ABSTRACT

Title: MYOSTATIN RELATED GENE ASSOCIATIONS WITH MUSCLE MASS AND STRENGTH IN HUMANS

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INTRODUCTION: The gradual decline in muscle mass with age is known as sarcopenia, and has been associated with an increased risk of falls, hip fractures, and functional decline. However, there is large inter-individual variability in this decline, even among people of a similar age and sex. Heritability studies have shown that genetic factors can account for up to 90% of this variation in muscle mass and ~65% in muscle strength. Myostatin is a negative regulator of skeletal muscle and plays a key role in muscle development and the maintenance of muscle mass. However, DNA sequence variation within this gene has not been consistently associated with skeletal muscle mass nor muscle strength in humans. PURPOSE: The purpose of this dissertation was to examine genetic variation in follistatin and Activin RIIB (ACVR2B), two myostatin related genes, to explore associations with skeletal muscle related phenotypes. METHODS: Three hundred fifteen Caucasian males and 278 Caucasian females aged 19-90 years from the Baltimore Longitudinal Study of Aging were genotyped to determine respective haplotype groupings. Whole-body soft tissue composition was measured by dual-energy X-ray absorptiometry. Peak torque (strength) was measured using an isokinetic dynamometer. RESULTS: Women heterozygous for ACVR2B haplotype groups 1 and 2

exhibited significantly less concentric quadriceps muscle strength than women homozygous for haplotype group 2 (108.7 ± 2.2 vs 118.6 ± 4.1 N·m, .52rad/sec, respectively, $p < 0.05$). No significant association was observed in men. However, men homozygous for follistatin haplotype group 1 exhibited significantly greater total leg FFM than men heterozygous for follistatin haplotype groups 1 and 3 (17.8 ± 0.2 vs 16.7 ± 0.4 kg, respectively, $p < 0.05$) and significantly greater total leg FFM than non-carriers of follistatin haplotype group 1 (17.8 ± 0.2 vs 16.5 ± 0.5 kg, respectively, $p < 0.05$). Moreover, male carriers of follistatin haplotype group 3 exhibited significantly less total leg FFM than non-carriers (16.6 ± 0.3 vs 17.5 ± 0.2 kg, respectively, $p < 0.05$). No significant associations between these groups were observed in women. CONCLUSIONS: The data indicate that the ACVR2B and follistatin loci may contribute to the inter-individual variation in skeletal muscle mass and strength.

# MYOSTATIN RELATED GENE ASSOCIATIONS WITH MUSCLE MASS AND STRENGTH IN HUMANS

By

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# LIST OF ABBREVIATIONS

**ACVR2A** - activin-type II receptor A

**ACVR2B** - activin-type II receptor B

**ANCOVA –** analysis of co-variance

**ANOVA –** analysis of variance

**BLSA** - Baltimore Longitudinal Study of Aging

**CNTF** - ciliary neurotrophic factor

**CON** - concentric

**COX** - cytochrome *c* oxidase

**COX$^-$** - complete loss of detectable COX activity

**DEXA** - dual-energy X-ray absorptiometry

**DNA** - deoxyribonucleic acid

**DZ** - dizygotic

**ETS** - electron transport system

**FFM** - fat free mass

**GH** - growth hormone

**HAP** - haplotype

**HGP** - human genome project

**IGF-1** - insulin like growth factor 1

**IL-6** - interleukin-6

**LD** - linkage disequilibrium

**MZ** - monozygotic

**MRI** - magnetic resonance imaging

**mtDNA** - mitochondrial DNA

**NIA** - National Institute of Aging

**POLG** - mitochondrial DNA polymerase g

**PCR** - polymerase chain reaction

**RDA** - Recommended Dietary Allowance

**ROS** - reactive oxygen species

**SD** - standard deviation

**SE** - standard error

**SNPs** - single nucleotide polymorphisms

**TGF-β** - transforming growth factor-β

**TNF-α** – tumor necrosis factor-α

# INTRODUCTION

Skeletal muscle mass gradually declines starting at about age 45 years (61) and it is estimated that after the fifth decade 6% of muscle mass is lost per decade until the eighth decade of life in men (80). This loss of muscle mass that occurs with healthy aging is commonly known as sarcopenia, a Greek term coined by Rosenberg referring to the "poverty of flesh" (106) denoting this tissue loss with age. $18.5 billion dollars was the estimated direct healthcare cost attributable to sarcopenia in 2000 and it is currently estimated that ~45% of the older population ( > 60 yrs of age) is sarcopenic (61) with sarcopenic women having 3.6 times higher rates of disability and men 4.1 times higher rates of disability than non-sarcopenic individuals (8). Furthermore, the loss of muscle strength is an independent predictor of mortality in the elderly (88; 101). The negative economic impact of sarcopenic related disability at the societal level is clear, while the cost of the reduction in quality of life for these individuals can't be easily calculated.

Despite its relatively short history, myostatin has quickly become an established target of study for skeletal muscle researchers interested in identifying mechanisms of muscle development and therapies for muscle-related disorders. First reported in 1997 by McPherron et al. (85), myostatin (growth and differentiation factor-8) was identified in mice as a transforming growth factor-β (TGF-β) family member that acts as a negative regulator of skeletal muscle growth. Soon after the initial report of myostatin's discovery, several groups identified mutations in the myostatin gene in naturally-bred "double-muscled" cattle breeds (41; 86), providing additional evidence for a critical role for myostatin in muscle development, and thus establishing myostatin as the key target it has become for muscle researchers.

Identifying how myostatin influences skeletal muscle and what processes regulate myostatin expression and activity has dominated the myostatin literature in the past few years. Following up their initial discovery, Lee and McPherron (74), established putative myostatin receptors (activin-type II receptors A and B; ACVR2A and B) and negative regulators (the myostatin propeptide and follistatin), and formalized a basic model of myostatin regulation. With the identification of ACVR2B as a primary myostatin receptor and the myostatin propeptide (124; 139) and follistatin as negative regulators of myostatin activity, Lee and McPherron (74) put forth the following model of myostatin regulation in 2001: the myostatin C-terminal dimer remains in a latent complex with the inhibitory propeptide. This latent complex can be further negatively regulated by binding with follistatin, and upon release of the negative regulators, myostatin is free to signal through its receptors, primarily ACVR2B. Lee and McPherron (74) demonstrated that myostatin binding to ACVR2B receptors was specific and saturable, and transgenic mice with increased muscle expression of a dominant negative form of ACVR2B had increased muscle weights, with individual muscles weighing up to 125% more than those of control nontransgenic animals.

Several studies have shown that follistatin can function as a potent myostatin antagonist and plays an important role in vivo. First, follistatin is capable of blocking myostatin activity in both receptor binding and reporter gene assays (74; 142). Secondly, genetic studies in mice have shown that overexpression of follistatin in muscle can cause dramatic increases in muscle growth. Lee and McPherron (74) generated transgenic mice in which the myosin light chain promoter/enhancer was used to drive the expression of follistatin and dramatic effects on skeletal muscle were seen. In one animal muscle

weights were increased by 194-327% relative to control animals; thus it appears, follistatin appears to be a potent myostatin antagonist (74).

Despite myostatin's remarkable influence on skeletal muscle and the well-established effect of myostatin gene mutations in double-muscled cattle breeds, studies of myostatin genetic variation in humans have shown little association with muscle phenotypes. Ferrell and coworkers (33) identified several common polymorphisms in the human myostatin gene, with six nucleotide changes observed, of which five are predicted to lead to amino acid sequence changes, but in follow-up work by that group and others only minor associations have been observed for the most common of these polymorphisms with muscle mass or strength (58; 118). Scheulke and colleagues (116) reported a novel loss-of-function mutation in the myostatin gene of a young child who when born appeared extraordinarily muscular, with protruding muscles in his thighs and upper arms. However, the mutation is not common and is considered rare. The mutation was absent in 200 alleles from control subjects with a similar ethnic background. Recent work has focused on genetic variation in myostatin pathway genes (55; 56), specifically genes involved in the signaling cascade once myostatin signaling has been initiated. However, myostatin pathway genes upstream of this signaling cascade have yet to be examined.

Therefore, the purpose of this study was to examine genetic variation in follistatin and ACVR2B to determine associations with skeletal muscle mass and skeletal muscle strength.

# HYPOTHESES

**Hypothesis 1:** Polymorphic genetic variation in a primary myostatin receptor, ACVR2B, will be associated with significantly different levels of muscle mass and strength.

**Hypothesis 2:** Polymorphic genetic variation in follistatin will be associated with significantly different levels of muscle mass and strength.

# METHODS

## Subjects

The subjects included in this study were Caucasian and came from the Baltimore Longitudinal Study of Aging (BLSA), an ongoing NIA-funded investigation of normal aging. All volunteer subjects enrolled in this study receive a complete physical examination, a bone scan, a joint pain assessment questionnaire, a physical-activity questionnaire, and a functional assessment, among other tests; those with clinical cardiovascular and musculoskeletal disease are excluded, as are subjects with active neck and back pain, frequent and severe joint pain, prior joint surgery, prior bone scan below normal for their age, any recent (6 mo) major surgery, or other condition that might be aggravated by testing. All subjects are asked questions on a physical-activity questionnaire concerning their involvement in weight training exercise. The average number of minutes per week is recorded, analyzed, and compared among the various age groups. Only a very small percentage of subjects (<1%) participate in any type of regular resistive exercise, and there is no significant difference in participation by age or gender (77). Before the study, all subjects receive a complete explanation of the purpose and procedures of the investigation and give their written informed consent.

## Body Composition

Data collected for body composition variables were obtained using methods previously approved by the BLSA (77). Body mass and height were measured to the nearest 0.1 kg and 0.5 cm, respectively, using a Detecto medical beam scale. Total body fat and soft tissue fat free mass (FFM) and total leg fat and FFM (both legs combined)

were assessed by dual-energy X-ray absorptiometry (DEXA) (model DPX-L Lunar Radiation, Madison, WI) using previously described methods (77). Soft tissue FFM was used as a valid indicator of muscle mass based on previous work (39; 136).

**Measurement of Muscle Strength**

Data collected for muscle strength variables were obtained using methods previously approved by the BLSA (77). Peak torque (strength) was measured using the Kinetic Communicator isokinetic dynamometer (Kin-Com model 125E, Chattanooga Group, Chattanooga, TN). Concentric (Con) peak torque was measured at angular velocities of 0.52 rad/s (30 deg/sec) and 3.14 rad/s (180 deg/sec) for the dominant knee extensors. All subjects performed a 5-min warm-up on a stationary cycle ergometer, followed by mild stretching of the hamstring and quadriceps muscle groups. Three graded submaximal practice repetitions were performed prior to each test. For each test, subjects performed three maximal efforts, separated by 30-s rest intervals. Each test is separated by a 2-min rest period. The best of the three maximal efforts was used as peak torque. Peak torque 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 (50). However, Lindle et al. (77) performed a test-retest reliability study using 10 older men to determine reliability of the specific test machine and protocol used by the BLSA. Subjects were tested twice, separated by a 1-wk interval. For concentric torques at both test velocities in the knee extensor muscle groups, intra-class correlation coefficients ranged between 0.96 and 0.99. Coefficients of variation ranged between 1.5 and 7.5%, with a mean coefficient of variation value for all tests of 5%. Detailed

procedures regarding subject positioning and stabilization, gravity correction, and Kin-Com calibration are described elsewhere (77; 80).

