		p=0.42		p=0.4		p=0.004	
Grip (Kg)	24.5±1.4	25.8±0.3	26.9±1.0	25.6±0.3	Race*haplotype Interaction		25.1±0.6	25.8±0.4	Race*haplotype Interaction	
	(23)	(306)	(48)	(281)			(90)	(239)		
	p=0.35		p=0.25		p=0.061 & 0.043		p=0.36		p=0.014 & 0.014	
Isokinetic 30°/s (Nm)	105.2±5.6	98.6±1.6	98.4±4.8	98.9±1.6	97.7±2.9	99.7±1.8	100.2±3.0	94.1±1.8	94.5±1.7	99.6±3.9
	(40)	(373)	(62)	(351)	(117)	(296)	(106)	(307)	(310)	(103)
	p=0.25		p=0.92		p=0.56		p=0.09		p=0.24	
Isokinetic 180°/s (Nm)	67.7±4.0	67.7±1.1	Race*haplotype Interaction		64.1±2.0	66.6±1.3	67.1±2.1	65.4±1.2	65.4±1.2	67.4±2.7
	(39)	(369)			(116)	(292)	(104)	(304)	(305)	(103)
	p=0.99		p=0.015 & 0.004		p=0.3		p=0.47		p=0.5	
Isometric 120° (Nm)	394.7±25.3	372.9±6.3	374.8±18.6	372.2±6.5	359.1±11.4	378.9±7.2	370.2±12.0	366.7±7.2	364.7±6.8	371.1±15.3
	(32)	(342)	(58)	(316)	(108)	(266)	(96)	(278)	(280)	(94)
	p=0.4		p=0.9		p=0.14		p=0.8		p=0.7	
Isometric 140° (Nm)	302.4±21.9	274.6±5.5	276.3±16.3	276.6±5.7	273.6±10.0	277.4±6.3	267.5±10.3	271.1±6.1	267.2±5.8	278.9±13.1
	(32)	(339)	(58)	(313)	(107)	(264)	(95)	(276)	(279)	(92)
	p=0.22		p=0.99		p=0.75		p=0.76		p=0.42	

Comparisons are for carriers vs. non-carriers of each *TNF* promoter haplotype; LmArm, LmLeg, LmTrunk, FFM, ASM were all adjusted for age, age*age (if significant), height, FM; ASMI was adjusted for age, age*age (if significant), FM; Grip, Isokinetic 30°/s, Isokinetic 180°/s, Isometric 120°, Isometric 140° were all adjusted for age, age*age (if significant), height, FM, LmArm (for Grip only) and LmLeg; Interaction: statistically significant race by genotype interaction.

Table 19. Race stratification analyses for relevant haplotypes and muscle phenotypes in females-1

		CCCGA (N=11)	p	TCTGG (N=10)	p	TCCAG (N=32)	p	TCCGG (N=38)
Black Female	LmLeg	11.8±0.7	0.0509					13.2±0.3
	ASM	15.8±0.9	0.0947					17.4±0.4
	ASMI	5.9±0.3	0.1026					6.5±0.1
	Grip					25.3±1.1	0.0106	29.0±1.0
	Knee isokinetic 180°/s			49.8±6.1	0.0061			68.8±3.1
		CCCGA (N=34)	p	TCTGG (N=62)	p	TCCAG (N=102)	p	TCCGG (N=84)
White female	LmLeg	13.0±0.4	0.0366					12.0±0.2
	ASM	16.8±0.5	0.0503					15.7±0.3
	ASMI	6.3±0.2	0.0358					5.9±0.1
	Grip					25.2±0.7	0.2622	24.1±0.7
	Knee isokinetic 180°/s			67.3±2.5	0.4967			65.0±2.3

Comparisons are for variant haplotype carriers and wild-type haplotype (TCCGG) homozygotes; LmLeg and ASM were adjusted for age, age*age (if significant), height, FM; ASMI was adjusted for age, age*age and FM; Grip was adjusted for age, age*age (if significant), height, FM and lmArm; Knee Isokinetic 180°/s was adjusted for age, age*age (if significant), height, FM and LmLeg.

Table 20. Race stratification analyses for relevant haplotypes and muscle phenotypes in females-2

Black female	CCCGA Carriers (N=11)	CCCGA Non-carriers (N=116)	p	TCTGG Carriers (N=10)	TCTGG Non-carriers (N=117)	p	TCCAG Carriers (N=32)	TCCAG Non-carriers (N=95)	p	TCCGG Carriers (N=108)	TCCGG Non-carriers (N=19)	p
LmLeg	11.7±0.6	13.0±0.2	0.036				19.7±0.5	18.4±0.3	0.032	13.1±0.2	11.6±0.4	0.001
ASM	15.6±0.8	17.2±0.2	0.053							17.4±0.2	15.6±0.5	0.003
ASMI	5.9±0.3	6.5±0.1	0.050							6.5±0.1	5.9±0.2	0.005
Grip										27.4±0.6	22.5±1.6	0.003
Isokinetic 180°/s				50.1±6.6	68.6±1.9	0.007						

White female	CCCGA Carriers (N=34)	CCCGA Non-carriers (N=318)	p	TCTGG Carriers (N=62)	TCTGG Non-carriers (N=290)	p	TCCAG Carriers (N=102)	TCCAG Non-carriers (N=250)	p	TCCGG Carriers (N=257)	TCCGG Non-carriers (N=95)	P
LmLeg	12.9±0.3	12.1±0.1	0.020				17.9±0.3	18.4±0.3	0.862	12.1±0.1	12.2±0.2	0.677
ASM	16.8±0.5	15.8±0.1	0.045							15.8±0.2	16.0±0.3	0.507
ASMI	6.3±0.2	5.9±0.1	0.032							5.9±0.1	6.0±0.1	0.432
Grip										24.5±0.4	24.1±0.7	0.674
Isokinetic 180°/s				67.2±2.7	63.9±1.3	0.266						

Comparisons are for carriers and non-carriers of each haplotype.

LmLeg, ASM were all adjusted for age, age*age (if significant), height, FM.

ASMI was adjusted for age, age*age (if significant), FM.

Grip was adjusted for age, age*age (if significant), height, FM and lmArm

Knee Isokinetic 180°/s was adjusted for age, age*age (if significant), height, FM and LmLeg.

Table 21. Adjusted means of muscle mass and strength for variant haplotype carriers and wild-type haplotype homozygotes in male BLSA subjects.

	CCCGA Carrier	p	TCTGG Carrier	p	TCCAG Carrier	p	CACGG Carrier	p	TCCGG Homozygote
LmArm (Kg)	6.8±0.2 (33)	0.73	Race*haplotype Interaction	0.014	6.9±0.1 (127)	0.48	6.3±0.2 (93)	0.07	6.7±0.1 (102)
.LmLeg (Kg)	18.9±0.6 (33)	0.5	18.4±0.5 (59)	0.62	18.9±0.3 (127)	0.37	17.9±0.4 (93)	0.18	18.5±0.3 (102)
LmTrunk (Kg)	27.6±0.8 (33)	0.5	27.0±0.8 (59)	0.91	27.2±0.4 (127)	0.66	25.9±0.5 (93)	0.17	27.0±0.4 (102)
FFM (Kg)	57.3±1.1 (36)	0.91	57.5±1.0 (64)	0.9	57.6±0.6 (136)	0.68	56.4±0.7 (104)	0.6	57.4±0.5 (109)
ASM(Kg)	25.5±0.7 (33)	0.89	25.4±0.7 (59)	0.98	25.9±0.3 (127)	0.27	24.2±0.5 (93)	0.11	25.4±0.3 (102)
ASMI	8.2±0.2 (33)	0.79	8.2±0.2 (59)	0.91	8.3±0.1 (127)	0.25	7.9±0.15 (93)	0.29	8.1±0.1 (101)
Grip (Kg)	40.4±1.7 (30)	0.27	Race*haplotype Interaction	0.227 & 0.028	39.2±0.8 (124)	0.45	40.2±1.3 (87)	0.16	38.3±0.8 (92)
Isokinetic 30°/s (Nm)	146.9±10.4 (41)	0.62	139.7±9.2 (61)	0.83	136.8±4.8 (153)	0.61	139.2±6.1 (114)	0.98	141.1±5.1 (115)
Isokinetic 180°/s (Nm)	106.8±6.6 (42)	0.66	99.5±6.0 (61)	0.57	99.8±3.1 (150)	0.53	105.0±3.9 (114)	0.48	103.6±3.2 (114)
Isometric 120° (Nm)	490.7±32.1 (36)	0.68	477.9±31.7 (52)	0.84	466.4±15.9 (132)	0.6	472.7±19.8 (105)	0.89	475.8±16.4 (99)
Isometric 140° (Nm)	320.3±25.0 (36)	0.25	343.8±25.0 (53)	0.63	338.3±12.8 (130)	0.44	338.9±15.8 (104)	0.53	352.7±12.7 (98)

Comparisons are for variant allele carriers vs. wild-type allele homozygotes for each *TNF* promoter SNP

LmArm, LmLeg, LmTrunk, FFM, ASM were all adjusted for age, age*age (if significant), height, FM.

ASMI was adjusted for age, age*age (if significant), FM.

Grip was adjusted for age, age*age (if significant), height, FM and lmArm

Isokinetic 30°/s, Isokinetic 180°/s, Isometric 120°, Isometric 140° were all adjusted for age, age*age (if significant), height, FM and LmLeg.

Interaction: statistically significant race by genotype interaction

Table 22. Adjusted means of muscle mass and strength for carriers and non-carriers of 5 major *TNF* haplotypes in male BLSA subjects.

	CCCGA		TCTGG		TCCAG		CACGG		TCCGG	
	Carrier	Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier	Carrier	Non-carrier
LmArm (Kg)	6.7±0.3	6.7±0.1	Race*haplotype Interaction		6.9±0.1	6.6±0.1	6.3±0.2	6.8±0.1	6.7±0.1	6.5±0.2
	(33)	(348)			(127)	(254)	(93)	(288)	(280)	(101)
	p=0.84		P=0.008		p=0.067		p=0.008		p=0.36	
LmLeg (Kg)	18.5±0.6	18.4±0.2	18.1±0.5	18.4±0.2	Race*haplotype Interaction		17.8±0.4	18.5±0.2	18.4±0.2	18.1±0.4
	(33)	(348)	(59)	(322)			(93)	(288)	(280)	(101)
	p=0.82		p=0.59		p=0.049		p=0.07		p=0.5	
LmTrunk (Kg)	27.3±0.9	26.6±0.2	26.7±0.7	26.7±0.2	27.0±0.4	26.5±0.3	25.7±0.5	26.9±0.3	26.6±0.3	26.4±0.6
	(33)	(348)	(59)	(322)	(127)	(254)	(93)	(288)	(280)	(101)
	p=0.4		p=0.94		p=0.38		p=0.048		p=0.69	
FFM (Kg)	56.7±1.2	56.9±0.3	57.0±0.99	56.8±0.3	57.2±0.6	56.7±0.4	56.4±0.7	57.0±0.4	56.8±0.3	56.8±0.8
	(36)	(375)	(64)	(347)	(136)	(275)	(104)	(307)	(301)	(110)
	p=0.92		p=0.89		p=0.52		p=0.42		p=0.99	
ASM(Kg)	25.2±0.8	25.0±0.2	25.1±0.6	25.1±0.2	25.7±0.4	24.8±0.3	24.1±0.5	25.3±0.2	25.1±0.2	24.7±0.5
	(33)	(348)	(59)	(322)	(127)	(254)	(93)	(288)	(280)	(101)
	p=0.81		p=0.95		p=0.050		p=0.025		p=0.42	
ASMI	8.2±0.2	8.1±0.1	8.1±0.2	8.1±0.1	8.3±0.1	8.0±0.1	7.9±0.2	8.2±0.1	8.1±0.1	8.0±0.2
	(33)	(347)	(59)	(321)	(127)	(253)	(93)	(287)	(279)	(101)
	p=0.8		p=0.99		p=0.09		p=0.082		p=0.45	
Grip (Kg)	38.3±0.8	40.1±1.8	Race*haplotype Interaction		38.1±0.8	38.2±0.6	Race*haplotype Interaction		37.9±0.5	39.8±1.3
	(30)	(321)			(124)	(227)			(255)	(96)
	p=0.34		0.112 & 0.020		p=0.94		0.024 &0.011		p=0.16	
Isokinetic 30°/s (Nm)	144.9±9.2	137.0±2.7	134.5±8.2	133.4±2.8	135.1±4.5	138.9±3.2	139.0±6.0	137.5±2.9	142.2±2.7	139.5±6.7
	(41)	(404)	(61)	(384)	(153)	(292)	(114)	(331)	(327)	(118)
	p=0.42		p=0.9		p=0.49		p=0.82		p=0.71	
Isokinetic 180°/s (Nm)	104.6±6.1	99.8±1.8	97.0±5.5	100.3±1.8	98.0±3.0	100.9±2.1	100.8±1.8	102.9±4.0	96.6±1.9	106.3±4.4
	(42)	(400)	(61)	(381)	(150)	(292)	(114)	(328)	(324)	(118)
	p=0.45		p=0.56		p=0.43		p=0.64		p=0.043	
Isometric 120° (Nm)	491.2±30.4	467.3±9.0	463.9±26.3	461.2±8.9	457.1±14.5	462.1±10.3	458.7±19.6	461.1±9.4	456.7±9.3	475.2±20.8
	(36)	(354)	(52)	(338)	(132)	(258)	(105)	(285)	(287)	(103)
	p=0.45		p=0.92		p=0.78		p=0.91		p=0.42	
Isometric 140° (Nm)	318.0±24.9	341.3±7.4	338.0±22.3	339.4±7.5	337.1±12.2	340.5±8.7	340.2±16.0	340.0±7.9	334.0±7.8	365.0±17.6
	(36)	(351)	(053)	(334)	(130)	(257)	(104)	(283)	(285)	(102)
	p=0.37		p=0.95		p=0.82		p=0.99		p=0.11	

Comparisons are for carriers vs. non-carriers of each *TNF* promoter haplotype; LmArm, LmLeg, LmTrunk, FFM, ASM were all adjusted for age, age*age (if significant), height, FM; ASMI was adjusted for age, age*age (if significant), FM; Grip, Isokinetic 30°/s, Isokinetic 180°/s, Isometric 120°, Isometric 140° were all adjusted for age, age*age (if significant), height, FM, LmArm (for Grip only) and LmLeg; Interaction: statistically significant race by genotype interaction.

Table 23. Race stratification analyses for relevant haplotypes and muscle phenotypes in males.

		TCTGG carrier	TCCGG homozygote	p	TCTGG Carrier	TCTGG Non-carrier	p	CACGG Carrier	CACGG Non-carrier	p	TCCAG Carrier	TCCAG Non-carrier	p
Black male		N=9	N=38		N=9	N=98		N=19	N=88		N=33	N=74	
	LmArm	7.9±0.4	6.9±0.2	0.041	7.8±0.4	6.9±0.1	0.029						
	LmLeg										19.7±0.5	18.4±0.3	0.032
	Grip	34.2±2.7	39.3±1.4	0.088	33.4±2.6	38.7±0.9	0.05	43.4±2.3	37.4±0.9	0.015			
White male		N=64	N=95		N=64	N=343		N=112	N=295		N=141	N=266	
	LmArm	6.3±0.2	6.6±0.1	0.135	6.2±0.2	6.5±0.1	0.09						
	LmLeg										17.9±0.3	17.9±0.2	0.86
	Grip	40.5±1.2	38.3±1.0	0.129	39.6±1.1	37.9±0.5	0.176	37.7±0.9	38.4±0.5	0.459			

LmArm was adjusted for age, age*age (if significant), height and FM

LmLeg was adjusted for age, age*age (if significant), height and FM.

Grip was adjusted for race, age, age*age (if significant), height, FM and lmArm

Sarcopenia

ASMI values of less than 5.45 kg/m^2 for female and less than 7.26 kg/m^2 for male were used to define sarcopenia (12). In subjects 55 years and older (297 women and 328 men), 61 female and 88 male subjects were identified as sarcopenic. T test analyses was performed to compare characteristics of normal and sarcopenic subjects in each sex and the results indicated that in both sexes, sarcopenic subjects were older, lower in weight, BMI, FFM, all regional lean mass measures (arm, leg, trunk), ASM, and ASMI. It was noted that the difference in FM between normal and sarcopenic males did not reach significant level (p=0.10; Table 24).

χ^2 tests were performed to assess the differences in the frequencies of *TNF* genotypes and haplotypes between sarcopenic and non-sarcopenic groups in females and males respectively. The results indicated that *TNF* polymorphisms were not associated with presence of sarcopenia in either female or males (Table 25).

Logistic regression analysis was conducted to further analyze the association between *TNF* promoter polymorphisms and sarcopenia. In this analysis, female and male subjects were combined and sex was included in the model as a fixed factor and subjects of other race were excluded. Interactions between sex and genotype/haplotype (sex*genotype/haplotype) and between race and genotype/haplotype (race*genotype/haplotype) were not significant and were removed sequentially from the model (final model: sarcopenia = genotype/haplotype + sex + race + age + FM). Logistic analyses including potential confounding factors in the model indicated that *TNF* promoter genotypes or haplotypes were not associated with sarcopenic status of the subjects (Table 26).

Table 24. Characteristics of non-sarcopenic and sarcopenic female and male subjects.

Variable	Female (N=297)			Male (N=328)		
	Non-sarcopenic (N=236)	Sarcopenic (N=61)	p	Non-sarcopenic (N=240)	Sarcopenic (N=88)	p
Age (years)	68.98±0.64(236)	74.44±1.46(61)	0.0002	71.92±0.59(240)	78.48±0.96(88)	<.0001
Weight (Kg)	73.03±0.93(236)	59.34±1.24(61)	<.0001	86.02±0.93(240)	76.25±1.24(88)	<.0001
Height (m)	1.62±0(236)	1.61±0.01(61)	0.2034	1.75±0(240)	1.73±0.01(88)	0.2157
BMI (Kg/m^2)	27.73±0.33(236)	22.85±0.43(61)	<.0001	28.11±0.27(240)	25.25±0.3(88)	<.0001
FM (Kg)	30.57±0.72(236)	22.37±0.92(61)	<.0001	25.77±0.66(240)	24.06±0.8(88)	0.1009
LmArm (Kg)	4.09±0.04(236)	3.07±0.05(61)	<.0001	6.75±0.07(240)	5.25±0.09(88)	<.0001
LmLeg (Kg)	12.8±0.11(236)	10.18±0.14(61)	<.0001	18.72±0.16(240)	14.88±0.22(88)	<.0001
LmTrunk (Kg)	20.33±0.16(236)	17.87±0.4(61)	<.0001	27.87±0.23(240)	24.61±0.39(88)	<.0001
FFM (Kg)	40.44±0.28(236)	34.82±0.42(61)	<.0001	25.47±0.2(240)	20.13±0.29(88)	<.0001
ASM (Kg)	16.89±0.14(236)	13.25±0.17(61)	<.0001	8.33±0.05(240)	6.67±0.06(88)	<.0001
ASMI (Kg/m^2)	6.41±0.04(236)	5.1±0.04(61)	<.0001	57.39±0.4(240)	49.03±0.62(88)	<.0001
Grip (Kg)	24.8±0.46(210)	20.07±0.79(56)	<.0001	38.12±0.61(222)	31.47±0.94(79)	<.0001
Isokinetic 30°/s (Nm)	87.9±2.05(197)	70.24±3.03(45)	<.0001	131.18±2.62(204)	104.51±4.39(68)	<.0001
Isokinetic 180°/s (Nm)	62.02±1.37(197)	50.2±2.02(47)	<.0001	94.84±1.97(205)	76.38±3.06(68)	<.0001
Isometric 120° (Nm)	353.47±7.59(193)	277.02±11.07(47)	<.0001	452.82±8.19(199)	370.12±15.64(66)	<.0001
Isometric 140° (Nm)	261.42±6.14(193)	204.33±12.14(47)	<.0001	316.37±6.84(197)	264.76±11.78(65)	0.0002

Values are means± standard error (sample size)

p values are for mean comparison between non-sarcopenic and sarcopenic subjects within sex

Table 25. Genotype and haplotype frequencies for sarcopenic and non-sarcopenic women and men.

			Sarcopenic	Non-sarcopenic	Odds (95% CL)	p
Female	-1031	CC+CT	25(23.81)	80(76.19)	1.22(0.86-1.73)	0.28
		TT	35(18.52)	154(81.48)	0.89(0.70-1.12)	
	-863	AA+AC	20(20.83)	76(79.17)	1.02(0.68-1.53)	0.93
		CC	41(20.4)	160(79.6)	0.99(0.81-1.21)	
	-857	TT+TC	7(12.73)	48(87.27)	0.56(0.27-1.17)	0.10
		CC	54(22.59)	185(77.41)	1.11(1.00-1.25)	
	-308	AA+AG	14(16.28)	72(83.72)	0.75(0.46-1.24)	0.25
		GG	47(22.27)	164(77.73)	1.11(0.94-1.30)	
	-238	AA+AG	7(28)	18(72)	1.50(0.66-3.44)	0.33
		GG	54(19.85)	218(80.15)	0.96(0.87-1.06)	
	CCCGA	Non-carrier	54(19.57)	222(80.43)	0.94(0.86-1.04)	0.13
		Carrier	7(33.33)	14(66.67)	1.93(0.82-4.58)	
	TCTGG	Non-carrier	56(22.22)	196(77.78)	1.11(1.01-1.22)	0.09
		Carrier	5(11.11)	40(88.89)	0.48(0.20-1.17)	
	TCCAG	Non-carrier	47(22.27)	164(77.73)	1.11(0.94-1.30)	0.25
		Carrier	14(16.28)	72(83.72)	0.75(0.46-1.24)	
	CACGG	Non-carrier	44(20.09)	175(79.91)	0.97(0.82-1.16)	0.75
		Carrier	17(21.79)	61(78.21)	1.08(0.68-1.70)	
	TCCGG	Non-carrier	13(20.31)	51(79.69)	0.99(0.57-1.69)	0.96
		Carrier	48(20.6)	185(79.4)	1.00(0.87-1.16)	
Male	-1031	CC+CT	33(28.21)	84(71.79)	1.06(0.77-1.46)	0.71
		TT	55(26.32)	154(73.68)	0.97(0.80-1.16)	
	-863	AA+AC	26(26)	74(74)	0.97(0.67-1.41)	0.88
		CC	60(26.79)	164(73.21)	1.01(0.86-1.19)	
	-857	TT+TC	24(34.78)	45(65.22)	1.45(0.94-2.23)	0.10
		CC	64(24.81)	194(75.19)	0.90(0.78-1.03)	
	-308	AA+AG	28(26.42)	78(73.58)	0.97(0.68-1.39)	0.87
		GG	60(27.27)	160(72.73)	1.01(0.86-1.20)	
	-238	AA+AG	9(27.27)	24(72.73)	1.02(0.49-2.11)	0.95
		GG	79(26.78)	216(73.22)	1.00(0.92-1.08)	
	CCCGA	Non-carrier	79(26.51)	219(73.49)	0.98(0.91-1.07)	0.68
		Carrier	9(30)	21(70)	1.17(0.56-2.45)	
	TCTGG	Non-carrier	69(25)	207(75)	0.91(0.81-1.03)	0.09
		Carrier	19(36.54)	33(63.46)	1.57(0.94-2.61)	
	TCCAG	Non-carrier	60(26.91)	163(73.09)	1.00(0.85-1.19)	0.96
		Carrier	28(26.67)	77(73.33)	0.99(0.69-1.42)	
	CACGG	Non-carrier	65(26.42)	181(73.58)	0.98(0.85-1.13)	0.77
		Carrier	23(28.05)	59(71.95)	1.06(0.70-1.61)	
	TCCGG	Non-carrier	27(30.68)	61(69.32)	1.21(0.82-1.77)	0.34
		Carrier	61(25.42)	179(74.58)	0.93(0.79-1.09)	

p values are for χ^2 tests for differences in genotype/haplotype frequencies between sarcopenic and non-sarcopenic groups within each sex.