**Haplotype Block Determination**

A graphical genome browser maintained by the International HapMap Project website (http://www.hapmap.org) was used to navigate to the particular regions surrounding the candidate genes of interest and retrieve HapMap genotype data for all genotyped markers in the selected regions in a format accepted by *Haploview*, a software program designed to provide a number of tools for haplotype analysis (6). Haploview calculated pairwise measures of linkage disequilibrium (LD) among the polymorphisms in each region and created haplotype blocks based on the definition of haplotype blocks provided by Gabriel and colleagues, one of several commonly used block definitions used to partition the region of interest into segments of strong LD (6). Block structures and specific polymorphisms for each gene are shown in the results section.

**Haplotype Grouping**

One issue inherent in haplotype association studies is haplotype complexity. Although haplotypes may be more informative than single markers, the power of haplotype analysis is reduced by the potentially large number of haplotypes that are present in large haplotype blocks. A statistical approach known as the sliding window has been developed to address this issue (9; 20; 126; 141). The sliding window helps to focus on the candidate region of interest and assess evidence for association within each window, helping to reduce the dimensionality of haplotype analysis (141). Therefore, given the size and complexity of the block containing the follistatin gene, the four single nucleotide polymorphisms (SNPs) closest to the follistatin gene that fell within the

greater haplotype block structure were used as the window for analysis regarding this haplotype in order to create three haplotype groups rather than five groups. The rationale for inclusion of these four SNPs is that all four of these SNPs fall within the follistatin gene, therefore any true causal variants within the follistatin gene would be linked to one of the three haplotype groups analyzed.

**Genotyping**

All subjects participating in the present study consented to and provided DNA for genetic analysis of muscle-related phenotypes. Standard procedures were used to obtain a 10 ml blood sample from consented subjects, and genomic DNA was prepared from the EDTA-anticoagulated whole blood samples by standard salting-out procedures (Puregene DNA Extraction, Gentra Systems Inc.). Genotyping of SNPs was performed using the 5' nuclease allelic discrimination or TaqMan assay (79) for high-throughput genotyping.

Each 12.5 µL polymerase chain reaction (PCR) contained 1.5 µL (10-20 ng) of genomic DNA, 0.625 µL of 20X diluted SNP mix (SNP rs #'s are shown in the results section), 4.125 µL DNAse free $dH_20$, and 6.25 5 µL of 2X TaqMan Universal PCR master mix (Perkin-Elmer, Applied Biosystems Division), which is a solution containing buffer, Uracil-N-glycosylase, deoxyribonucleotides, uridine, passive reference dye (ROX), and TaqGold DNA polymerase. The PCR cycling protocol consisted of the following: 50º for 2 minutes, 95 º for 10 minutes, 70 cycles of 92 º for 15 seconds and 60 º for 1 minute. Fluorescence in each well was measured using an ABI 7300 Real Time PCR System machine (Perkin Elmer, Applied Biosystems Division). Analysis of raw data to determine genotypes was performed by the ABI 7300 Sequence Detection System

software. For quality control purposes, twelve samples were directly sequenced for each assay and used in each 96 well plate as sequencing controls.

**Statistical Analyses**

Statistical analysis relied on analysis of co-variance (ANCOVA) to compare means among haplotype groups for all outcome variables controlling for possible confounding factors by adding covariates such as age, height, and weight when significant. Given the complexity of the follistatin haplotype structure, specific haplotype groups were collapsed to improve statistical power (Appendix C). Analyses were performed within each sex group. Data presented are least squares means ± standard error (SE), except where noted. Analysis of variance (ANOVA) models were used to test for differences in physical characteristics among ACVR2B and follistatin haplotype groups. Physical characteristic data are presented as means ± SE. Statistical significance for the ANCOVA and ANOVA models was accepted at $p < 0.05$. For all non-significant results, the omnibus p-value is shown in the tables; for all significant results, the specific contrast p-values are reported.

All analyses shown were performed in Caucasians only. Although the BLSA cohort is of mixed race (though predominantly Caucasian), differences in allele frequencies for each of the three SNPs in the present study between Caucasians and African Americans prevented combining race groups for statistical analysis. Although underpowered due to small sample size, exploratory analyses performed in African Americans only did not reveal any significant associations (data not shown).

# RESULTS

## ACVR2B Receptor Haplotype

HapMap database research into the haplotype structure of the genome sequence surrounding the myostatin receptor gene, ACVR2B, revealed the following haplotype (Hap) block and frequencies:

**Table 1: ACVR2B Haplotype Structure.**

| | Snp1 | Snp3 | Snp5 | Snp8 | Snp10 | Snp11 | Snp14 | Snp15 | Snp16 | Freq |
|---|---|---|---|---|---|---|---|---|---|---|
| **Hap 1** | A | T | T | T | A | A | A | T | T | 0.615 |
| **Hap 2** | C | C | C | C | G | G | C | C | G | 0.273 |
| **Hap 3** | C | C | C | C | G | A | A | T | G | 0.05 |
| **Hap 4** | A | T | T | T | A | G | A | T | T | 0.034 |
| **Hap 5** | C | C | C | C | A | G | C | C | G | 0.01 |

Genotyping SNP 8 (rs: #2268757), located in intron 1 of the ACVR2B gene, captured the majority of the information of this haplotype block and created two related Haplotype Groups. Hap1 (.615) along with Hap 4 (.034) were considered one group (Haplotype Group 1) due to their high degree of similarity, sharing eight of nine alleles, and Hap 2 (.273), Hap 3 (.05), and Hap 5 (.01) were considered a second haplotype group (Haplotype Group 2), sharing a minimum of five of nine alleles to a maximum of eight of nine alleles.

In men, haplotype group frequencies were 25.1 % for homozygous haplotype group 1, 58.4 % for heterozygous haplotype groups 1 & 2, and 16.5 % for homozygous haplotype group 2, while in women, haplotype group frequencies were 23.7 % for homozygous haplotype group 1, 58.1% for heterozygous haplotype groups 1 & 2, and 18.2 % for homozygous haplotype group 2. Subject characteristics are shown by ACVR2B haplotype group in Table 2. No significant differences existed by ACVR2B

haplotype group for any physical characteristic, however, women heterozygous for haplotype groups 1 and 2 tended to be younger in comparison to the other haplotype groupings.

**Table 2: Subject characteristics by ACVR2B haplotype for Caucasian Men and Women.**

| Haplotype Group | Homozygous Haplotype Group 1 | Heterozygous Haplotype Groups 1 & 2 | Homozygous Haplotype Group 2 | P-value |
|---|---|---|---|---|
| **MEN** | | | | |
| N | 78 | 181 | 51 | |
| Age (y) | 64.6 ± 1.9 | 62.1 ± 1.2 | 59.4 ± 2.4 | 0.228 |
| Height (cm) | 176.6 ± 0.8 | 176.2 ± 0.6 | 176.1 ± 1.0 | 0.92 |
| Weight (kg) | 86.2 ± 1.5 | 83.6 ± 1.0 | 83.9 ± 1.8 | 0.34 |
| **WOMEN** | | | | |
| N | 70 | 172 | 54 | |
| Age (y) | 59.4 ± 1.9 a | 55.0 ± 1.2 b | 59.4 ± 2.1 c | a vs b 0.051<br>b vs c 0.073 |
| Height (cm) | 163.3 ± 0.8 | 163.3 ± 0.5 | 163.4 ± 0.9 | 0.99 |
| Weight (kg) | 68.4 ± 1.5 | 67.5 ± .9 | 65.7 ± 1.7 | 0.48 |

Data are means ± SE.

As shown in Table 3, women heterozygous for haplotype groups 1 and 2 exhibited significantly less concentric quadriceps muscle strength than women homozygous for haplotype group 2 (108.7 ± 2.2 vs 118.6 ± 4.1 N·m, 30º/sec, respectively, p= 0.036). In a combined analysis of carriers of haplotype group 1 versus homozygous haplotype group 2 the same association was observed (109.2 ± 1.9 vs 118.6 ± 4.1 N·m, 30º/sec, respectively, p= 0.039). No significant strength differences among ACVR2B haplotype groups were observed in men.

**Table 3: Concentric knee extensor peak torque values by ACVR2B haplotype in Caucasian Men and Women.**

| Haplotype Group | Homozygous Haplotype Group 1 | Heterozygous Haplotype Groups 1 & 2 | Homozygous Haplotype Group 2 | P-value |
|---|---|---|---|---|
| **MEN** | | | | |
| N | 80 | 184 | 49 | |
| Concentric (N·m, 30º/sec) | 175.7 ± 4.5 | 167.7 ± 3.0 | 172.8 ± 5.8 | 0.307 |
| Concentric (N·m, 180º/sec | 117.6 ± 2.9 | 114.2 ± 2.0 | 118.0 ± 3.7 | 0.495 |
| **WOMEN** | | | | |
| N | 61 | 158 | 48 | |
| Concentric (N·m, 30º/sec) | 110.5 ± 3.6 | 108.7 ± 2.2 a | 118.6 ± 4.1 b | a vs b 0.036 |
| Concentric (N·m, 180º/sec | 72.8 ± 2.4 | 70.4 ± 1.5 | 75.4 ± 2.7 | 0.250 |

Data are least squares means ± SE. Age and height were included in the model as significant covariates.

No significant differences were observed in total body FFM or total leg FFM in either men or women by ACVR2B haplotype group, however there was a tendency for ACVR2B haplotype to be associated with these phenotypes in both men and women. As shown in Table 4, men heterozygous for haplotype groups 1 and 2 tended to have slightly lower total body FFM ($p = 0.066$) and lower total leg FFM ($p = 0.07$) than men homozygous for haplotype group 1, while women homozygous for haplotype group 2 tended to have slightly lower total body FFM than women homozygous for haplotype group 1 ($p = 0.10$).

**Table 4: Soft tissue FFM variables by ACVR2B haplotype in Caucasian Men and Women.**

| Haplotype Group | Homozygous Haplotype Group 1 | Heterozygous Haplotype Groups 1 & 2 | Homozygous Haplotype Group 2 | P-value |
|---|---|---|---|---|
| **MEN** | | | | |
| N | 78 | 181 | 51 | |
| Total Body FFM (kg) | 57.8 ± 0.5 a | 56.5 ± 0.3 b | 57.8.0 ± 0.7 | a vs b 0.066 |
| Total Leg FFM (kg) | 17.7 ± 0.3 a | 16.9 ± 0.2 b | 17.5 ± 0.4 | a vs b 0.07 |
| **WOMEN** | | | | |
| N | 70 | 172 | 54 | |
| Total Body FFM (kg) | 40.1 ± 0.4 a | 39.4 ± 0.2 | 39.1 ± 0.5 b | a vs b 0.10 |
| Total Leg FFM (kg) | 11.3 ± 0.2 | 11.3 ± 0.1 | 11.2 ± 0.3 | 0.891 |

Data are least square means ± SE. Age and height were included in the model as significant covariates.