Table 26. Sarcopenic status and *TNF* promoter polymorphisms in entire population (Logistic regression analysis)

		Non-sarcopenic	Sarcopenic	Odds Ratio (95%CL)	p
	C(222)	164(34.75)	58(39.19)	0.87 (0.58-1.30)	0.51
-1031	T(398)	308(65.25)	90(60.81)		
	A(196)	150(31.65)	46(31.29)	1.10 (0.72-1.68)	0.66
-863	C(425)	324(68.35)	101(68.71)		
	C(124)	93(19.7)	31(20.81)	1.05 (0.64-1.70)	0.86
-857	T(497)	379(80.3)	118(79.19)		
	A(192)	150(31.65)	42(28.19)	1.17 (0.76-1.81)	0.47
-308	G(431)	324(68.35)	107(71.81)		
	A(58)	42(8.82)	16(10.74)	0.84 (0.44-1.60)	0.59
-238	G(567)	434(91.18)	133(89.26)		
	carriers(51)	35(7.35)	16(10.74)	0.74 (0.38-1.43)	0.36
CCCGA	Non-carriers(574)	441(92.65)	133(89.26)		
	carriers(97)	73(15.34)	24(16.11)	1.01 (0.59-1.72)	0.97
TCTGG	Non-carriers(528)	403(84.66)	125(83.89)		
	carriers(191)	149(31.3)	42(28.19)	1.06 (0.67-1.66)	0.82
TCCAG	Non-carriers(434)	327(68.7)	107(71.81)		
	carriers(160)	120(25.21)	40(26.85)	0.98 (0.63-1.52)	0.92
CACGG	Non-carriers(465)	356(74.79)	109(73.15)		
	carriers(473)	364(76.47)	109(73.15)	1.01 (0.67-1.66)	0.82
TCCGG	Non-carriers(152)	112(23.53)	40(26.85)		

Adjustment was made for age and FM.

DISCUSSION

A large body of data has indicated the catabolic effect of TNF-α in skeletal muscle metabolism and the key role of TNF-α in the pathogenesis of various muscle pathologies including sarcopenia. The promoter polymorphisms in the *TNF* gene have been implicated in the regulation of TNF-α production and been associated with a wide spectrum of inflammatory and infectious diseases, though results from different studies are not consistent. We hypothesized that SNPs in the promoter region of *TNF* gene, which are potentially of biological significance, would be associated with muscle phenotypes in healthy adults. To our knowledge, our investigation is the first study attempting to evaluate the association between genetic polymorphisms of the *TNF* gene and muscle phenotypes in an adult population of various ethnic backgrounds. The results from the present study indicated that *TNF* promoter polymorphisms are associated with muscle phenotypes in a gender and race specific manner.

Genotype and allele frequencies of the five *TNF* SNPs in the entire cohort of the present study are similar to those reported by a previous study (150). The frequencies for the individual race groups are generally within the ranges of the frequencies reported from the public dbSNP database (NCBI). Linkage disequilibrium (LD) among the 5 SNPs was noted in this study. Consistent with previous studies (8; 64; 123; 150), the LD between -1031T/C and -863C/A is very strong, but otherwise limited LD was observed. The race differences in genotype distribution observed in the present study agreed with the finding that *TNF* SNPs occur with distinct patterns in ethnically distinct populations and the relatively low frequency of the -857T allele in African-Americans has been reported in a previous study (8).

For analyses on muscle phenotypes, we included eleven muscle traits including four mass measurements (arm lean mass, leg lean mass, trunk lean mass, FFM), two derived mass parameters (ASM and ASMI), and five strength measurements (hand grip strength, knee extensor isokinetic peak torque at 30°/s, knee extensor isokinetic peak torque at 180°/s, knee extensor isometric peak torque at 120°, knee extensor isometric peak torque at 140°). All the phenotypic variables are correlated (r=17.9%-89%), but their associations with *TNF* promoter polymorphisms are disparate. In the present study, the most consistent findings are that the -1031C/-863A allele is associated with lower muscle mass, especially muscle mass of the arms, in males; the association of -863A with higher muscle strength independent of muscle mass was also suggested in females. In the present study, although both -1031T/C and -863C/A were shown to be associated with muscle mass in males, we postulate that -863C/A is the contributing factor, whereas the association of -1031T/C is due to linkage to -863C/A, because -863C/A also showed associations with muscle phenotypes in females, whereas -1031T/C did not.

The -863A allele has been indicated to be physiologically relevant, enhancing TNF-α expression in humans in some (64; 65; 170) but not all studies (73; 150). Higuchi et al. (64) found that the transcriptional promoter activity of the -1031C/-863A allele in response to concanavalin A (Con A) stimulation was 2.0 fold higher than that of the wild-type allele, and that the levels of TNF-α production by Con A-activated peripheral blood mononuclear cells from the -1031C/-863A carrying subjects was 1.8 fold higher than that from the variant allele non-carriers. Hohjoh et al. (65) suggested that allele-specific binding of nuclear factor OCT-1 with -863A could contribute to the modulation of *TNF* expression. Skoog et al. (150), however, found that -863A was functional but in the opposite direction such that the variant allele was associated

with lower transcriptional activity and reduced circulating levels of TNF-α in comparison with the wild-type allele. Skoog et al. discussed that use of different cell lines, constructs and stimulants may yield contradictory results in similar reporter gene studies (150). TNF-α has been recognized as a potent catabolic factor, able to cause muscle loss by directly enhancing muscle protein breakdown and cell death, and indirectly promoting insulin resistance, reducing dietary energy intake, and inhibiting expression of insulin-like growth factors (36; 39; 97; 125; 134; 143) (fully reviewed in Review of Literature). Given the catabolic nature of TNF-α in skeletal muscle, our study lends support to the hypothesis that -863A is associated with higher transcriptional activity of the *TNF* gene promoter, and people carrying -863A have higher basal production of TNF-α, and thus lower muscle mass. Of course, lack of data on gene expression and protein production prevents us from drawing such a conclusion in the present study. Further studies including those data are needed to unravel the mechanisms underpinning the relationship between muscle mass and *TNF* gene polymorphisms.

Regarding the observed associations between *TNF* genotype and muscle strength independent of muscle mass, we can not provide a clear explanation. One issue is that muscle strength as measured in the present study is a behavioral trait involving not only the musculoskeletal system, but also the nervous system. TNF-α is a pleiotropic cytokine, which has the ability to perform a broad range of effects on different cell types, and is involved in normal functioning and development of the central nervous system (CNS), and pathology in some circumstances (114). Also worth noting is that TNF-α expression and effect in skeletal muscle appears to be fiber type dependent (125; 128) and fiber type composition in turn affects muscle

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performance. Therefore, further studies are needed to clarify the relationship between genetic variation in the *TNF* locus and muscle performance at the tissue level.

In the present study, we did not find any significant association between muscle phenotypes and *TNF* promoter SNPs at positions -857 and -308. Reports regarding the functional relevance of these two SNPs and their associations with various diseases are inconclusive and contradictory (15). The observations from our study provide no support for the significance of these two SNPs for muscle traits. The relationship between -857T/C and muscle mass in males suggested by the raw data was not confirmed by the later stringent analyses which accounted for confounding factors. -238G/A showed significant interactions with race in the relationship with muscle mass in females; however, the low frequency of -238A in our population makes the present study underpowered to detect associations between this SNP and muscle phenotypes in the race and gender stratified subgroups.

The genotype and phenotype associations found in our study have gender and race specificity. For example, variant allele 'A' at position -863 was associated with higher muscle strength independent of muscle mass in females; whereas in males, this allele was associated with lower muscle mass, especially for muscle mass in the arms. Interaction of race with -238G/A was present in females for muscle mass, whereas interaction of race with -1031T/C was present in males for muscle strength.

Regarding the gender specificity of our results, similar findings have been made by other studies. For example, Pedersen et al. (124), found that plasma level of TNF-α was associated with lower ASM (DXA) and body cell mass (total body potassium counting) in elderly men, but not in elderly women. Roth et al. found that genotype of another cytokine-encoding gene,

interleukin-6 (*IL6*), was associated with FFM in men, but not women; muscle strength was not associated with genotype in either men or women (137). There is limited information in the literature explaining this gender specificity. One possible explanation is that estradiol levels are higher in females than in males and have a direct inhibitory action on *TNF* transcription (5). It has also been suggested that with advancing age, the losses of muscle mass and strength may follow different regulatory pathways in men and women (13).

In the present study, significant interactive effects of race with *TNF* genotype and haplotype were extensively observed for muscle mass and strength in both females and males. Comparisons within race stratified subgroups revealed that the relationship between -1031T/C and muscle strength was stronger in blacks than in whites; in fact, the difference in grip strength in white men between genotype groups was not significant. Race stratified analyses on -238G/A, and multiple haplotypes, which showed significant race interactions, also suggested a greater genotypic association in blacks than in whites. Unfortunately, the lower allele frequency of -238A and smaller sample size of blacks in the present study make the interpretation of these findings difficult. The race effect on the *TNF* promoter genotypic association with muscle phenotypes is in line with a previous study (177), in which Visser et al. reported a race effect on the association between cytokine levels and muscle mass and strength. They found that the relationship between levels of TNF-α and IL-6 and muscle phenotypes were stronger in blacks than in whites: for each standard deviation increase in TNF-α level, black men had a greater decrement in thigh muscle area than white men, and black women had a greater decrement in knee extensor strength than white women (177).

Although muscle mass and muscle strength are highly correlated, as documented in the literature and also shown in the present study (e.g., r=0.75 between grip strength and arm lean mass), the genotypic associations of -1031C/863A with muscle mass and muscle strength were not reciprocally supportive. -863C/A was associated with muscle strength in females after adjustment for age, height, fat mass as well as menopause and HRT status, and this association became stronger after controlling for regional muscle mass. We have no explanation for the disparateness between muscle mass and strength in their association with *TNF* promoter SNPs. However, in a previous study (165), Toth et al. observed in heart failure patients that circulating levels of TNF-α were consistently related to skeletal muscle mass, especially leg and appendicular skeletal muscle mass, and no relationship was found for knee isokinetic and isometric knee extensor strength, though they did find a significant relationship for isometric grip strength.

Among all the muscle mass measures and parameters, arm lean mass showed the strongest association with -1031C/-863A. In fact, after adjustment for confounding factors including race, age, body size and total body fat, as well as chronic diseases and smoking status, genotypic associations of -1031C/-863A with arm lean mass in males remained significant, whereas the associations with trunk lean mass and leg lean mass either turned to be statistically marginal or lost significance. This finding may suggest that the upper limb, compared with lower limb and trunk, is more controlled by genetic factors as indicated recently by Prior et al. (130) or more affected by TNF-α.

It has been suggested that combining adjacent SNPs into composite multilocus haplotypes offers a robust and powerful approach to the genetic study of a specific region

because haplotypes are more informative than single SNPs by taking into account the regional linkage disequilibrium information (3). We constructed *TNF* promoter haplotypes from the overall data using PHASE software. In our study, the haplotype profile (composition and frequencies) was similar to a previous study (171). We found that the -1031C and -863A containing haplotype, 'CACGG', was significantly associated with lower muscle mass in male subjects, which is consistent with the findings from single SNP analysis that both -1031C and -863A were associated with lower muscle mass in males. However, no corresponding findings were observed in females for leg muscle strength. The functional relevance of this haplotype has been suggested in a previous study, in which Park et al. (123) found that the CACGG haplotype was associated with an increased risk of Behcet's disease, a chronic multi-system inflammatory disorder of unknown etiology. That no association with muscle mass was observed for another -1031C carrying haplotype 'CCCGA' may suggest again that -863C/A was the primary contributing factor in the relationship of -1031C/-863A with muscle mass. It is of note that in the present study, because of race interactions and low sample sizes for race and gender stratified subgroups, especially for blacks, as well as the possibility that both high-productive and low-productive alleles are present in one haplotype, more complex and ambiguous results could be expected. Further studies with larger sample size and balanced design in terms of racial composition are needed to confirm the findings from the present study.

To investigate the independence of the association between *TNF* promoter SNPs and muscle phenotypes, adjustment was made for age, body height and total body fat in the first model; in a second model, additional controllong was considered for smoking status and chronic diseases, as well as menopause status and HRT conditions in females. Adipocytes produce

inflammatory cytokines and the correlation between body fatness (assessed by BMI or total fat mass) and inflammation cytokine levels has been reported (28). The aging effect on muscle has been well established and both linear and non-linear correlations between muscle mass (and strength) and age have been reported (13). The importance of controlling for skeletal size when studying the factors associated with variation in muscle phenotypes has also been suggested (13). Adjustment for confounding factors altered the relationships between *TNF* promoter polymorphisms and muscle phenotypes, but the findings regarding the associations between -1031C/-863A, as well as haplotype 'CACGG', with muscle phenotypes were consistent, which may suggest the presence of a role for *TNF* gene polymorphisms (likely influencing TNF-α production) in modulating skeletal muscle phenotypic traits.

TNF-α has been implicated as a mediator of sarcopenia, so we attempted to explore the relationship between sarcopenia and *TNF* promoter genotypes in a sub- population of older adults aged 55 years and older. Using Baumgartner's definition for sarcopenia (12), the prevalence of sarcopenia in our cohort is 20.54% and 26.83% for females and males respectively, which is similar to prevalence rates reported by Kenny et al. (76) and Iannuzzi-Sucich et al. (67). Kenny et al. (76), reported a sarcopenia prevalence of 22.6% in older postmenopausal women and Iannuzzi-Sucich et al. (67), reported a prevalence of 22.6% and 26.8% in Caucasian women and men 64 years and older. Our case-control analyses did not show any significant relationship between sarcopenic status and *TNF* genotypes or haplotypes. This result was confirmed by our logistic regression analyses in the entire population. This negative result is, however, not unexpected, since from the muscle mass analyses, we found minimal genotypic effect on ASMI, on which sarcopenia in this study is based.

In summary, the findings of the present study indicate that the putative high-productive alleles of SNPs at positions -1031 and -863 are associated with lower muscle mass in males, but higher muscle strength in females, partially supporting the primary hypothesis that *TNF* promoter SNPs of potential biological significance are associated with muscle phenotypes in humans. In an attempt to quantify the contribution of the *TNF* promoter genotype/haplotype to the variation in the muscle phenotypes, we examined the ANCOVA model developed for arm lean mass of males, in which -1031T/C, or -863C/A, or haplotype 'CACGG' was the fixed factor. In addition to race, age, age*age, FM, and body height, *TNF* promoter genotype/haplotype explained another 1-2% of the variation in arm lean mass (R^2=0.01~0.02). The findings of the present study that *TNF* promoter genotypes/haplotypes contribute to the variations in muscle phenotypes, though minor, may imply that TNF-α has a potential role in regulating body composition in even healthy adults and that *TNF* gene may be an interesting candidate gene locus for further studies on genetic aspect of muscle phenotypes.

To better understand the potential role of *TNF* genetics on muscle physiology, several suggestions can be made for further studies. One is to consider the wider MHC region. Since to date no human *TNF* genetic variation has been consistently and reliably identified to result in substantial alterations in *TNF* expression, and no definitive disease-related *TNF* polymorphism has been characterized (15; 144), the SNPs found to be related to muscle phenotypes in the present study may represent markers of other functional polymorphisms which are important to muscle. Broad mapping of the MHC region has been suggested to be a powerful strategy to identify candidate functional SNPs in *TNF* locus (15). Another suggestion is to incorporate in one study genetic variations in other locus, which are involved in regulating the expression of

TNF gene and activity of TNF-α, for instance, the gene encoding TNF-α receptors (TNFR), which control the availability of bioactive TNF-α levels by binding and neutralizing secreted or membrane-bound TNF-α, and the gene encoding anti-inflammatory cytokine IL-10, which inhibits *TNF* expression, and *LTA*, which located adjacent to *TNF* and produces the cytokine, lymphotoxin alpha, which shares receptors and many functions with TNF-α (114; 144).

CONCLUSIONS

Single nucleotide polymorphisms in the promoter region of the *TNF* gene, which encodes the catabolic cytokine, tumor necrosis factor-alpha (TNF-α), are associated with muscle phenotypes in adult healthy men and women. Putative high TNF-α-producing alleles at positions -1031 and -863 individually or in combination in haplotype '1031C-863A-857C-308G-238G' are associated with lower muscle mass in males, and with higher muscle strength in females by unknown mechanism.

REVIEW OF LITERATURE

Skeletal Muscle, Health and Physical Function

Skeletal muscle is a very important tissue in the body of humans, playing a central role to overall health of individuals at all ages (185): it is responsible for maintaining body posture and making all body movements; it plays an important role in keeping joint stability and maintaining normal body temperature; and skeletal muscle provides a principal reservoir for amino acids to maintain protein synthesis in vital tissues and organs especially when the amino acid absorption from the gastrointestinal system is restricted in certain illness and trauma. Associations between muscle dysfunction and many common chronic diseases, such as obesity, diabetes and osteoporosis have been recognized, which may be explained by the metabolic function of skeletal muscle in energy balance and glucose disposal (185). For example, in a prospective study, Jurca et al. found that muscular strength was inversely associated with incidence of metabolic syndrome (72).

In elderly people, loss of muscle mass and strength has been associated with functional impairment, physical disability (69; 135), risk of falls, and bone fractures (158). Szulc et al. (158) found that in elderly men, decreased relative appendicular skeletal muscle mass (ASM) was associated with narrower bones, thinner cortices, decreased resistance of bones to bending, impaired balance, and increased risk of falls. After hip fracture, loss of muscle strength was related to poorer mobility recovery (175). Prospective studies found that muscle strength is an independent predictor of functional limitations and disability (133), as well as mortality (118).

Environmental Factors Affecting Muscle Mass and Strength

Age, Gender and Race

Age, gender and race have been found to exert independent influences on skeletal muscle mass. For example, Gallagher et al. (51) found that after adjustment for height, body weight and age, men had larger muscle mass then women in both African-American and Caucasian subjects across the entire age range; after adjustment for height, body weight, age and gender, African-American women and men had greater muscle mass than Caucasian women and men; after adjustment for height, body weight, gender and race, age was negatively associated with muscle mass, assessed either by DXA or by total body potassium.

In a study including health ambulatory Caucasians across the adult age span, fat free mass (FFM) and appendicular skeletal muscle mass (ASM), estimated by DXA, and body cell mass, estimated by total body potassium, were all significantly lower in older men and women than young men and women, and an accelerated decline was observed in men and women after 60 y of age (89). Newman et al. (116) reported that across the eighth decade of age, leg lean mass (by DXA) was decreased by about 0.1kg per year of age in both men and women. Age-associated losses of muscle mass have been confirmed by several longitudinal studies (31; 55; 176).

Gender and age differences in muscle strength have also been reported frequently. Lindle et al. (99) reported that peak torque values for men were significantly higher than those of women across all ages, velocities, and type of muscle action. Highgenboten et al. (63) measured knee extensor strength using the Kinetic Communicator (Kin-Com) and found that males' weight-adjusted torque values (averaged across range of motion or peak torque) were

significantly greater than the females'; younger (15 to 24 years) male and female subjects produced significantly greater torques for shortening contractions than older subjects (25 to 34 years). Lynch et al. (104) reported that in general, muscle strength peaks in the 20s and 30s, remains stable or declines slowly into the 40s, and declines ~12 – 14% per decade after 50 yr of age. Newman et al. (116) reported that across the eighth decade of age, leg strength (assessed as knee extension peak torque by Kin-Com dynamometer at angular velocity of 60°/s) dropped at a rate of 2.5 Nm per year for men and 1.8 Nm per year for women. Gender difference in age-related muscle loss has also been documented in the literature. Greater decreases of muscle mass and strength with aging were found in men than in women (13; 89). Lynch et al. (104) found the same trend for muscle quality (strength per kilogram of regional FFM) both in arms and in legs.

Nutrition and Physical Activity

Skeletal muscle is a dynamic tissue with protein synthesis and degradation processes being regulated by intricate signaling pathways that alter their activities in response to stimuli such as nutritional changes, hormones, specific drugs, and mechanical tension or contraction (9). Energy intake has been positively associated with muscle mass in men and women (13). However, the stimulatory effect of carbohydrate on muscle protein balance is minimal compared to the stimulation exerted by amino acids (186). Increasing systemic availability of amino acid, especially essential amino acids, either by infusion or ingestion, stimulates the synthesis of muscle protein and promotes muscle protein synthesis in a does-dependent manner (40; 186). The effect of resistance exercise on muscle protein metabolism and the effect of amino acids are synergistic, so that the net anabolic response to amino acids following exercise is greater than the sum of the amino acids effects and the exercise effects alone (186).

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With progression of age, food intake gradually declines; insufficient calorie and protein intake makes a significant contribution to loss of muscle mass and function (179). It has been suggested that the current recommended dietary allowance (RDA) of 0.8 g protein. kg^{-1}. d^{-1} is inadequate to maintain lean body mass in individuals older than 65 y; such that the amount of protein needed to maintain lean body mass is likely below that needed to optimize physical and metabolic functions of muscle (185). Elderly women who consumed a weight-maintenance but low-protein diet (one half the RDA) in 9 weeks experienced significant losses in lean tissue mass and muscle function, which was in contrast to no change in muscle mass and an improvement in muscle function in women who consumed a high-protein diet (higher than the RDA) (26). Low serum levels of albumin, a marker of poor protein nutrition status, have been associated with an increased risk of muscle loss with aging in older people (176). Essential amino acids (EAAs) and carbohydrate supplementation have also been shown to be able to ameliorate muscle protein loss as a consequence of prolonged muscular inactivity in humans (121).