**Follistatin Haplotype**

HapMap database research into the haplotype structure of the genome sequence surrounding the follistatin gene revealed the following haplotype block and frequencies:

**Table 5: Follistatin Haplotype Structure.**

| | Snp1 | Snp2 | Snp3 | Snp4 | Snp5 | Snp6 | Snp7 | Freq |
|---|---|---|---|---|---|---|---|---|
| **Hap 1** | A | C | C | C | T | A | T | 0.367 |
| **Hap 2** | A | C | C | T | C | A | C | 0.217 |
| **Hap 3** | A | A | T | C | T | A | C | 0.20 |
| **Hap 4** | G | C | C | C | T | G | T | 0.133 |
| **Hap 5** | G | C | C | C | T | A | T | 0.042 |

SNPs 2, 3, 4 & 5 comprise all the SNPs within the follistatin gene within this block and are all intronic. SNP 2 (rs: # 3797297) is located 7,677 base pairs downstream of SNP 1 (rs: # 10080213) while SNP 4 (rs: #12152850) resides 233 base pairs upstream of SNP 5 (rs: # 12153205). By genotyping SNP 2 (rs: # 3797297) and SNP 4 (rs: # 12152850) the majority of the information of this haplotype structure could be gained. Genotyping SNP 2 (rs: # 3797297) separated Hap 3 (Haplotype Group 3) from the rest of the haplotypes and by genotyping SNP 4 (rs: #12152850) Hap 2 (Haplotype Group 2)

was separated from the remaining haplotypes creating a final group of Hap 1, Hap 4, and Hap 5 (Haplotype Group 1) that share a minimum of four of six alleles. In some analyses, different combinations of these three haplotype groups were collapsed to improve statistical power.

In men, haplotype group frequencies were 40.6 % for homozygous haplotype group 1, 1.3 % for homozygous haplotype group 2, 3.8 % for homozygous haplotype group 3, 21.3 % for heterozygous haplotype groups 1 & 2, 26.3 % for heterozygous haplotype groups 1 & 3, and 6.7 % for heterozygous haplotype groups 2 & 3, while in women, haplotype group frequencies were 38.8 % for homozygous haplotype group 1, 3.6 % for homozygous haplotype group 2, 6.1 % for homozygous haplotype group 3, 19.1 % for heterozygous haplotype groups 1 & 2, 23.4 % for heterozygous haplotype groups 1 & 3, and 9.0 % for heterozygous haplotype groups 2 & 3. Subject characteristics are shown by follistatin haplotype group in Table 6. Men homozygous for haplotype group 1 were significantly younger than men heterozygous for haplotype groups 1 & 2 ($p=0.015$) and men heterozygous for haplotype groups 1 & 3 ($p= 0.004$). Women homozygous for haplotype group 1 weighed significantly less than women heterozygous for haplotype groups 1 & 2 ($p=0.005$), while non-carriers of haplotype group 1 also weighed significantly less than women heterozygous for haplotype groups 1 & 2 ($p=0.045$).

**Table 6: Subject characteristics for Follistatin haplotype group 1 homozygous carriers, haplotype group 1 heterozygous carriers, and non-carriers of haplotype group 1 in Caucasian Men and Women.**

| Haplotype Group | Homozygous Haplotype Group 1 | Heterozygous Haplotype Groups 1 & 2 | Heterozygous Haplotype Groups 1 & 3 | Non-Carriers of Haplotype Group 1 | P-value |
|---|---|---|---|---|---|
| **MEN** | | | | | |
| N | 128 | 67 | 83 | 37 | |
| Age (y) | 59.4 ± 1.5 a | 65.4 ± 2.0 b | 66.1 ± 1.8 c | 58.9 ± 2.7 d | a vs b 0.015<br>a vs c 0.004<br>b vs d 0.052<br>c vs d 0.027 |
| Height (cm) | 176.9 ± 0.7 | 175.3 ± 0.9 | 176.1 ± 0.8 | 175.9 ± 1.2 | 0.533 |
| Weight (kg) | 85.4 ± 1.1 | 82.8 ± 1.6 | 83.1 ± 1.4 | 85.6 ± 2.1 | 0.388 |
| **WOMEN** | | | | | |
| N | 108 | 53 | 65 | 52 | |
| Age (y) | 58.8 ± 1.5 | 55.7 ± 2.2 | 55.8 ± 2.0 | 57.3 ± 2.3 | 0.583 |
| Height (cm) | 163.2 ± 0.7 | 164.9 ± 0.9 | 163.1 ± 0.8 | 162.3 ± 0.9 | 0.258 |
| Weight (kg) | 65.4 ± 1.2 a | 71.3 ± 1.7 b | 68.9 ± 1.5 c | 66.4 ± 1.7 d | a vs b 0.005<br>a vs c 0.066<br>b vs d 0.045 |

Data are means ± SE.

As shown in Table 7, men homozygous for haplotype group 1 exhibited significantly more total leg FFM than men heterozygous for haplotype groups 1 and 3 (17.8 ± 0.2 vs 16.7 ± 0.4 kg, respectively, p=0.007) and exhibited significantly more total leg FFM than non-carriers of haplotype group 1 (17.8 ± 0.2 vs 16.5 ± 0.5 kg, respectively, p=0.023). Also, when men homozygous for haplotype group 1 were compared to men heterozygous for haplotype groups 1 and 3 there was tendency for higher levels of total body FFM (57.4 ± 0.4 vs 56.1 ± 0.5 kg, respectively, p=0.061). No significant differences were observed for muscle strength phenotypes in men (Appendix E-Table A). No significant associations were observed for FFM or muscle strength phenotypes in women for follistatin haplotype groups.

**Table 7: Soft tissue FFM variables for Follistatin haplotype group 1 homozygous carriers, haplotype group 1 heterozygous carriers, and non-carriers of haplotype group 1 in Caucasian Men and Women.**

| MEN | | | | | |
|---|---|---|---|---|---|
| Haplotype Groups | Homozygous Haplotype Group 1 | Heterozygous Haplotype Groups 1 & 2 | Heterozygous Haplotype Groups 1 & 3 | Non-Carriers of Haplotype Group 1 | P-value |
| Total Body FFM (kg) | 57.4 ± 0.4 a | 56.8 ± 0.6 | 56.1 ± 0.5 b | 57.9 ± 0.8 | a vs b 0.061 |
| (N) | (128) | (67) | (83) | (37) | |
| Total Leg FFM (kg) | 17.8 ± 0.2 a | 17.1 ± 0.4 | 16.7 ± 0.3 b | 16.5 ± 0.5 c | a vs b 0.007 |
| (N) | (116) | (62) | (72) | (36) | a vs c 0.023 |
| **WOMEN** | | | | | |
| Haplotype Groups | Homozygous Haplotype Group 1 | Heterozygous Haplotype Groups 1 & 2 | Heterozygous Haplotype Groups 1 & 3 | Non-Carriers of Haplotype Group 1 | P-value |
| Total Body FFM (kg) | 39.5 ± 0.4 | 39.7 ± 0.4 | 39.5 ± 0.3 | 39.5 ± 0.4 | 0.903 |
| (N) | (108) | (53) | (65) | (52) | |
| Total Leg FFM (kg) | 11.1 ± 0.2 | 11.4 ± 0.3 | 11.6 ± 0.3 | 11.4 ± 0.3 | 0.606 |
| (N) | (99) | (49) | (61) | (46) | |

Data are least square means ± SE. Age and height were included in the model as significant covariates. Weight was added as an additional significant covariate for the female analysis.

Due to statistical power limitations a sub-analysis was performed by collapsing all carriers of haplotype group 2 into one group and all non-carriers of haplotype group 2 into a second group. No significant differences were observed for any muscle mass or muscle strength phenotypes in the sub-analysis of haplotype group 2, data not shown (Appendix E-Tables C & D). Similarly, a sub-analysis was performed for haplotype group 3 by collapsing all carriers of haplotype group 3 into one group and all non-carriers of haplotype group 3 into a second group. Subject characteristics are shown in Table 8. As shown in Table 9, male carriers of haplotype group 3 exhibited significantly less total FFM than non-carriers of haplotype group 3 (16.6 ± 0.3 vs 17.5 ± 0.2 kg, respectively, p= 0.012) and displayed a tendency to have lower total body FFM as well (56.3 ± 0.5 vs 57.4 ± 0.3 kg, respectively, p= 0.055). No significant differences were observed in either men

or women for muscle strength for the haplotype 3 sub-analysis (Appendix E-Table E).

**Table 8: Subject characteristics for Follistatin haplotype group 3 carriers and non-carriers of haplotype group 3 in Caucasian Men and Women.**

| Haplotype Group | Carriers of Haplotype Group 3 | Non-Carriers of Haplotype Group 3 | P-value |
|---|---|---|---|
| **MEN** | | | |
| N | 111 | 204 | |
| Age (y) | 64.3 ± 1.6 | 61.3 ± 1.1 | 0.123 |
| Height (cm) | 176.3 ± 0.7 | 176.2 ± 0.5 | 0.877 |
| Weight (kg) | 83.8 ± 1.2 | 84.5 ± 0.9 | 0.636 |
| **WOMEN** | | | |
| N | 107 | 171 | |
| Age (y) | 56.2 ± 1.5 | 57.9 ± 1.2 | 0.387 |
| Height (cm) | 162.7 ± 0.7 | 163.7 ± 0.5 | 0.236 |
| Weight (kg) | 67.8 ± 1.2 | 67.3 ± 0.9 | 0.718 |

Data are means ± SE.

**Table 9: Soft tissue FFM variables by carriers and non-carriers of haplotype group 3 in Caucasian Men and Women.**

| Haplotype Group | Carriers of Haplotype Group 3 | Non-carriers of Haplotype Group 3 | P-value |
|---|---|---|---|
| **MEN** | | | |
| Total Body FFM (kg) (N) | 56.3 ± 0.5 (111) | 57.4 ± 0.3 (204) | 0.055 |
| Total Leg FFM (kg) (N) | 16.6 ± 0.3 (93) | 17.5 ± 0.2 (187) | 0.012 |
| **WOMEN** | | | |
| Total Body FFM (kg) (N) | 39.7 ± 0.3 (107) | 39.3 ± 0.3 (171) | 0.458 |
| Total Leg FFM (kg) (N) | 11.5 ± 0.2 (97) | 11.2 ± 0.2 (158) | 0.276 |

Data are least square means ± SE. Age and height were included in the model as significant covariates.