Physical activity level (job-related and/or leisure-time activities) has been positively correlated with muscle mass (13) in men and women. Muscular inactivity associated with bed rest as a consequence of illness or injury induces losses of muscle mass and strength (121), and decreases muscle protein synthesis (45), whereas rehabilitative resistance exercise training is able to reverse this catabolic process of inactivity (45; 71). In space flight, because of the absence of gravity, use of the lower limbs for postural support and locomotion is diminished; the reduced loading on the lower limbs causes muscle atrophy (95). It has been noticed that those crewmembers who engaged in the most intense exercise exhibited the least loss in leg strength and volume (95).

Inactivity is implicated in the age-associated loss of muscle mass and function (i.e., sarcopenia) (113; 160). Data from the Third National Health and Nutrition Examination Survey (NHANES III) revealed that inactive old people were twice as likely to be afflicted with clinically significant sarcopenia than their peers who maintained leisure time physical activity like walking, jogging, swimming and cycling at least 3 times per week (69).

Diseases

In clinical situations, prolonged inactivity, anorexia, and elevation of catabolic hormones including cortisol, epinephrine and inflammatory cytokines can work in concert to induce a massive loss of lean body mass (119). When an organism is afflicted by an injury, infection or illness, the immune system is activated; the immune system has the capacity to radically change body protein and energy metabolism, and therefore body composition over time (138). Skeletal muscle wasting is prevalent in patients with chronic inflammatory diseases including cancer, rheumatoid arthritis, AIDS, congestive heart failure and chronic obstructive pulmonary disease (140; 143; 165). Toth et al. (165) found in patients with chronic heart failure that circulating levels of inflammatory markers of immune activation including TNF-α, IL-6 and their soluble receptors were consistently associated with muscle mass and strength.

Heritability of Skeletal Muscle Mass and Strength

Skeletal muscle is heritable. Heritability values for various muscle phenotypes have been reported. Thomis et al. (163) studied genetic influences on upper limb maximal torques produced in static, shortening and lengthening contractions, and arm circumferences in young adult male twins, and they found that the genetic determination of maximal isometric torques was as high as

66%~78% at small angles; the contribution of genetic factors to torques produced in lengthening contractions was larger than in shortening contractions; heritability for arm cross-sectional area was over 85%. Heritability for muscle fiber composition estimated in young people for males and females were as high as 99.5% and 92.8% respectively (82), though a more recent study found a more modest heritability of 45% for type I fiber proportion (147). The longitudinal change of muscle strength with age has also been reported and the heritability was estimated to be ~60% (190). The genetic influence on skeletal muscle declines with aging; it is believed that muscle development and function are predominantly determined by genes in the first 30 to 40 years of human life, with environmental factors gradually taking on the dominant role in determining muscle phenotypes with advancing age (46). In a study in postmenopausal women, heritability estimates for lean body mass, leg extensor strength, and grip strength as determined by comparison of twins were 0.52, 0.46 and 0.30, respectively (6). In a recent study, the heritability of muscle phenotypes was studied in a population of African descent, and it was found that heritability of muscle phenotypes differed as a function of age and sex (130). Extensive efforts have been made to seek genes and genetic variations which may contribute to the heritability of muscle phenotypes, however findings in this area are controversial and no conclusions can be draw at present (156).

Sarcopenia

Sarcopenia definition and criteria

Sarcopenia is used to refer to the gradual loss of skeletal muscle mass and strength that occurs with advancing age (12). Sarcopenia is a major geriatric problem, happening even among relatively healthy older people (13).

At present, there are no established criteria for identifying sarcopenia. Various ways have been published in the literature by which researchers defined sarcopenia in their studies. Baumgartner et al. (12) took an approach analogous to that used to define obesity from body mass index to define sarcopenia as index of relative skeletal muscle mass two standard deviations below the sex-specific means of the reference data for young adults. Using data from 229 non-Hispanic white male and female participants of the Rosetta Study (18-40 years of age) as the reference data, Baumgartner et al. calculated cutoff values for sarcopenia for each sex: 7.26 kg/m^2 for males and 5.45 kg/m^2 for females (12). The sarcopenia definition established by Baumgartner et al. (12) has been frequently used in geriatric research (67; 76; 94).

In one study, four definitions of sarcopenia were examined based on four muscle parameters: handgrip, lower extremity muscle power, calf muscle cross-sectional area (CSA), and knee extension torque, and for each parameter the sarcopenia was defined as 2 standard deviations below the means for young healthy men or women (94). In this study, Lauretani et al. (94) found that lower extremity muscle power was most sensitive to age-related change; lower extremity muscle power, knee-extension torque and handgrip had similar good discriminating power to identify persons with poor mobility; calf muscle CSA is a weak and inconsistent predictor of mobility limitations. They suggested that the handgrip should be the measure of

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choice used for the screening of sarcopenia in clinical geriatric practice because it is easy, rapid and relatively inexpensive (94).

Various indicators of muscle mass are available and choosing what indicators to use can affect the estimation of sarcopenia prevalence. FFM (by BIA) was used to estimate prevalence of sarcopenia in the Rancho Bernardo cohort, and the prevalence of 6.2% for men and 5.9% for women were reported for Caucasian aged 55-98 years (27). Using appendicular skeletal muscle mass (ASM) as muscle mass indicator, Baumgartner et al. reported prevalence of 13-24% for Hispanic and non-Hispanic white men and women under 70 and a prevalence of over 50% for persons over 80 years of age(12); Kenny et al. and Iannuzzi-Sucich et al. (67; 76) reported that sarcopenia was present in around 20% of healthy independent Caucasian women 59 years and older.

Using different reference population could also introduce variation in prevalence rate estimated. For example, in elderly Chinese people, the prevalence of sarcopenia was estimated to be low (12.3% and 7.6% in Chinese men and women 70 years and older), which was suggested to be partially due to the selection of the reference population: the normative values built on a sample of Chinese men and women aged 20 to 40 years were lower as compared with values from other studies (93).

Besides the definition proposed by Baumgartner et al. (12), other methods have also been used to define sarcopenia in research, but the independent replication of these methods has been rare.

Janssen et al. (69) used the data from the Third National Health and Nutrition Examination Survey (NHANES III) to establish the prevalence of sarcopenia in older

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Americans. They defined sarcopenia at three levels: muscle mass index (SMI=skeletal muscle mass /body mass × 100) greater than one standard deviation below the sex-specific mean for young adults (aged 18-39) (37.0%) was defined as normal; SMI within one to two standard deviations lower than young adult values (37.0% - 31.5%) was defined as class I sarcopenia; SMI below two standard deviations of young adult values (31.5%) was defined as class II sarcopenia (69). Using this approach, 45% and 59% older ($\geq$ 60) men and women were identified as having class I sarcopenia and 7% and 10% of the older men and women were identified as having class II sarcopenia (69). Using the same data, the same group determined skeletal muscle cutpoints for identifying elevated physical disability risk in older adults (68). They defined skeletal muscle mass index (SMI) as SMI = whole body muscle mass (kg) /height $(m)^2$, SMI $\leq 6.75 kg/m^2$ for women and SMI$\leq 10.75 kg/m^2$ for men were related to moderately increased risk of physical disability and selected as the norm for moderate sarcopenia and SMI$\leq 5.75 kg/m^2$ for women and SMI$\leq 8.75 kg/m^2$ for men were related to high-risk of physical disability and thus established as the norm for severe sarcopenia (68). Using these norms, 9.4% and 11.2% of older women and men were identified to have severe sarcopenia, and 21.9% and 53.1% of the older women and men were identified to have moderate sarcopenia (68). Janssen et al. (68) pointed out that if the ASM by DXA is converted to whole-body muscle using a published algorithm, the cut off values (5.45 for women and 7.26 for men) determined by Baumgartner et al. (12) are similar to the high-risk sarcopenia cutpoints established in their study.

Sarcopenia has also been defined by using gender-specific lowest tertile, quartile, etc. of lean body mass (27), by examining the distributions of residuals obtained from a regression analysis of skeletal muscle measure or the distribution of a certain skeletal muscle measure in the

population under study (28; 117). Newman et al. (117) used the sex-specific lowest 20% of the distribution of the appendicular skeletal muscle index (skeletal muscle mass adjusted for height squared) in the participants of the Health Aging and Body Composition (Health ABC) Study, and found that cutpoints obtained this way were comparable to cutoff values created by Baumgartner et al. (117). However, since overweight or obese individuals were less likely to be classified as sarcopenic using the ASM/ht^2 methods, the residuals method used with regression analyses including height and total fat mass in the model was suggested to be more proper (117).

In summary, although no gold-standard definition of sarcopenia has been established, the definition developed by Baumgartner et al. (12) has been widely accepted and the cutoff values they calculated based on ASM index have been justified by several studies using different methods to be valid in detecting age-associated functional limitations or disabilities.

Mechanisms of Sarcopenia

Sarcopenia is a normal aspect of the aging process. Many age-related factors have been found to contribute to the development and progression of sarcopenia in humans. These include a loss of α-motor neutrons, decreased levels of steroid hormones (both reproductive and the hypothalamic-GH-insulin like growth factor-1 axis), a reduction in calorie and protein intake, a decreased level of physical activity, an increased production of catabolic cytokines and oxidative stress, as well as a decreased tissue sensitivity to anabolic stimuli in skeletal muscle (12; 13; 69; 160).

The progressive degeneration of the nervous system accompanies the aging process, which is reflected by a loss of motor neurons, a reduction in both the numbers and diameters of motor axons, and a decrease in the number of functioning motor units, after age of 60 yr (96). It

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is believed that as age extends beyond 60 yr, human muscle undergoes continuous denervation and reinnervation, with denervation gradually exceeding reinnervation resulting in muscle fibers being permanently denervated and subsequently replaced by fat and fibrous tissue (96).

The declines in appetite and food intake, termed as 'anorexia', are common in elderly people (61). Inadequate intake of calories and protein over a prolonged period of time results in a decrease in the rate of whole body protein turnover and an accelerated loss of muscle mass in elderly people (40). It has been reported that around 30% of old people consume protein at the level of Recommended Dietary Allowance (RDA) or less, while it has been suggested that the RDA, 0.8 grams.kg^{-1}.d^{-1}, is not adequate for the physiological needs of elderly people (40). Older men and women fed on eucaloric diets containing 0.8 g protein.kg^{1}.day^{-1} developed a metabolic accommodation within 14 weeks which was associated with a decrease in mid-thigh muscle area (25).

Anabolic effects of testosterone, insulin-like growth factor (IGF), and growth hormone (GH) on muscle tissue have been well known. Aging is associated with a gradual and progressive decline in serum testosterone (T) at a rate of approximately 1% per year after 30 yr (107). ~20% of men older than 60 and ~50% of men older than 80 years of age have serum total T levels below the normal range for young men; in terms of the biologically active fraction of circulating T, this percentage is even higher due to the age-associated increase in concentration of the T binding protein, sex hormone-binding globulin (SHBG) (107). In women, natural menopause has been associated with reduced energy expenditure and accelerated loss of fat-free mass, as well as increased central adiposity(129). Sex hormone replacement therapy (HRT) with estrogen or

estrogen combined with progesterone has been suggested to be protective against muscle loss in post-menopausal women by some studies (153), but not by others (52; 77; 169).

The GH-IGF-1 axis plays an essential role for the development and maintenance of muscle mass and function with aging (46; 58). Significant inverse correlations of serum levels of IGF1 with age have been observed in men (13). Serum total and free IGF-1 have been found to decrease in old men and women compared to their younger peers and the levels of IGF-1 were significantly associated with fractional synthesis rate of myosin heavy chain proteins (10).

There is cumulative evidence for an increase in catabolic stimuli with aging. Roubenoff et al. (141) reported increased production of both IL-6 and IL-1 receptor antagonist (IL-1ra) by peripheral blood mononuclear cells (PBMC) in elderly subjects. Pedersen et al. (124) found higher circulating levels of TNF-alpha and IL-6 in elderly subjects. The higher plasma levels of inflammatory markers including TNF-α, IL-6, and CRP in the elderly have been associated with lower ASM (28; 124; 177), lower body cell mass (BCM) (124), lower strength (177), and greater risk of sarcopenia (47; 145).

Regardless of the contributing factors, the reduced muscle mass seen in older people is considered to be the result of losses of muscle fibers and a reduction in size of the remaining fibers (58). Muscle fiber loss has been speculated to be due to apoptosis, whereas the myofibrilar protein loss may result from depressed protein synthesis, or increased proteolysis, or both (7). Balagopal et al. (10; 11) found that synthesis rates of mixed muscle protein and myosin heavy chain (MHC) decreased progressively from young to middle-aged and old people.

Loss of muscle strength is mostly accounted for by the loss of muscle mass; in addition to loss of muscle mass, decreased skeletal muscle innervations and capillary density and the

selective atrophy of type II muscle fibers also contribute to loss of muscle strength with aging (12). Verdijk et al. (174) found that the proportion of the type II fibers was significantly lower in the elderly subjects than in the younger subjects; the mean cross-sectional area of the type II fibers was less in the elder than in the young, which was in contrast to no age difference for the type I fibers; interestingly, satellite cell content was also found to be reduced specifically in the type II fibers of the elderly (174).

Increases in apoptosis of myonuclei in aged skeletal muscles has been shown in rodents (126) and humans (183). Both the intrinsic apoptotic pathway (mitochondria mediated apoptosis via the release of cytochrome C) (35) and the extrinsic pathway of apoptosis (activated by binding of a TNF-α to type I TNFR) have been shown to be active in aged muscle, especially in type II muscle fibers (36; 38; 125; 126).

Interventions against Sarcopenia

The major causes of sarcopenia including the age-related decline in testosterone, lack of physical activity, inadequate caloric and protein intake are all potentially reversible, and exercise (particularly progressive resistance exercise) is believed to be the most efficacious way to increase muscle mass and strength (105; 113; 160).

Testosterone (T) replacement therapy has been consistently found to be able to increase lean body mass and decrease fat mass, whereas the effects of T supplementation in older men on muscle strength were more variable (78; 107; 148; 151). Recently it was found that testosterone-induced skeletal muscle hypertrophy in older men is associated with increased satellite cell replication and activation (148). In a controlled study (44), Ferrando et al. found that T administration improved net protein balance in skeletal muscle, which translated into a

significant increase in muscle mass and muscle strength in elderly male subjects who received T administration for 6 months, as opposed to no change in the control group.

Since several unfavorable side effects have been associated with use of T supplementation, it was recommended that when considering T replacement therapy in older men, the potential benefits of T treatment must be weighed against possible risks (107). Risks of T administration in older men include increased blood viscosity and therefore risk of thrombotic complications, induction of obstructive sleep apnea, worsening of peripheral edema, and development of gynecomastia; long-term use of T administration in older men has been associated with the incidence of clinically significant prostate cancer. In addition, T replacement in older men may increase the risk of cardiovascular disease, though favorable effects of T on cardiovascular disease have also been reported (54; 107). Most studies agreed on the idea that T therapy should be limited and only men with decreased testosterone levels and symptoms of hypogonadism are likely to receive overall beneficial effects from T therapy (54).

Elderly people have an increased need for dietary protein probably due to decreased insulin sensitivity in the skeletal muscle or to inefficient utilization of the available amino acids (AAs); the current RDA of 0.8 gram.kg^{-1}.d^{-1} does not appear to be adequate to maintain skeletal muscle function (40). It has been recommended that to preserve skeletal muscle, elderly people should increase their intake of high quality protein or use amino acids supplements. Amino acids, especially essential amino acids, not only are precursors for protein synthesis, but also stimulate protein synthesis directly (50). The anabolic responsiveness to AAs (122) or protein-rich meal (157) is well preserved in the elderly, though a lower efficiency of protein synthesis may be present in the elderly. One possible problem implicated in the protein/nutritional supplement is

that the elderly taking supplements tend to reduce voluntary food intake and therefore the supplements function primarily as a food replacement (48; 75). Studies have been done to explore the energetically efficient protein/nutritional supplement aiming at optimizing the anabolic effect while reducing the caloric load for the elderly. Essential amino acids have been indicated to be more energetically efficient than whey protein (120). Ingestion of 15 g of EAAs or whey protein stimulated muscle protein synthesis in the elderly, but the increase in muscle protein fractional synthesis rate following whey protein ingestion was only half of that induced by EAA ingestion (120). Among the EAAs, leucine has been found to play a unique role in the stimulation of muscle protein synthesis in the elderly (75). EAA at a submaximal dose of 7 g failed to increase muscle protein synthesis in the elderly, however EAA enriched with leucine on the same dose induced accretion of muscle protein synthesis successfully (75).

Although, interventions using testosterone supplementation and diet supplementation with amino acid have been shown to be able to induce some gains in muscle mass, compared to exercise training, these treatments are less effective, costly, and associated with adverse side effects in the case of hormone supplementation (160).

Data from NHANES III (1988-1994) revealed that older people who were engaged in regular resistance exercise (>=1 per week) were fewer than 2% (69), whereas resistance exercise has been well established as a potent stimuli to induce muscle growth.

Hypertrophic effects of resistance exercise training on skeletal muscle have been well established. Numerous studies have consistently shown that high intensity progressive resistance exercise training induces significant increases in muscle mass and strength (11; 17; 19; 115; 188). In frail, community-dwelling elderly men and women aged 78 years and older, 9-month

supervised progressive resistance training with moderate intensity induced significant increments in total and regional FFM, and isokinetic muscle strength (17). In Hispanic older adults with type 2 diabetes, 16 week strength training including upper back, chest press, leg press, knee extension and flexion (3 sets, 8 repetition, 66~75% of baseline 1 RM, 3 times/wk), successively increased whole-body lean body mass (DXA) and muscle quality (strength per unit volume of muscle), and skeletal muscle hypertrophy was observed in both type I and type II muscle fibers (19). In a study by Balagopal et al. (11), 3-months of resistance exercise training increased mRNA levels of MHC and enhanced synthesis rates of mixed muscle protein and of myosin heavy chain. In a recent study (111), skeletal muscle biopsies taken before and after a six-month resistance exercise-training program from young and old adults were analyzed using gene expression profiling, and it was found that most genes that were affected by both age and exercise were markedly reversed back to that of younger levels.

It has been stated that for elderly people, once or twice weekly training on the major muscle groups at a moderate intensity is sufficient for an improvement in muscle function (160).

Tumor necrosis factor-alpha (TNF-α) as a contributing factor to sarcopenia

TNF-α may play a role in age-related muscle loss and functional decline (139; 160). It has been found in humans (124) and animals (125) that circulating levels of TNF-α were increased compared with their younger counterparts. However, age-associated elevation of TNF-α production was not demonstrated in all studies (141). The discrepancy may be explained by variations in sensitivity of the assays used, by differences in age of the populations under study, or by the differences in health status of the subjects (20). In elderly people, higher levels of TNF-α have been associated with lower muscle mass and muscle strength (177); a lower muscle

protein synthesis rate has also been associated with increased levels of sTNFR-II (166); and the muscle hypertrophic response to strength training has been inversely related with baseline levels of sTNFR-I (23). sTNFR has been suggested to act as long-term marker of TNF-α because the receptors are more stable in the circulation than TNF-α itself (20).

Studies using muscle biopsy have shown that in aged people (53) and aged rats (125), TNF-α expression was increased as indicated by both mRNA and protein content. Interestingly, it has been found that the age-associated increase in TNF-α production was inhibited by anti-aging interventions such as strength training in humans (53) and caloric restriction in rats (125), and accompanying this change was the improvement of muscle phenotypes. In aged muscle, signaling pathways initiated by TNF-α leading to muscle loss have also been found to be active. For example, the pro-apoptotic signaling downstream of the TNFR1 has been shown to be active in aged skeletal muscle, especially in type II myofibers (125; 126).

Tumor Necrosis Factor-Alpha (TNF-α)

TNF-α protein

Tumor necrosis factor-alpha (TNF-α) is a member of a large cytokine family, the tumor necrosis factor (TNF) superfamily (178). It is mainly produced by macrophages, but also by other tissues including lymphoid cells, endothelial cells, fibroblasts and neuronal tissue (86; 178). TNF-α is first produced as a 26-kD transmembrane protein (mTNF) arranged in stable homotrimers; after cleavage by the metalloprotease disintegrin, TNF alpha converting enzyme (TACE), the soluble 17- kD sTNF is generated and released from this membrane-integrated form

of TNF-α protein (144). sTNF functions in homotrimeric structure and is unstable at low concentrations and tends to dissociate at concentrations below the nanomolar range (178).

TNF-α plays a key role in the host defense against microbial infection. However, high concentrations of TNF-α induce shock-like symptoms, and the prolonged exposure to low concentrations of TNF-α can result in a wasting syndrome (cachexia) (56). Therefore, biosynthesis and processing of TNF-α are under rigid control in the body (162). The regulation of TNF-α production occurs at several levels including gene transcription, message translation, and protein processing (84). TNF-α gene transcriptional control appears to be cell-type and stimuli-specific with different transcription factors contributing differentially in different contexts (162). *TNF* gene transcription can be induced by several stimuli such as lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA), TNF-α itself, interferon-γ (IFN- γ), as well as transfoming growth factor β (TGFβ) (162). At a post-transcriptional level, the 3' untranslated region (UTR) of the TNF-α mRNA has been found capable of repressing the translation of mRNA in normal conditions and markedly enhancing translation of mRNA upon activation (59). At a protein processing level, TNF-α down-regulates its own bioactivity by altering the number of cell receptors and by increasing the amount of circulating inhibitors including sTNFR, and IL-10 (86).

TNF-α, both mTNF and sTNF, functions through interactions with their membrane receptors, TNF-R1 (TNF receptor type 1, human p55) and TNF-R2 (TNF receptor type 2, human p75) (144). The extracellualr domains of both receptors can be proteolytically cleaved, and the soluble receptor fragments (sTNFR1 and sTNFR2) yielded have the capacity to neutralize TNF-α, thus antagonizing the activity of TNF-α (144). TNFR1 is constitutively expressed in most

tissues, and binds with both mTNF and sTNF; whereas TNFR2 is only found in cells of the immune system, and can only be fully activated by mTNF (178). Accordingly, TNFR1 appears to be the key mediator of TNF-α signaling in the vast majority of cells, whereas TNFR2 seems to play a major role in the lymphoid system (178).

Beside the effect as a key player in innate immunity, TNF-α is involved in regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation (2). Three major signaling pathways have been proposed for TNF-α functioning: TNF-α -induced activation of NF-κB; TNF-α -induced activation of JNK and P38-MAPK; and TNFR1 induced cell death through apoptosis (178). It has been found that the pathway leading to NF-κB activation and that leading to apoptosis antagonize each other and thus it has been speculated that it is a balance between induction of NF-κB and the induction of apoptosis that ultimately determines the cell's fate (86).