# DISCUSSION

The principal findings of the present study indicate that the ACVR2B and follistatin loci may contribute to the inter-individual variation in muscle mass and strength phenotypes. The present study is one of the first to explore haplotype structure of candidate genes and their associations with skeletal muscle phenotypes. The use of haplotype structure has previously served as a tool for human genetic research with the initial step of first finding an association to a haplotype, and then subsequently identifying the causal mutation(s) that it carries (123). This tool has played a key beginning role in helping identify causal genes for mendelian diseases such as diastrophic dysplasia (48) and cystic fibrosis (64), and recently for complex disorders such as age-related macular degeneration (29; 45; 65; 123). Previous work has shown that there is inter-individual variability in the loss of muscle strength with age (77) indicating a potential role for genetic variability in the risk for sarcopenia (107). Given the complexity of skeletal muscle mass and strength, the present results regarding the haplotype structure of ACVR2B and follistatin will need to be verified in other populations with subsequent studies aimed at identifying causal mutations.

To our knowledge, the present study is the first to explore associations between the haplotype structures of the follistatin and ACVR2B genes with skeletal muscle mass and strength phenotypes. Men heterozygous for follistatin haplotype groups 1 & 3 exhibited significantly less total leg FFM than men homozygous for follistatin haplotype group 1 and there was a tendency for the same observation in regards to total body FFM. Similarly, male carriers of follistatin haplotype group 3 also showed significantly less total leg FFM than non-carriers. It appears that the influence of follistatin haplotype

group 3 has a negative impact on skeletal muscle mass in men. Sowers and colleagues have shown that the loss of 2.5 kg of total lean mass over a 3 year period resulted in lower levels of physical functioning (119). When examining the difference in skeletal muscle mass with follistatin haplotype structure, in specific total leg FFM differences, follistatin haplotype structure was associated with a 1.3 kg difference in FFM. We feel that leg muscle mass could be a stronger indicator of physical functioning than total lean mass. Lower extremity function has been shown to accurately predict disability across diverse populations (44). Work by Rolland et al. (105) has shown that older individuals with lower calf circumferences have greater disability and lower physical function leading the authors to suggest "that topography of muscle loss is more associated than global muscle mass loss with physical function and disability". Although we don't have physical function measures as in the Sowers et al. study, given the importance of muscle mass to physical functioning, a difference of 1.3 kg of total leg FFM is likely physiologically significant for physical function.

Women carriers of ACVR2B haplotype group 1 exhibited significantly less skeletal muscle strength than women homozygous for ACVR2B haplotype group 2 when measured as isokinetic peak torque at 30º /sec. The difference of ~10 N·m between groups is important when considering the age of these subjects. In a longitudinal study by Hughes et al. (53) involving older female subjects (60.4 ± 7.4 yrs) the women experienced a 12% decrease in knee extensor strength (-11 N·m) over an ~ 10 year follow-up period, measured as isokinetic peak torque at 60º/sec. The difference in muscle strength associated with ACVR2B haplotype is similar to losses of skeletal muscle strength that occurred over a decade in women in the study by Hughes et al.

Given the well documented impact that the loss of skeletal muscle strength has on the slowing of gait speed, increased risk in falls and hip fractures (4; 15; 100), and as an independent predictor of mortality in the elderly (88; 101), the difference in skeletal muscle strength observed in this present study may have important implications. These findings, if verified, may allow for the identification of women who are genetically susceptible to low levels of muscle strength.

The present study is not the first to examine genes within the myostatin pathway for an influence on skeletal muscle phenotypes. Despite myostatin's remarkable influence on skeletal muscle in animal models, studies of myostatin genetic variation in humans have shown little association with muscle phenotypes (58; 118). This has led researchers to explore variation in genes involved in the regulation/signaling of myostatin. Two linkage analyses have been performed examining myostatin pathway genes with knee strength in humans (55; 56). Huygens et al. (55) single point linkage analysis provided suggestive evidence that GDF8, CDKN1A and MYOD1 may explain a portion of the inter-individual variance of knee strength. However, Huygens and colleagues (56) multipoint linkage analysis failed to replicate these findings but did observe significant linkage for the CDK2 and RB1 genes as potent quantitative trait loci for muscle strength. While Huygens and colleagues examined genes downstream of myostatin (e.g., after myostatin signaling has been initiated), we chose to focus on genes upstream of this signaling cascade. The two candidate genes chosen for the present study were follistatin and ACVR2B; follistatin has been shown to be a potent antagonist of myostatin (3; 74), capable of binding myostatin and inhibiting its ability to bind to its primary receptor, ACVR2B (74; 142).

The molecular basis for the associations observed for the ACVR2B and follistatin genes with skeletal muscle phenotypes in the present study is uncertain and cannot be addressed by the current study. However, follistatin has been shown to have a strong affinity for myostatin and can completely prevent myostatin receptor activation and downstream phosphorylation of Smad3 (3). Phosphorylation of Smad3, a key step in the myostatin cascade in negatively regulating skeletal muscle, induces binding of Smad3 to MyoD and represses the activity of the MyoD family of transcription factors resulting in inhibition of myoblast differentiation (70; 78). We speculate that perhaps a causal mutation yet to be identified within the follistatin haplotype group 3 leads to a decreased ability of follistatin to bind and inhibit the activity of myostatin, leading to a greater phosphorylation of Smad3, and therefore a reduction in skeletal muscle mass in men.

In regards to ACVR2B haplotype structure and its observed association with muscle strength in women, the absence of myostatin in myostatin null mice has been shown to lead to an overall faster and more glycolytic muscle phenotype in these animals (40). The soleus muscle of myostatin null mice display a larger proportion of fast twitch type II fibers and a reduced proportion of slow type I fibers compared with wild-type animals (40). Although speculative and without fiber type data in support, perhaps ACVR2B haplotype structure influences the ability of myostatin to interact with its receptor and therefore impacts the resulting signaling cascade leading to differences in fiber type distribution. Specifically, perhaps myostatin's interaction with its receptor in women homozygous for ACVR2B haplotype group 2, leads to a greater proportion of fast twitch muscle fibers, helping to explain the greater level of muscle strength observed in these individuals.

There is no obvious explanation for the sex differences observed in the present study (i.e., a relationship between follistatin and muscle mass in men, and a relationship between ACVR2B and muscle strength in women). Previous candidate gene association studies involving skeletal muscle phenotypes by our group (110; 134) and others (18; 129; 130) have also observed sex-specific differences. Although speculative, perhaps the sex differences that have been observed in several studies are partially due to sex-specific gene x hormonal environment interactions.

The present study is not without limitations. Due to statistical power limitations regarding the follistatin haplotype analysis, specific haplotype groupings needed to be collapsed in order to increase statistical power. Specifically, due to the low number of both homozygous follistatin haplotype group 2 individuals and homozygous follistatin haplotype group 3 individuals, sub-analyses were performed for each of these haplotype groupings by creating two groups, carriers of the respective haplotype or non-carriers. Another limitation is that this study examines the relationship between only two genes with skeletal muscle phenotypes and we don't do a combined analysis of the two genes. Skeletal muscle mass and strength are complex phenotypes and most likely are influenced by multiple genes, environmental factors, and gene x environment interactions. To date, variation in the ciliary neurotrophic factor (109), ciliary neurotrophic factor receptor (108), vitamin D (111), interleukin-6 (110), androgen receptor (134), type I collagen (131), alpha-actinin-3 (19), glucorticoid receptor (66; 129), insulin like growth factor-1 (66), and insulin like growth factor-2 (66; 115) genes have all been shown to be associated with skeletal muscle mass and/or strength. The current study adds the ACVR2B and follistatin genes to a growing list of genes that have

been tentatively identified as contributing to inter-individual variation in skeletal muscle phenotypes. The importance of all of these genes will need to be confirmed and the interactions among them will also need to be examined. Moreover, for genes with a verified influence on muscle, potential interactions with therapeutic strategies (e.g., strength training or hormonal therapy) will need to be explored, as the ultimate goal of this research is to allow for optimization of individual prescriptions for maintaining muscle function throughout the age span.

The strong role for myostatin in both muscle development and the maintenance of muscle mass in adults (82) provides a rationale to address whether genetic variation in members of its pathway (e.g., ACVR2B and follistatin) influence muscle phenotypes. Although the heritability of both muscle mass and strength have been well established, the identification of specific genes and allelic variants contributing to these phenotypes is in its infancy (111). Understanding the genetic factors that underlie these phenotypes will require researchers to continue to identify the genes important to muscle phenotypes, identify polymorphic variation within those genes, determine the influence of the observed genetic variation on muscle phenotypic variation, with subsequent studies aimed at assessing the mechanisms and interactions of observed associations and developing clinically-relevant strategies for at-risk individuals with low levels of skeletal muscle mass and strength. Although the present study is not specifically examining sarcopenia, such genetic work may prove important for helping to explain the inter-individual variation in muscle mass and strength. Considering the continued increase in the proportion of older men and women in the U.S. population and the high economic cost associated with sarcopenia (61), results from this project and others may allow for

the early identification of individuals genetically susceptible to low levels of muscle mass and strength, thus allowing the introduction of interventions prior to the onset of associated infirmities.

In conclusion, this is the first study to explore associations between the haplotype structures of the ACVR2B and follistatin genes with skeletal muscle mass and strength phenotypes. These data indicate that the ACVR2B and follistatin loci may contribute to the inter-individual variation in skeletal muscle mass and strength. These results will help generate the direction of hypotheses for future studies and in combination with previous and future studies will contribute to the growing understanding of the role of genetic variation and its influences on skeletal muscle mass and strength.

# CONCLUSIONS

***Hypothesis 1:*** *Polymorphic genetic variation in a primary myostatin receptor, ACVR2B, will be associated with significantly different levels of muscle mass or strength.*

This hypothesis was only partially supported. ACVR2B haplotype structure was significantly associated with skeletal muscle strength in men but not in women. No significant association was found between ACVR2B genetic variation and muscle mass.

***Hypothesis 2:*** *Polymorphic genetic variation in follistatin will be associated with significantly different levels of muscle mass or strength.*

This hypothesis was only partially supported. Follistatin haplotype structure was significantly associated with skeletal muscle mass in men but not women. No significant association was found between follistatin genetic variation and muscle strength.

# REVIEW OF LITERATURE

The following review of literature is divided into three main areas and provides background information on sarcopenia, myostatin including myostatin related genes, and the genetic analysis developed for this dissertation. Across these three areas the review will focus on the following topics: 1) the importance of muscle mass and strength to health and function 2) mechanisms of sarcopenia, 3) prevention and treatment of sarcopenia through strength training, 4) the heritability of skeletal muscle mass/strength, 5) candidate gene studies, 6) myostatin, 7) regulation of myostatin-ACVR2B and follistatin as candidate genes, 8) background of the genetic analysis-haplotype, 9) *Haploview* software program, 10) sliding window analysis, and 11) TaqMan genotyping assays.