TNF-α and Skeletal Muscle

TNF-α has long been recognized as a major mediator of muscle pathologies in inflammatory or infectious diseases such as sepsis, cancer, AIDS, and rheumatoid arthritis (140; 143; 165; 167; 168). Production of TNF-α was consistently found to be elevated in chronic inflammatory diseases such as rheumatoid arthritis (143), chronic heart failure (165), and HIV (140) . In patients with chronic heart failure, circulating concentration of TNF-α was negatively related to muscle mass and muscle strength (165). In HIV patients, production of TNF-α by peripheral blood mononuclear cells predicted loss of lean body mass (140). TNF-α was originally named 'cachectin' in recognition of its catabolic property; it has been demonstrated

that chronic administration of recombinant human TNF-α to rats induces cachexia, the depletion of whole-body protein and lipid stores (168).

The mechanisms underpinning the loss of lean mass or muscle mass induced by TNF-α have been extensively explored. Higher levels of TNF-α has been associated with anorexia, hypermetabolism and insulin resistance (143; 167), and all of these could contribute to loss of lean body mass. Local production of TNF-α and paracrine/autocrine activity of TNF-α in skeletal muscle have also been demonstrated and suggested to contribute most to inflammation-associated muscle loss (53; 160). Basal expression of TNF-α in human skeletal muscle tissue has been demonstrated using muscle biopsies from healthy male subjects and the expression appears to be fiber type specific with the expression solely found in type II muscle fibers (128). Expression of both TNFR1 and TNFR2 have been observed in C2C12 myotubes in response to LPS administration (43). In rodents, an increase in TNF-α expression was observed after injection of LPS in rat's tibialis anterior muscle (43).

Several mechanistic pathways have been proposed for catabolic action of TNF-α on skeletal muscle (134). TNF-α induces activation of NF-κB, which signals muscle protein degradation via the ubiquitin/proteasome system (134). Treating C2C12 myotubes and rat primary muscle cells with TNF-α at concentrations of 1 to 6 ng/ml induced dose-dependent loss of muscle protein with preferential loss occurring to myosin heavy chain (MHC); accompanying the loss of muscle protein was the augmentation of NF-κB activation and I-κB degradation (98). In transdominant negative C2C12 myotubes which produced undegradable I-κB, TNF-α-induced activation of NF-κB and reduction of MHC and total protein were abolished (97). TNF-α alters expression and production of anabolic hormones such as IGF-1and affects tissue sensitivity to

anabolic hormones such as insulin (134). Direct administration of TNF-α strongly inhibited

IGF-1 gene and protein expression in differentiated C2C12 myotubes, whereas such an effect

was not observed for other cytokines including IL-1β, IL-6 and IFN-γ (43).

TNF-α inhibits myoblast differentiation (134). TNF-α has been implicated as a mediator

of muscle wasting through inhibition of myogenic differentiation via the action of nuclear factor

kappa B (NF-κB) (91; 92). TNF-α induces loss of myonuclei by apoptosis via interaction with

TNFR1 (36). It has also been reported that TNF-α can affect protein transcription efficacy in

skeletal muscle (90).

Although these is little doubt that TNF-α is involved in muscle wasting in many

pathological conditions, more complex effect of this cytokine on skeletal muscle cells have also

been reported. The observations that in response to skeletal muscle damage (29) or strenuous

exercise (103), TNF-α expression in skeletal muscle is upregulated, may suggest a beneficial role

for TNF-α in muscle regeneration, tissue repair and suppression of catabolic processes. Indeed,

both anabolic and catabolic effects of TNF-α have been observed on L6 myotubes in a study by

E. Naggar et al. (39), in which incubation of the L6 myotubes with 1 to 10 U/ml of TNF-α

resulted in a significant increase of total and myofibrillar protein contents, whereas incubation

with TNF-α at doses of 0.001-0.1 U/ml or 100-300 U/ml resulted in a decrease of protein

content. However, contradictory results were generated in another study (4), in which at

concentrations of 1 U/ml or less, TNF-α decreased the total and myofibrillar protein content in

C2C12 myotubes, while at relatively high concentrations of 100 U/ml or more, TNF-α increased

protein content. Inflammation is crucial to new tissue formation upon tissue injury and TNF-α

has been found to play an essential role in tissue regeneration of liver (187), but it may not be

required in muscle regeneration as evidence by the finding that the absence of TNF-α did not change the onset or pattern of muscle regeneration in mice (29). Therefore, TNF-α might have a beneficial role in skeletal muscle in some circumstances as specified by the amount of TNF-α, time of exposure, cell type, culture conditions, as well as the differentiation stage of the cell, there is still much confusion with regard to the specificity of the condition favoring anabolic effect of TNF-α.

TNF gene (official symbol TNF, Aliases: DIF, TNFA, TNFSF2, TNF-alpha)

In the human genome, the *TNF* is located on chromosome 6p21.3; along with two related genes, LTα and LTβ, *TNF* occurs within the major histocompatibility complex (MHC) class III cluster (144). MHC is a highly polymorphic region and encodes numerous genes involved in immunologic responses (144). *TNF* is approximately 750 kb in size, consisting of 4 exons and 3 introns (2; 43). Genetic factors contribute substantially to production of cytokines (56; 182). Healthy individuals exhibit a considerable variation in their levels of mitogen-induced TNF production (56; 182). Heritability for TNF-α production was estimated to be 60% (182); however, the heritability of the in vivo baseline levels of TNF-α in elderly twins was much lower (0.26) (32). It has been suggested that the responsiveness of TNF-α may have a stronger genetic component than the baseline circulating levels of TNF-α; also the contribution of genetic factors could be smaller in the elderly than in the young because of the presence of surplus and variety of inflammatory triggers, such as reactive oxygen species (ROS) and subclinical conditions (32).

Polymorphisms in and around the *TNF* gene have been suggested to contribute to the variations in TNF-α production (56). Six microsatellites and many single nucleotide polymorphisms (SNPs) in the *TNF* locus have been described (56). The promoter of the *TNF*

gene plays a key role in the transcriptional control of TNF-α expression (162). In cells of monocyte/macrophage lineage, binding elements for various transcription factors have been identified across the *TNF* gene promoter; they include NF-κB, Ets, NF-AT, activating protein 1 (AP-1), cAMP response element-binding protein, signal transducers and activators of transcription (STAT1) and LPS-induced TNF-α factor (LITAF) (162). The promoter region of the *TNF* gene is highly polymorphic with many SNPs located, which are thought to influence *TNF* transcription rate and likely to be of direct functional significance in regulating TNF-α production (144). 5 SNPs at the positions -1031 (-1031 T/C, rs1799964), -863 (-863 C/A, rs1800630), -857 (-857 C/T, rs1799724), -308 (-308 G/A, rs1800629), and -238 (-238 G/A, rs361525), relative to transcriptional start site, have been extensively studied and suggested to be of potential biological significance.

The -863C/A polymorphism has been suggested to influence TNF-α expression through selective binding of two NF-κB complexes and chromatin structure remodeling under basal and LPS-stimulated conditions in human macrophages (149). Both p50-p50 homodimer and p50-p65 heterodimer bind to the C-allele whereas the A-allele only binds to the p50-p65 complex and hypersensitivity to DNase I was increased in the promoter harboring the variant A-allele, particular after LPS stimulation (149). The -863 A-allele showed increased promoter activity in transient transfection experiments compared with the C-allele during LPS stimulation (149). *TNF* -1031T/C has been consistently found to be in linkage disequilibrium (LD) with the adjacent promoter SNP -863C/A (64). Peripheral blood mononuclear cells (PBMC) from subjects carrying -1031C/-863A were found to produce more TNF-α under stimulation than PBMCs from the donors with the dominant alleles (64).

The transcription factor OCT1 was found specifically to bind to the *TNF* -857T allele and TNF-α production in whole blood was increased in *TNF* -857C homozygotes (171). It was speculated that OCT1 binding at site -857 affects NF-κB binding at the adjacent site of -863 in the *TNF* promoter region. OCT1 can physically interact with NF-κB and inhibit its transactivating effects, and thus the *TNF* -857C allele, with no binding affinity for OCT1, might act to further augment the NF-κB -mediated inflammatory response (171).

Kaijzel et al. (73) argued against the functional relevance of *TNF* promoter SNPs by showing that there was no difference in the contribution of distinct *TNF* alleles in *TNF* pre-mRNA production upon in vitro and physiological stimulation in both heathy and rheumatoid arthritis patients.

In a reporter gene assay, *TNF* -308A showed a much stronger transcriptional activity than the wild-type allele -308G in a human B cell line (184). The finding that this site was bound with an unknown nuclear protein may indicate that -308 G/A has direct effects on *TNF* gene regulation (184). However, this finding was not confirmed in another study, in which, the two G-to-A transition polymorphisms at positions -308 and -238 in the *TNF* gene promoter were not associated with TNF-α production in a whole blood stimulation assay ex-vivo (182).

Overall, the results from studies on the functional significance of the above *TNF* promoter SNPs, as well as others, are not consistent; the discrepancy may be attributable to the different experimental approaches and different materials used in different studies (56).

Polymorphisms in the promoter region of the *TNF* gene have been implicated in the susceptibility to, or severity or outcomes of various infectious and autoimmune diseases such as multiple sclerosis, chronic hepatitis, asthma, parkinson's disease and rheumatoid arthritis (56),

but again the findings from different studies are not consistent. One source of this variation is the heterogeneity of the diseases studied, such that certain genetic factors may not associate with the absence or presence of a disease but with its severity or course (144). *TNF* promoter polymorphisms have also been associated with various aging phenotypes, such as longevity (101), and age-related diseases including dementia (22), Alzheimer's disease (100), coronary heart disease (173) type-2 diabetes (87) and metabolic syndrome (152), although negative results have also been reported (66; 79; 136; 152; 189). Linkage disequilibrium among the SNPs may explain part of the existing conflicts among different studies (56). Studies of multiple *TNF* SNPs in a large sample have been recommended over studies of an isolated SNP or studies using small samples (56).

Measurements of Skeletal Muscle Mass by DXA and Strength by Kin-Com

Many methods and technologies have been developed to measure regional and total-body skeletal muscle mass. At present, the most accurate in vivo methods are computed tomography (CT) and magnetic resonance imaging (MRI), however they are not practically available in routine clinical practice and body composition research because of high cost and limited access to the instruments (180). What is more, considerable radiation is involved in a CT scan, making it not feasible for special populations like adolescents and pregnant women (180).