## Importance of Muscle Mass and Strength to Health and Function

Skeletal muscle mass gradually declines starting at about age 45 years (61) and it is estimated that after the fifth decade 6% of muscle mass is lost per decade until the eighth decade of life in men (80). This loss of muscle mass that occurs with healthy aging is commonly known as sarcopenia, a Greek term coined by Rosenberg referring to the "poverty of flesh" (106) denoting this tissue loss with age. $18.5 billion dollars was the estimated direct healthcare cost attributable to sarcopenia in 2000 and it is currently estimated that ~45% of the older population ( > 60 yrs of age) is sarcopenic (61) with sarcopenic women having 3.6 times higher rates of disability and men 4.1 times higher rates than non-sarcopenic individuals (8). Furthermore, the loss of muscle strength is an independent predictor of mortality in the elderly (88; 101). The negative economic impact of sarcopenic related disability at the societal level is clear, while the cost of the reduction in quality of life for these individuals can't be easily

calculated. By the year 2030 approximately 30% of the United States population will be elderly and will potentially experience some health problems as well as loss of independence. This loss of independence occurs on many levels, but one undoubtedly important component is loss of mobility, due in part to losses in both muscle mass and strength. Therefore, sarcopenia presents a major public health concern to our aging population.

Many studies have shown a correlation between muscle mass and strength (34; 37). Muscle strength has been reported to reach peak values between 25 and 35 yrs of age, is maintained or is slightly lower between 40-49 yrs of age, and then is ~12-14 % less per decade after 50 yrs of age (71; 77; 87). Even when corrected for the decreased muscle mass that occurs with age, there is significant decline in peak torque, suggesting that the quality of skeletal muscle or efficiency of muscle strength per muscle mass is reduced. Reduction in muscle strength is determined by not only a decrease in muscle mass but also due to changes in the quality of the muscle.

It seems intuitive that loss of muscle quantity, quality, and strength would result in a quantifiable loss of function in task performance. A number of groups have studied and quantified this loss of function. Brown et al. (11) studied 16 elderly (aged 75-88) healthy community volunteers and showed that composite lower extremity strength correlated with gait speed and negatively correlated with the time required to perform five stand-ups. Wolfson et al. (138) compared the strength, balance, and gait speed of 17 nursing home residents that had two or more documented falls in the preceding year (fallers) with 17 matched non-falling controls. Ankle and knee flexion and extension were significantly weaker in fallers than in controls. Bassey et al. (7) measured muscle strength and the amount and speed of customary walking in a large sample of men and

women older than 65 yrs. They found an age-related decline in muscle strength and a significant negative correlation between strength and chosen normal walking speed for both sexes. Fiatarone et al. (34) showed that in frail institutionalized men and women over the age of 86 there is a close correlation between the loss of quadriceps muscle strength, loss of skeletal muscle mass, and the slowing of gait speed. These data suggest that with advancing age muscle strength is a critical component of walking ability.

The New Mexico Study (8) lends further insight into the relationship between sarcopenia and function. Sarcopenic women had 3.6 higher rates of disability, and men 4.1 times higher rates compared with study participants with normal muscle mass. There were significantly greater risks of use cane or walker and a history of falling in the sarcopenic subjects as well. These odd ratios were significant after adjustments for age, race, obesity, income, alcohol intake, physical activity, current smoking, and comorbidity. Thus, the authors concluded that sarcopenia is independently associated with important health outcomes and disabilities in this relatively healthy ambulatory population.

**Mechanisms of Sarcopenia**

Determinants of sarcopenia are likely to be a combination of both intrinsic factors such as hormonal changes, mitochondrial damage due to oxidative stress, denervation, and extrinsic/environmental factors, such as nutrition, physical inactivity. Thus, it follows that the etiology of sarcopenia will turn out to be multifactorial (113). The relative importance of each of these factors is extremely complex and variable and has yet to be completely resolved. Each area is reviewed below.

*Hormones and Aging*

Aging causes loss of many of the anabolic signals to muscle that are present in young adulthood. Recent research suggests that there is also an increase in catabolic signals with age. Loss of muscle with age may be caused by the loss of anabolic factors such as testosterone and growth hormones as well as an increase in catabolic factors such as inflammatory cytokines; or by a combination of the two.

Levels of bioavailable "free" testosterone are known to decrease with age. Cross sectional population based studies have shown varying rates of decline. Van den Beld et al. (128) showed that between the ages of 73-94 there is about a 3% per year decline in free testosterone levels. Cross-sectional and longitudinal data from Harman and colleagues (47) on relatively healthy men in the BLSA demonstrated unequivocally that testosterone concentrations are not only lower in older men, but that they decline progressively with aging, beginning in the third decade of life. This decrease is thought to parallel the decrease in lean muscle mass and the loss of strength that occurs in aging men. Low bioavailable testosterone concentrations have been associated with low fat free mass and decreased strength of knee extension (96; 114) and Katznelson and colleagues demonstrated that low testosterone concentrations were associated with decreased fat free mass in healthy hypogonadal men compared with human adult controls (63).

Circulating levels of growth hormone (GH) and its peripheral mediator, insulin-like growth factor-1 (IGF-1), decrease with age (52) and many studies have demonstrated that GH levels begin to decline in the fourth decade of life and progressively continue to

decline over ensuing years. This loss of GH and IGF-1 is thought to be causally related to the loss of muscle mass and strength that occurs with age (42).

Interleukin-6 (IL-6) is one of several proinflammatory cytokines. Under normal circumstances in young individuals, its expression is tightly regulated by the interplay of several transcription factors and hormonal factors including the secondary sex steroids. IL-6 expression increases late in life, this increase is thought to result from loss of the normally inhibiting sex steroids. Ershler and Keller (30) point out that testosterone, which diminishes with age, is a key factor that downregulates IL-6 gene expression and propose that the age associated increase in IL-6 could help to account for the phenotypic changes that occur in the elderly, including decreased lean body mass. Roubenoff (112) has suggested that IL-6 and other cytokines could function through direct catabolic effects or, more indirectly by inducing anorexia, lowering GH and IGF-1 concentrations, or by triggering loss of muscle cells in the elderly—even in the absence of overt inflammatory disease. Visser and colleagues (132) reported that higher plasma concentrations of IL-6 were associated with lower muscle mass and strength in healthy older individuals.

The aging-associated inflammation affecting skeletal muscle also includes elevated circulating levels of tumor necrosis factor-α (TNF-α) as well as the local expression of TNF-α by skeletal muscle (97). This age-related increase in circulating levels of TNF-α in skeletal muscle may be a contributing stimulus for apoptosis and fiber loss. Chronic exposure of skeletal muscle to TNF-α can cause apoptosis in both myoblasts and myofibers. TNF-α causes existing differentiated muscle fibers to degenerate and at the same time limit the ability for regeneration via proliferation and

fusion of myoblasts. Myoblasts exposed to chronically elevated TNF-α have also been shown to undergo apoptosis (26; 120).

Because sarcopenia develops over many decades, only a small change in the balance of muscle protein catabolism and anabolism is needed to effect a large change in body composition over such a long time span. The combination of the withdrawal of anabolic stimuli, such as testosterone and growth hormone, and the possible increase in catabolic stimuli, such as IL-6 and TNF-α , may thus weave a complex web of signals whose ultimate result is a decline in muscle mass and strength that we now recognize as sarcopenia (113).

*Oxidative Stress and Mitochondrial Damage*

Dr. Denham Harman in 1956 (46) developed the Free Radical Theory of Aging. The basic tenet of this theory is that reactive oxygen species (ROS) underlie the fundamental changes found in aging. Harman hypothesized a correlation between aerobic metabolism, cumulative oxidative damage, and senescence. Due to the high level of oxygen consumption seen in skeletal muscle compared to other tissues along with the attention that has been given to mitochondria because of their role in oxidative phosphorylation (the electron transport system (ETS) consumes ~ 85% of all the oxygen used in the cell) and the fact that the vast majority of cellular ROS, with some estimates as high as 90%, can be traced back to the mitochondria, recent research has begun to investigate the role that ROS may play in mitochondrial damage and sarcopenia.

Mitochondrial DNA (mtDNA) is the only extrachromosomal DNA in mammalian cells. The mtDNA contains 37 genes, encoding 13 proteins (all of which are respiratory chain subunits), 22 tRNAs, and two rRNAs. Mutation in the regulatory regions of

mtDNA could interfere with replication, transcription, or processing of mitochondrial transcripts. Deletion or mutation of mtDNA has been found to be responsible for dysfunction of energy production and an increase in necrosis of skeletal muscle fibers.

It has been proposed that mtDNA, which encodes a small number of critical mitochondrial genes and may be exceptionally sensitive to oxidative damage (14; 21; 103). High levels of oxidative damage and mutation in mtDNA have been ascribed to location of the DNA near the inner mitochondrial membrane where oxidants are formed, lack of protective histones, and lack of DNA repair activity. Damage to mtDNA by mitochondrial oxidants, it has been suggested, may result in faulty mitochondrial proteins, decreased oxidative phosphorylation capacity, increased mitochondrial oxidant generation, and increased mtDNA damage. This hypothetical positive feedback loop is generally referred to as the "vicious cycle"

mtDNA somatic mutation $\longrightarrow$ defective mtDNA-encoded proteins $\longrightarrow$

defective ETS $\longrightarrow$ generation of mutagenic oxidants $\longrightarrow$ mtDNA somatic mutation

This vicious cycle of mutation and oxidant production eventually leads to cellular catastrophe, organ failure, and senescence (81).

McKenzie and colleagues (84) hypothesize that oxidative damage to the mitochondrial genome has the potential to trigger a mitochondrial deletion event. Accumulation of these mtDNA deletion mutations would cause a decline in the energy production of the affected cells, result in abnormal electron transport enzyme phenotypes,

and the fibers would no longer be capable of self maintenance, resulting in intrafiber atrophy, and ultimately, lead to fiber loss.

Work by Aiken and colleagues (1) involving rhesus monkeys, rats, and mice have proposed the following mechanism for muscle fiber loss with age. An early event is a mtDNA replication error that results in the deletion of a large region of the mitochondrial genome. This smaller genome apparently out-replicates the wild-type genome, becoming the predominant species in an expanding region of the muscle fiber. The high abundance of the deletion mutation in a specific region of a muscle fiber results in a focal decline in cytochrome *c* oxidase (COX) activity. A common ETS-abnormal phenotype observed in aging skeletal muscle is the complete loss of detectable COX activity ($COX^-$). The nuclear response to this decline in ETS efficiency appears to result in nuclear upregulation of mitochondrial biogenesis, further exacerbating the problem and producing the $COX^-$ phenotype. This process expands along the length of the muscle fiber until the resulting energy deficit triggers the fiber atrophy and, eventually, fiber breakage. This fiber breakage could produce the fiber loss observed with age. Therefore given the increase in oxidative damage that accumulates with age, it is conceivable mitochondrial DNA deletion events that occur as a result of oxidative damage thereby helps to promote muscle fiber loss with age, thus sarcopenia.

Work by Kujoth et al. (68) have also examined mitochondrial DNA mutations and their role in cell death. The researchers showed that mice expressing a proofreading-deficient version of the mitochondrial DNA polymerase g (POLG) accumulate mtDNA mutations and display features of accelerated aging. The authors cloned the mouse POLG locus, *PolgA*, and used gene targeting in embryonic stem cells to introduce an AC

to CT two-base substitution. This mutation results in a critical residue substitution in the conserved exonuclease domain of POLG, impairing its proofreading ability. Transgenic mice homozygous for this mutation were generated and given the name D257A. Young D257A mice were indistinguishable from wild-type littermates, but long-term follow-up revealed a striking premature aging phenotype beginning at ~9 months of age, consisting of hair loss, graying, and kyphosis. Consistent with a causal role for mtDNA mutations in sarcopenia, the D257A mice displayed age-related loss of skeletal muscle. At 3 months of age, muscle weight in the D257A mice was similar to that of wild-type mice; at 9 months of age, however, the mutant mice showed a significant reduction in the weight of both gastrocnemius (~10% decrease) and quadriceps (~10% decrease) muscles. Therefore, the authors concluded that age-related accumulation of mtDNA mutations is likely to contribute to sarcopenia (68).

*Denervation*

If there is a single most important endogenous cause of sarcopenia, it is probably the loss of alpha motor neuron input to muscle that occurs with age (12). This decline of muscle innervation may be one of the key events in the sarcopenic process since innervation is crucial to the maintenance of muscle mass, as well as strength. In the elderly there is a decrease in the number of functional motor units associated with a concomitant enlargement of the remaining units. This motor unit remodeling seems to be caused by selective denervation of muscle fibers with re-innervation by axonal sprouting from juxtaposed innervated units (10). This process leads not only to a net loss of fibers and functional motor units but also an increase in motor unit size (28). This age related net loss of muscle fibers has been demonstrated to be fiber type specific and has been

shown to involve a greater loss of fast fiber cross sectional area. Lexell et al. (76) in a comprehensive study of the entire vastus lateralis in 43 male cadavers aged 15-83, showed that there is an age related loss in fiber number and based on measurements of cross sectional area, there is a selective loss of fast twitch type II fibers as compared to slow twitch type I fibers. The end result is a decrease in the number of fast twitch motor units and fast twitch muscle fibers and the skeletal muscle appears to compensate for this reduction in motor units by hypertrophy of existing smaller and slower motor units that attempt to reinnervate faster fibers and transform them into slower fiber types (25; 28).

*Cycling of Denervation-Reinnervation*

Investigators reason that the normal cycling of denervation-reinnervation that is evident in younger muscle is impaired in aged muscle. Consequently, there is a net effect of denervation affecting skeletal muscle of the elderly, which significantly contributes not only to the loss of motor units, but also to the loss of muscle fibers that mainly accounts for sarcopenia (76).

The mechanism(s) of the denervation occurring during aging has yet to be fully elucidated, however ciliary neurotrophic factor (CNTF) has been identified as an important molecule in the survival of motor neurons (94). Recent research has shown that relative to young motor neurons, CNTF production is attenuated in aged peripheral nerves (43). In work performed by Guillet and colleagues (43), a strong correlation was established between the decline of CNTF expression and muscle strength measured among aged rats.

*Nutrition-Anorexia*

The concept that there is a physiological decline in food intake from the ages of 20-80 yr is now well established in both large populations and in highly healthy persons (90). It is well recognized that aging is associated with a decline in food intake and this has been termed the "anorexia of aging" and in most cases is an appropriate response to the decrease in physical activity that occurs over the lifespan (91). However physiological anorexia in older men and women may outstrip the reduction of physical activity, leading to weight loss and sarcopenia in the elderly (89). Inadequate intake of calories, particularly sufficient protein, results in a negative nitrogen balance and muscle breakdown and loss.

For muscle to maintain its mass, the rate of protein synthesis must be in balance with the rates of degradation to amino acids in combination with dietary absorption maintaining the difference in amino acid utilization. For sarcopenia to occur, only small imbalances between synthesis and degradation over many years are necessary to eventually result in a significant loss of muscle mass (92). With advancing age combined with inadequate dietary intake of amino acids can decrease the rates of protein synthesis and ultimately exacerbate the onset of sarcopenia.

The current Recommended Dietary Allowance (RDA) for protein to meet the needs of adults over the age of 19 years is 0.8 grams/kg/day. However, recent studies suggest that the protein requirements of older individuals may be higher (~ 1g/kg/day). Campbell et al. (16) examined studies in the literature examining nitrogen-balance in elderly people and recalculated the balance data using currently accepted values for miscellaneous losses. The conclusion of this retrospective analysis and new balance data

presented was that the current RDA is inadequate to meet the dietary protein needs of most elderly people. Campbell (17) then confirmed this conclusion in a study that examined the long term consequences of consumption of the protein RDA. This study enrolled healthy elderly men and women and provided the 0.8 gr/kg/day over a 14 week period in a metabolic ward. Using computerized tomography, the researchers demonstrated that a significant reduction in the cross sectional area of thigh muscle. Thus, the authors concluded that the data suggest that the protein RDA for elderly people is not adequate, even while consuming a weight maintenance diet.

*Physical Inactivity*

The extent to which lifelong activity patterns and training can prevent declines in muscle mass and strength has not been prospectively examined (27). Therefore it is difficult to causally determine the relative importance of physical inactivity in the development of sarcopenia, especially regarding the cross-sectional nature of many studies in this area. However, it has been well demonstrated that elderly persons who are less physically active have less strength and lean mass than do active elderly individuals (69; 99) and it is very well known that short term muscle inactivity severely reduces muscle mass and strength even in young individuals (133). A typical example of this is bed rest (31). However, it has been shown that these muscle changes can be counteracted by exercise, typically resistance exercise (32), which perhaps provides the most convincing evidence of the importance of physical activity and maintenance of skeletal muscle mass and strength.

**Prevention and Treatment of Sarcopenia through Strength Training**

By the year 2030 approximately 30% of the United States population will be elderly and will potentially experience some health problems as well as loss of independence. This loss of independence occurs on many levels, but one undoubtedly important component is loss of mobility, due in part to losses in both muscle mass and strength. Therefore, sarcopenia represents a major public health concern to our aging population. Prevention of muscle loss before it occurs would be one of the most important interventions we could hope to make. Exercise trials have provided some of the most promising data for both prevention and treatment of loss of muscle mass and strength.

Although sarcopenia is a multi-factorial process, decreases in physical activity throughout the lifespan may play a key role. Hunter and colleagues (54) argue the reduction of strength and muscle function that occurs with age are mediated by decreases in physical activity. The result is a positive feedback loop as reduced physical activity further decreases strength, ease of physical activity, and participation in physical activity. These authors state that interrupting this feedback loop is a vital step toward maintaining the quality of life and health of an aging population. A growing body of evidence supports resistance training as an effective means of disrupting this deleterious loop. Latham et al. in 2004 (72) reviewed the literature on progressive resistance training in older adults. Their analysis of 41 randomized controlled trials revealed that progressive resistance training results in improvements in muscle strength and some aspects of functional limitation, such as gait speed, in older adults.

Although some clinicians are reluctant to recommend high-intensity resistance training, many reports have shown that resistance training can be performed safely in an

elderly population. Sullivan et al. (122) performed a 10 week study of lower body resistance training in a group of 19 recuperating nursing home patients whose mean age was 83 years. 1-RM strength increased by 74% and maximum gait speed increased in 53% of subjects without any adverse effects. Similarly, Hauer et al. (49) studied 28 elderly subjects (mean age 81 years) with a history of injurious falls. These subjects performed 12 weeks of lower body resistance training at 70-90% 1-RM and obtained increases of 22-87% in 1-RM strength with no training related medical problems. A review of the literature by Fielding (35) demonstrates that a training stimulus of appropriate intensity (70-90% of 1–RM) produces gains in muscle size and strength in healthy older individuals that are comparable with gains produced in young individuals. Work by Ivey and colleagues (59) further solidified this finding. Eleven young men, 11 young women, 12 older men, and 11 older women had bilateral quadriceps muscle volume measurements performed using magnetic resonance imaging (MRI) before and after strength training. The training consisted of five sets of knee extension exercise designed to include a combination of heavy resistance and high volume exercise. After nine weeks of training strength training induced muscle volume changes in the older subjects were not significantly different than in the young subjects.

Perhaps some of the most impressive evidence in support of resistance training in the elderly as an efficacious intervention comes from work by Fiatarone and colleagues (34) published in JAMA in 1990. Ten frail, institutionalized volunteers aged ~90 years undertook 8 weeks of high-intensity resistance training. Strength gains averaged 174% in the 9 subjects who completed training. Mid-thigh muscle area increased 9.0 % and mean tandem gait speed improved 48% after training. The authors concluded that high-

resistance weight training leads to significant gains in muscle strength, size, and functional mobility among frail residents of nursing homes up to 96 years of age.

Work by Lemmer and colleagues (34; 75) have now provided suggestive evidence for how much of the age-associated losses in strength and muscle mass can be reversed with strength training. Strength losses assessed from isokinetic peak torque values occur at the rate of about 12-14% per decade after the age of about 50 years and strength gains, assessed from 1-RM values, of >30% occur within the first couple of months of heavy resistance strength training in 65-75 year old men and women (75). Thus, about two months of strength training essentially reverses at least 2 decades of strength loss with advancing age. Similar reversals can be observed with muscle mass, which is lost at a rate of about 6% per decade after the age of 50 years and increases by about 12% within the first couple of months of strength training (127). Thus, 2 decades of age-induced muscle mass loss can be reversed with only about 2 months of strength training.

**Heritability of Skeletal Muscle Mass and Strength**

Skeletal muscle mass and strength are known to vary among individuals of a given age and sex. Differences in muscle mass and strength between individuals may be due to environmental or genetic factors or to both of these factors. Heritability (h2) refers to the proportion of the total variation that can be attributed to genetic effects (Vg/Vtot). Assessment of heritability is based on the basic genetic model that the total variation (Vtot) in traits such as muscular strength/muscle mass is partitioned into genetic (Vg), common environmental (Vc) and individual-specific environmental (Ve) variation components (Vtot = Vg + Vc + Ve). The most commonly used strategy to identify

genetic and environmental contributions to muscle strength and mass has been twin studies.

Arden et al. (5) examined the heritability of lean body mass in healthy postmenopausal women in 227 pairs of monozygotic (MZ) twins and 126 dizygotic (DZ) twins and concluded that genetic factors explain about half (.52) of the total variance of lean body mass in this cohort. Forbes et al. (36) found a heritability of 70% in 49 MZ and 38 DZ twin pairs. Seeman et al. (117) estimated the genetic component of lean body mass explained 80% of the total variance found in a twin study of pre and post menopausal women and concluded that greater muscle mass is likely to be determined by genes regulating tissue size.

Muscle strength is also a highly heritable phenotype. Arden et al. (5) examined the heritability of lean body mass in healthy postmenopausal women in 227 pairs of MZ twins and 126 DZ twins and concluded that genetic factors explain about one third (.30) of the total variance in grip strength in this cohort. Reed et al. (102) reported that genetic factors accounted for 65% of the variance in grip strength in a study of 260 MZ and DZ male twins from the National Heart, Lung, and Blood Institute (NHLBI) Twin Study who were 59-69 yr of age, even after adjusting for body weight, height and age.