DXA, as an alternative in vivo approach for measurement of total-body and regional skeletal muscle, is widely available, relatively inexpensive, and safe in terms of radiation (approximately 1% of a standard chest X-ray) and its effectiveness in measuring bone density has long been recognized (62). In body composition measurement, the DXA approach first separates the whole body into two compartments: soft tissue and bone, and then soft tissue can

be further separated into fat-free soft tissue and fat by use of the "R value", the ratio of X-ray attenuation at the system's two energy levels (62; 180). The R values correlate linearly with fat content in soft tissue and their relationships are established during calibration using phantoms of known fat content (62). To do regional measurement, the DXA operator needs to define the region of interest manually on the skeleton image produced during a whole body scan by using anatomic landmarks and a cursor provided by the DXA analysis software (62; 180). Estimation of whole body skeletal muscle mass is based on the assessment of appendicular skeletal muscle mass. To calculate appendicular skeletal muscle mass, a three-compartment model for appendages is generally used in the DXA software: appendicular mass=appendicular (skeletal muscle +fat + bone masses) (62). This model is built on an assumption that limb skin, connective tissue and bone marrow are negligible, though this assumption was not supported by Wang et al. (62; 180). Wang et al. found that inclusion of non-muscle compartments in regional fat-free soft tissue led to significant overestimation of regional skeletal muscle mass, as measured by CT (180). Skeletal muscle mass derived by DXA was highly correlated with that measured by other methods including anthropometry, CT and whole body neutron activation (62; 62; 80; 180). The reliability and precision of body composition analysis using DXA has been reported. 10 scans were made on 12 normal young adults over a period of 5~ 7 day and CV of DXA measurements on each individual was estimated to be ~1.5% for the total body lean tissue mass and ~2% for the regional lean tissue masses including arms, legs, trunk and head (110). Lindle et al. (99) examined reliability of DXA assessment of body composition in older adults, and found that the difference between the two scans (6 wk apart) was less than 1% for both FFM and fat mass. Over 5 days, repeated daily measurements in four subjects showed a coefficient of variation of 2.4 ±

0.5% for leg skeletal muscle mass, $7.0 \pm 2.4\%$ for arm skeletal muscle, and $3.0 \pm 1.5\%$ for appendicular skeletal muscle mass (51). However, DXA is reported to be less sensitive to detect changes in muscle mass as a result of strength training than 24-hour urinary creatinine, regional CT, hydrostatic weighting, whole-body potassium counting, and total body water (115). There is also report that DXA is not a sensitive method to capture age-associated skeletal muscle loss and is not an accurate method to assess skeletal muscle mass of old people because of age-associated increase in extracellular water and connective tissue (33; 131).

The Kinetic Communicator Exercise system (Kin-Com, Chattecx Corp., Chattanooga, TN), is a hydraulically driven, computer-controlled strength testing and exercise device (41). It allows measurement of force generated by muscle during both isometric and isokinetic contractions (41; 109). During a test, the user moves against the resistance provided by the device on its lever arm; the control system of the dynamometer monitors the position and speed of the lever arm and the force exerted by the user through transducers and provides corresponding resistance via the lever arm through feedback loops (41; 109). The reliability and validity of the Kin-Com device has been assessed. Comparisons of known weights, actual velocities and known positions with the Kin-Com-measured loads, user-set velocities and measured positions resulted in coefficients of determination all above 0.90 (41; 109); comparisons of measurements made between days revealed a high reproducibility of Kin-Com measurements with intraclass correlation coefficients approaching 1.00 (109). The reliability of measurements obtained with subjects has also been done. In one study, test-retest reliability was assessed using 10 older men, who accomplished two tests during a 1-wk interval for static, lengthening and shortening contractions at various velocities in the knee flexor and knee

extensor muscle groups, and it was found that intraclass correlation coefficients for all tests ranged between 0.96 and 0.99 and that coefficients of variation ranged between 1.5 and 7.5% (99).

Haplotype Structure and Analysis

A particular combination of alleles along a chromosome is termed a haplotype (1). Haplotypes can be obtained experimentally by genotyping related individuals or by direct sequencing, which is expensive, and can also be inferred from population genotypes using statistical approaches (155). There are three major statistical methods available to infer phase at linked loci from genotypes and thus reconstruct haplotypes: the

parsimony methods crated by Clark, maximum likelihood via the expectation-maximization (EM) algorithm, and the Bayesian approach developed by Stephens et al (PHASE) (155). The comparison of the three statistical methods of haplotype reconstruction with simulated data indicated that PHASE outperforms the other two algorithms with error rates being reduced by over 50%, and with the haplotypes of >80% of the sample being correctly reconstructed (155), though the out performance of PHASE over other algorithms was not consistently found in all studies (191). An updated version of PHASE (v2.0 and above) improved the performance of PHASE further by incorporating information about genetic distances between loci and the decay of linkage disequilibrium (LD) with distance (154). The PHASE v2 was proved to be the most accurate algorithm among the algorithms currently available for haplotype inference and was applied to phase haplotypes across the human genome by the International HapMap project (1).

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Genotyping Using TaqMan Allele Discrimination Assay

The TaqMan Allele Discrimination Assay is also called the 5' nuclease allelic discrimination assay, and it is one of the inexpensive and accurate technologies for high-throughput SNP genotyping (102; 132). Genotyping using this method is rapid because of no post-PCR processing and accurate with the error rate being estimated to be fewer than 1 in 2000 genotypes (132). The working mechanism for this assay has been described in the literature (102; 132). In a typical biallelic SNP assay, two primers (forward and reverse), used to amplify the polymorphic sequence of interest, and two TaqMan probes, used to distinguish between the two alleles, are included in the assay; the two TaqMan probes are each labeled with a fluorescent reporter dye at the 5' end and a quencher dye at the 3' end and they do not fluoresce when the ologonucleotide probe is intact. During a PCR amplification, each probe anneals specifically to its complementary sequence between the forward and reverse primer sites, and then is cleaved by the 5' nuclease activity of Taq polymerase; cleavage of the probe generates an increase in the fluorescence intensity of the reporter dye. Finally, the different fluorescence signals generated by PCR amplification are detected and measured, and a fluorescence plot is generated by specific software and distinct clusters of data points shown in the plot indicate individuals of different genotypes.

In TaqMan allele discrimination, utilization of a Minor groove binder (MGB) in the probe design improves sequence specificity and mismatch discrimination in probe annealing and primer extension, which make TaqMan allele discrimination a robust SNP genotyping methods (88).

Summary

Skeletal muscle is an important tissue in the body of humans, playing a central role to overall health of individuals at all ages (185). Regulation of skeletal muscle has both environmental and genetic components (46; 130; 156). The proinflammatory cytokine, TNF-α, is critical to immune response of the body, but its overproduction is detrimental to skeletal muscle, responsible for muscle wasting in diseases like cancer (140; 143; 165; 167; 168). Recently, TNF-α was implicated in age-associated muscle loss, sarcopenia (23; 24; 53; 124-126) . It has been stated that even transient increase in TNF-α production could lead to muscle frailty over a long period of time (46). TNF-α production is controlled at several levels. Single nucleiotide polymorphisms in the promoter region of *TNF* gene have been implicated in transcriptional control of TNF-α production, and have been associated with alteration in gene transcription activity and TNF-α production in the body, thereby with susceptibility and/or severity of various diseases (15; 56; 57). We conducted the present study to investigate if single nucleiotide polymorphisms in the promoter region of *TNF* gene are associated with muscle mass and strength in adult men and women, with risks of sarcopenina in elderly men and women. The present study will contribute to the growing understanding to the genetics of skeletal muscle and help to elucidate the mechanisms underlying sarcopenia.

APPENDIX A – Limitations of the Study

Our study has several limitations.

First, the BLSA participants are mainly healthy adults and may not be representative of the whole population.

Second, we were unable to include physical activity level as another covariate. Only a small number of subjects finished a physical-activity questionnaire in this cohort. Previous studies (99) on BLSA subjects indicated that only a very small percentage of subjects (<1%) participated in regular resistive exercise and there was no significant difference in participation by age or gender.

Third, we did not have any measure on TNF-α expression or production. This limitation prevents us from clarifying the physiological relationship between the genotypes and the muscle phenotypes. It would be tempting to include measurement on circulating levels of TNF-α in the study. However, TNF-α in circulation is unstable and its concentration is under multifactoral regulation, thus it is hard to use this information to interpret the relationship between genetic polymorphisms and muscle phoentypes. On the contrary, local TNF-α concentration is likely of greater importance and under more control by specific polymorphisms, and thus would provide more information.

Finally, another potential limitation associated with this study is the accuracy of the evaluation of muscle mass. It has been suggested that DXA underestimates the aging-related decrease in skeletal muscle mass (131), because DXA measures lean mass of the body comprising skeletal muscle protein (decrease with age), as well as connective tissue and water,

whose content increase with age. However this systemic error is not likely to affect our

conclusion because there is no significant age difference between any genotype group pairs and

this error can be assumed to be cancelled out in statistical mean comparisons.

APPENDIX B – Definition of Terms

Allele: an alternative form of a gene that is located at a specific locus in a specific chromosome.

Apoptosis: a form of programmed cell death in multicellular organisms.

Genotype: the genetic constitution of an individual or allelic makeup at a specific locus for an invidual.

Haplotype: a combination of alleles at multiple linked loci that are transmitted together, also refers to a set of single nucleotide polymorphisms on a single chromosome that are statistically associated.

Heritability: refers to the proportion of the total variation in a phenotype that can be attributed to genetic effects or the degree to which a given trait is controlled by inheritance.

Heterozygotes: an individual or an organism carrying different alleles of a gene on each of the homologous chromosomes.

Homozygotes: an individual or an organism carrying two identical copies of a gene on the two homologous chromosomes.

Linkage disequilibrium: the nonrandom association between two or more alleles such that certain combinations of alleles are more likely to occur together on a chromosome than other combinations of alleles.

Promoter: a regulatory region of DNA located upstream (towards the 5' end) of the transcription start site of a gene which contains specific DNA sequences that are recognized and bound by transcription factors thereby regulated DNA transcription occurs.

Sarcopenia: a condition characterized by the loss of muscle mass, muscle strength, and muscle quality with aging.

Single Nucleotide polymorphism: A DNA sequence variation involving the substitution of one nucleotide with a single different nucleotide.

APPENDIX C - Human Subjects Approval

UNIVERSITY OF
MARYLAND

INSTITUTIONAL REVIEW BOARD

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December 19, 2007

MEMORANDUM

Application Approval Notification

To:	Dr. Stephen M. Roth
	Dongmei Liu
	Ryan Sheppard
	Andrew Ludlow
	Grigory Gershkovich
	Department of Kinesiology
From:	Roslyn Edson, M.S., CIP
	IRB Manager
	University of Maryland, College Park
Re:	**IRB Application Number: # 02-0177**
	Project Title: "Genetic Influence on Aging Skeletal Muscle"
Approval Date:	**December 19, 2007**
Expiration Date:	**December 22, 2008**
Type of Application:	Renewal
Type of Research:	Nonexempt
Type of Review For Application:	Expedited

The University of Maryland, College Park Institutional Review Board (IRB) approved your IRB application. The research was approved in accordance with 45 CFR 46, the Federal Policy for the Protection of Human Subjects, and the University's IRB policies and procedures. Please reference the above-cited IRB application number in any future communications with our office regarding this research.

Recruitment/Consent: For research requiring written informed consent, the IRB-approved and stamped informed consent document is enclosed. The IRB approval expiration date has been stamped on the informed consent document. Please keep copies of the consent forms used for this research for three years after the completion of the research.

Continuing Review: If you intend to continue to collect data from human subjects or to analyze private, identifiable data collected from human subjects, after the expiration date for this approval (indicated above), you must submit a renewal application to the IRB Office at least 30 days before the approval expiration date.

Modifications: Any changes to the approved protocol must be approved by the IRB before the change is implemented, except when a change is necessary to eliminate apparent immediate hazards to the subjects. If you would like to modify the approved protocol, please submit an addendum request to the IRB Office. The instructions for submitting a request are posted on the IRB web site at: **http://www.umresearch.umd.edu/IRB/irb_Addendum%20Protocol.htm.**

Unanticipated Problems Involving Risks: You must promptly report any unanticipated problems involving risks to subjects or others to the IRB.Manager at 301-405-0678 or **redson@umresearch.umd.edu.**

Student Researchers: Unless otherwise requested, this IRB approval document was sent to the Principal Investigator (PI). The PI should pass on the approval document or a copy to the student researchers. This IRB approval document may be a requirement for student researchers applying for graduation. The IRB may not be able to provide copies of the approval documents if several years have passed since the date of the original approval.

Additional Information: Please contact the IRB Office at 301-405-4212 if you have any IRB-related questions or concerns.

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    Ref Type: Generic

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