Most recently, Huygens et al. (57) reported that heredity accounted for up to 90% of the inter-individual variation in muscle mass and ~60% in strength providing further evidence that genetic factors make important contributions to these muscle phenotypes. Although the heritability values for both muscle mass and strength have been well established, the identification of specific genes and allelic variants contributing to these phenotypes is in its infancy (111).

## Candidate Gene Studies

With the completion of the human genome project (HGP), a high-quality reference sequence of the gene rich portion of the human genome is now available. This has opened up the possibility to systematically identify all possible gene variants in different human populations, associate their presence with individual phenotypes, including disease susceptibility, and determine the functional impact of such variation. The most abundant source of genetic variation in the human genome comprises single nucleotide polymorphisms (SNPs) (121). Associating SNPs with human disease phenotypes has great potential for direct clinical application by providing new and more accurate genetic markers for diagnostic and prognostic purposes, and possibly novel therapeutic targets (121).

Association studies using a candidate gene approach looks for a statistical correlation between a specific genetic variant and a disease. The candidate gene approach can be defined as the study of genetic influences on a complex trait by: generating hypotheses about, and identifying candidate genes that might have a role in, the etiology of the disease; identifying variants in or near those genes that might either cause a change in the protein or its expression, or be in linkage disequilibrium (LD) with functional changes; genotyping the variants in a population; and then using statistical methods to determine whether there is a correlation between those variants and the phenotype. Given the possible promising aspects of association studies, association studies with candidate genes have been wide to study the genetic aspects of complex diseases. Before 1992, less than 10 genetic association studies were published per year, but by 2000, the number had increased to 120 (51).

**Myostatin**

Bullough (13) in 1962 proposed that tissue size is controlled by the activity of negative growth regulators that he dubbed chalones. According to this hypothesis, individual tissues secrete distinct chalones, which circulate throughout the body and act to inhibit the growth of the tissue producing the specific chalone. Recent work suggests that at least one tissue may, in fact, utilize this general type of regulatory mechanism to control tissue mass. This tissue is skeletal muscle, and the mediator appears to be myostatin. The identification of myostatin as a negative regulator of muscle growth has raised many important questions about the control of muscle growth. Understanding the mechanisms by which myostatin regulates muscle mass will be critical not only for understanding the control of tissue size in general but also for developing new strategies for increasing muscle growth for human therapeutic applications (73).

First reported in 1997 by McPherron et al. (85), myostatin (growth and differentiation factor-8) was identified in mice as a transforming growth factor-β (TGF-β) family member that acts as a negative regulator of skeletal muscle growth. Soon after the initial report of myostatin's discovery, several groups identified mutations in the myostatin gene in naturally-bred "double-muscled" cattle breeds (41; 86). Perhaps the most exciting research involving genetic variation within the myostatin gene comes from a case report published in JAMA in 2004. Scheulke and colleagues (116) reported a rare and novel variation in the myostatin gene in a young child, who when born appeared extraordinarily muscular, with protruding muscles in his thighs and upper arms. Results from ultrasonongraphy when the child was 4.5 years of age showed that cross-sectional plane of the patients quadriceps muscle was 7.2 SD above the mean for 10 age and sex

matched controls. All three exons and flanking intronic regions were sequenced and although no mutations were detected in the coding region a g-a transition in intron 1 was discovered and present in both alleles of the patient. This g-a transition results in a 108 base pair insertion adding a single lysine residue followed by a premature termination codon giving rise to a severely truncated protein. The authors attempted to measure myostatin levels in the patient's serum but failed to detect any. The authors concluded that the results strongly indicated that the patient has a loss of function mutation in the myostatin gene. This suggests the inactivation of myostatin has similar effects in humans, mice, and cattle, providing further evidence for a critical role for myostatin in muscle development in humans, and thus establishing myostatin as the key target it has become for muscle researchers.

Despite myostatin's remarkable influence on skeletal muscle and the well-established effect of myostatin gene mutations in double-muscled cattle breeds, studies of common myostatin genetic variation in humans have shown little association with muscle phenotypes. Ferrell and coworkers (33) identified several polymorphisms in the human myostatin gene, with six nucleotide changes observed, of which five are predicted to lead to amino acid sequence changes, but in follow-up work by that group and others only minor associations have been observed for the most common of these polymorphisms with muscle mass or strength (58; 118). For example, comparisons of myostatin variation, in two of the variants found to be common (A55T) and (K153R), in those who were rated in the top two categories to those who were rated in the bottom two categories for muscle mass increase in response to strength training showed no significant relationship between genotype and response to training (26; 33). Only suggestive

associations of the K153R polymorphism with muscular strength have been reported for older females. Seibert et al. (118) investigated polymorphism variation in the myostatin gene in a cohort of 286 older women and state that their data suggest an association of the R153 allele with lower strength in high-functioning older women. Finally, work by Ivey et al. (58) examined the effect of myostatin genotype on the hypertrophic response to heavy resistance strength training and myostatin genotype (K153R) did not explain the hypertrophic response to strength training when all 32 subjects were assessed.

*Myostatin Structure/Function*

Myostatin is synthesized in skeletal muscle as a 376 amino acid propeptide which gives rise to a 15kDa active, processed, and mature protein. Structurally, it contains all the characteristic features of the TGF-β family, such as a proteolytic processing signal site and an active carboxy terminal region. Myostatin exists as a large, latent complex with other proteins, including its propeptide. The function of myostatin was elucidated by gene targeting studies in mice (85). Mice carrying a deletion of the portion of the gene encoding the C-terminal domain of myostatin were shown to have dramatic and widespread increases in skeletal muscle mass, with individual muscles weighing about twice as much as those of wild type mice.

## Regulation of Myostatin-ACVR2B and Follistatin as Candidate Genes

Identifying how myostatin influences skeletal muscle and what processes regulate myostatin expression and activity have dominated the myostatin literature in the past few years. Similar to other TGF-β family members, myostatin is synthesized as a precursor protein that undergoes two proteolytic processing events in order to generate the biologically active molecule. The first event removes the N-terminal signal sequence,

most commonly referred to as the propeptide, and the second generates the C-terminal fragment which possesses receptor binding activity and is the biologically active species.

Myostatin exists as a large latent complex, including its propeptide. Following up their initial discovery, Lee and McPherron (74) demonstrated that activin type II receptors are involved in myostatin signaling. Myostatin binding to ACVR2B receptors was specific and saturable, and transgenic mice with increased muscle expression of a dominant negative form of ACVR2B had increased muscle weights, with individual muscles weighing up to 125% more than those of control nontransgenic animals. These results showed that expression of a truncated form of ACVR2B lacking the kinase domain and placed downstream of a skeletal muscle-specific myosin light chain promoter/enhancer can cause increases in muscle mass similar to those seen in myostatin knockout mice. In vitro work demonstrated that the propeptide blocked myostatin binding to its receptor. In vivo work by Lee and McPherron (74) showed that increased expression of the propeptide in transgenic mice resulted in increased muscle mass, thus, the propeptide acts as a myostatin inhibitor.

In addition to the propeptide, other proteins have also been shown to be capable of binding and inhibiting the activity of the myostatin C-terminal dimer. Several studies suggest that follistatin can function as a potent myostatin antagonist and plays an important role in vivo. First, follistatin is capable of blocking myostatin activity in both receptor binding and reporter gene assays (74; 142). Secondly, genetic studies in mice have shown that overexpression of follistatin in muscle can cause dramatic increases in muscle growth. Lee and McPherron (74) generated transgenic mice which the myosin light chain promoter/enhancer was used to drive the expression of follistatin and dramatic

effects on skeletal muscle were seen. In one animal muscle weights were increased by 194-327% relative to control animals; thus it appears, follistatin appears to be a potent myostatin antagonist (74).

With the identification of ACVR2B as the primary myostatin receptor and the myostatin propeptide and follistatin as negative regulators of myostatin activity, Lee and McPherron (74) put forth the following model of myostatin regulation in 2001: the myostatin C-terminal dimer remains in a latent complex with the inhibitory propeptide. This latent complex can be further negatively regulated by binding with follistatin, and upon release of the negative regulators, myostatin is free to signal through its receptors, acting primarily through ACVR2B.

*Downstream Targets*

Upon activation from its latent state, the myostatin C-terminal dimer is capable of binding to its receptor and activating a signal transduction cascade in the target cell. Myostatin signaling results in an upregulation of p21 which is an inhibitor of Cdk2. This causes a hypophosphorylation of retinoblastoma and a cell cycle arrest (G1) in proliferating myoblasts. Thus myoblast number and, hence, fiber number is regulated by myostatin (125). Myostatin can also inhibit differentiation by upregulation of Smad 3 proteins that bind to MyoD. This interaction represses MyoD transcriptional activity. As a result several regulatory factors are downregulated, which results in improper cell cycle withdrawal and inhibition of myoblast differentiation (70). In embryonic myogenesis, myostatin regulates myoblast number. In adult muscle, myostatin is specifically expressed in the regenerative satellite cells and myostatin null mice have greater numbers of both satellite cells per muscle fiber and activated satellite cells (82).

**Background of the Genetic Analysis-Haplotype**

Variation in the human genome sequence plays a powerful but poorly understood role in the etiology of common diseases. A comprehensive search for genetic influences on disease would involve examining all genetic differences in a large number of affected individuals and controls. It may eventually become possible to accomplish this by complete genome sequencing. In the meantime, it is increasingly practical to systematically test common genetic variants for their role in disease; such variants explain much of the genetic diversity in our species (2).

Systematic studies of common genetic variants are facilitated by the fact that individuals who carry a particular single nucleotide polymorphism (SNP) allele at one site often predictably carry specific alleles at other nearby variant sites. This correlation is known as linkage disequilibrium (LD); a particular combination of alleles along a chromosome is termed a haplotype (2).

Linkage disequilibrium plays a central role in association studies for identifying genetic variation responsible for common diseases (67; 93; 104; 137). The number of SNPs required for an association study depends on the pattern of LD. Recent studies showed that LD pattern varies greatly across the human genome with some regions of high LD interspersed by regions of low LD (22; 23; 38; 62; 95). These high LD regions are referred to as blocks in the literature. Only a small number of characteristic "tag" SNPs is sufficient to capture most of the haplotype structure of the human genome in high LD regions (62; 95). Thus, genotyping efforts could be greatly reduced without much loss of power for association studies (38; 140).

The correlations between causal mutations and the haplotypes on which they arose have previously served as a tool for human genetic research: first finding association to a haplotype, and then subsequently identifying the causal mutation(s) that it carries. This was pioneered in studies of the *HLA* region, extended to identify causal genes for mendelian diseases for example, cystic fibrosis (64) and diastrophic dysplasia (48), and most recently for complex disorders such as age-related macular degeneration (29; 45; 65).

## ***Haploview*-Software Program**

Given the increase in the number of association studies and the enormous amount of public genotype data from HapMap, tools for analyzing, interpreting and visualizing these data are of critical importance to researchers (6). The software program *Haploview* is designed to provide a number of tools for haplotype analysis. *Haploview* generates LD information, haplotype blocks, and population frequencies in a user friendly format.

*Haploview* calculates several pairwise measures of LD and the user has the option to select one of several commonly used block definitions (6; 38; 135). Alternatively, the user may manually select groups of markers for subsequent haplotype analysis. Once groups of markers are selected (either automatically or manually), the program generates haplotypes and their population frequencies (6).

*Background of the ACVR2B haplotype structure*

HapMap database research into the haplotype structure of the genome sequence surrounding the myostatin receptor gene, ACVR2B, has revealed the following haplotypes:

| | Snp1 | Snp3 | Snp5 | Snp8 | Snp10 | Snp11 | Snp14 | Snp15 | Snp16 | Freq |
|---|---|---|---|---|---|---|---|---|---|---|
| **Hap 1** | A | T | T | T | A | A | A | T | T | 0.615 |
| **Hap 2** | C | C | C | C | G | G | C | C | G | 0.273 |
| **Hap 3** | C | C | C | C | G | A | A | T | G | 0.05 |
| **Hap 4** | A | T | T | T | A | G | A | T | T | 0.034 |
| **Hap 5** | C | C | C | C | A | G | C | C | G | 0.01 |

By genotyping SNP 8 (rs: #2268757), the majority of the information of this haplotype (Hap) is gained by creating two related groups. Hap1 (.615) along with Hap 4 (.034) can be considered one group due to their high degree of similarity, sharing eight of nine alleles, and then Hap 2 (.273), Hap 3 (.05), and Hap 5 (.01) can be considered a second haplotype group, sharing a minimum of five of nine alleles to a maximum of eight of nine alleles

*Background of Follistatin haplotype structure*

HapMap database research into the haplotype structure of the genome sequence surrounding the follistatin gene has revealed the following haplotypes:

| | Snp1 | Snp2 | Snp3 | Snp4 | Snp5 | Snp6 | Snp7 | Freq |
|---|---|---|---|---|---|---|---|---|
| **Hap 1** | A | C | C | C | T | A | T | 0.367 |
| **Hap 2** | A | C | C | T | C | A | C | 0.217 |
| **Hap 3** | A | A | T | C | T | A | C | 0.20 |
| **Hap 4** | G | C | C | C | T | G | T | 0.133 |
| **Hap 5** | G | C | C | C | T | A | T | 0.042 |

**Sliding Window**

One issue inherent in haplotype association studies is haplotype complexity. Although haplotypes may be more informative than single markers, the power of haplotype analysis is reduced by the potentially large number of haplotypes that need to

be studied. A statistical approach known as the "sliding window" has been developed to address this issue (9; 20; 126; 141). The sliding window helps to focus on the candidate region of interest and assess evidence for association within each window, helping to reduce the dimensionality of haplotype analysis (141). The sliding window approach serves two purposes. First, the number of haplotype groups in each window may be significantly less than that in the whole region, so the association analysis involves fewer groups and likely has better power to detect an association between phenotype and haplotype. Second, it is anticipated that associations near true causal variants are stronger than those in other regions. Therefore, given the complexity of follistatin haplotype groups, by using SNPs 2, 3, 4, and 5, noted by the grey shading above, as the window for analysis regarding this haplotype in order to create three haplotype groups rather than five groups. The rationale for inclusion of SNPs 2, 3, 4, and 5 is that all four of these SNPs fall within the follistatin gene, therefore any true causal variants within the follistatin gene would be linked to one of the three haplotype groups analyzed.

By genotyping SNP 2 (rs: # 3797297) Hap 3 is separated from the rest of the haplotypes and genotyping SNP 4 (rs: #12152850) separates Hap 2 from the remaining haplotypes creating a final group of Hap 1, Hap 4, and Hap 5 that share a minimum of four of six alleles.

**Taqman Genotyping Assays**

Candidate gene studies are one of the most widely used approaches in the dissection of the genetic basis of disease. High-throughput methods for genotyping single nucleotide polymorphisms are necessary to perform large-scale association studies (83). Genotyping of SNPs in the present study was performed using the 5' nuclease allelic

discrimination or TaqMan assay (79) for high-throughput genotyping. In this method, the regions flanking the polymorphisms, typically 100 base pairs, are amplified in the presence of two probes each specific for one or the other allele. Probes have a fluor, called a "reporter," at the 5' end but do not fluoresce when free in solution because they have a "quencher" at the 3' end that absorbs fluorescence from the reporter. During polymerase chain reaction (PCR), the Taq polymerase encounters a probe specifically base-paired with its target and unwinds it. The polymerase cleaves the partially unwound probe and liberates the reporter fluor from the quencher, thereby increasing net fluorescence. In each PCR cycle, the cleavage of one or both allele-specific probes produces an exponentially increasing fluorescent signal by freeing the 5' fluorophore from the 3' quencher. The presence of two probes, each labeled with a different fluor, allows one to detect both alleles in a single tube. Moreover, because probes are included in the PCR, genotypes are determined without any post-PCR processing, a feature that is unavailable with most other genotyping methods (98).

The TaqMan SNP genotyping assay is read at the PCR endpoint. DNA samples are genotyped simultaneously on a 96 well plate and genotype calls for individuals are made by plotting the normalized intensity of reporter dyes in each sample well on a scatter plot. A clustering algorithm in the data analysis software assigns individual sample data to a particular genotype cluster (24).

Taqman SNP Genotyping Assays provide a number of significant technological advances (24): they require only a single enzymatic step, all assays use universal reactions and thermal cycling conditions, the location of primers is flexible in the region

surrounding the SNP site, and they are close-tubed assays and require no post PCR processing.

**Summary**

In summary, skeletal muscle mass gradually declines starting at about age 45 years and it is estimated that after the fifth decade 6% of muscle mass is lost per decade until the eighth decade of life in men (80). This loss of muscle mass, referred to as sarcopenia (106), has been associated with an increased risk of falls, hip fractures, functional decline, and mortality (4; 60; 88; 101). Skeletal muscle mass and strength are known to vary among individuals of a given age and sex. Heritability studies have shown that genetic factors can account for up to 90% of the inter-individual variation in muscle mass (57) and ~65% in strength (102) providing evidence that genetic variation makes important contributions to these muscle phenotypes. Myostatin, a negative regulator of skeletal muscle (73), has been shown to play a key role in both muscle development and the maintenance of muscle mass (41; 70; 74; 82; 85; 86; 116). However, variation within this gene has not been consistently associated with skeletal muscle mass nor muscle strength (33; 58; 118). Therefore, to further understand the inter-individual difference seen in muscle mass and strength research studies examining genetic variation in physiologically relevant candidate genes among myostatin related genes are needed to explore associations with skeletal muscle related phenotypes. Two genes whose variation has yet to be examined and explored with these phenotypes are follistatin, a known antagonist of myostatin, and the myostatin receptor, Activin IIRB (74).

# APPENDIX A: Limitations of the Study

**Delimitations**

1. The scope of this study will be delimited to 315 Caucasian males and 278 Caucasian females aged 19-90 yr. from the Baltimore Longitudinal Study of Aging (BLSA). The results are expected to apply only to populations with similar characteristics.

2. Variables such as age, height, and weight may have effects on muscle mass and strength. Therefore, statistical control for these variables was applied where appropriate.

3. Total body fat and soft tissue fat free mass (FFM) and total leg fat and FFM (both legs combined) was assessed by dual-energy X-ray absorptiometry (DEXA) (model DPX-L Lunar Radiation, Madison, WI).

4. Peak torque (strength) was measured using the Kinetic Communicator isokinetic dynamometer (Kin-Com model 125E, Chattanooga Group, Chattanooga, TN).

5. Genomic DNA was prepared from the EDTA-anticoagulated whole blood samples by standard salting-out procedures (Puregene DNA Extraction, Gentra Systems Inc.).

6. Genotyping of SNPs was performed using the 5' nuclease allelic discrimination or TaqMan assay for high-throughput genotyping. Fluorescence in each well was measured using an ABI 7300 Real Time PCR System machine (Perkin Elmer, Applied Biosystems Division). Analysis of raw data to determine genotypes was performed by the ABI 7300 Sequence Detection System software.

8. The International HapMap Project website's graphical genome browser was used to navigate to the particular regions surrounding the candidate genes of interest and retrieve HapMap genotype data for all genotyped markers in the selected regions in a format accepted by the software program *Haploview*. *Haploview* calculated pairwise measures

of linkage disequilibrium (LD) among the polymorphisms in each region and created haplotype blocks based on the definition of haplotype block provided by Gabriel and colleagues (38).

**Limitations**

1. The subjects in the study were generally healthy Caucasian volunteers from the Baltimore Longitudinal Study of Aging and not randomly selected from the general population.

2. Subjects self reported physical activity levels. Because physical activity was not directly measured the accuracy of these reports can not be verified. Inaccurate self reporting of physical activity could have confounded the results of this study.

3. Dietary intake and nutritional profiles of the subjects was not measured. Differences in caloric and macronutrient intake could have confounded the results of this study.

4. This study examines the relationship between only two genes with skeletal muscle phenotypes and combined association of these two genes was not assessed.

4. Genotypes other than the three SNPs genotyped in the present study that have previously been associated with muscle mass and strength were not genotyped or controlled for their potential influence on muscle phenotypes.

5. Due to statistical power limitations regarding the follistatin haplotype analysis, specific haplotype groupings needed to be collapsed in order to increase statistical power.

# APPENDIX B: Definitions

**Allele:** one of a pair of alternative forms of a gene that occur at a given locus in a chromosome.

**Genome:** the total set of genes in a nucleus of a cell.

**Genotype:** the genetic makeup of an individual. It may also apply to a specific locus or to a combination of alleles.

**Haplotype:** a set of closely linked genetic markers present on one chromosome which tend to be inherited together.

***Haploview:*** a software program designed to provide a number of tools for haplotype analysis.

**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.

**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.

**Loss-of Function Mutation**: a mutation that impairs or abolishes gene expression, or the function of a gene product.

**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.

**Sliding Window Analysis:** a statistical approach that helps to focus on the candidate gene region of interest and assesses evidence for association within each window, helping to reduce the dimensionality of haplotype analysis.

**TaqMan:** a high-throughput method for genotyping single nucleotide polymorphisms.

# APPENDIX C: Statistical Power Analysis

**Statistical power analyses**

Statistical power calculations were estimated for haplotype association with both muscle mass and strength phenotypes. These analyses were performed using effect size and standard deviation data obtained from previously published work. The effect size of 2.5 kg for FFM differences and the effect size of 11 N·m for skeletal muscle strength differences was chosen based on previous work. Sowers et al. (119) demonstrated in women that a loss of 2.5 kg of lean mass resulted in lower levels of functioning, slower walking velocity, and less leg strength. The value of 2.5 kg for FFM reflects a minimum difference we expect to see or consider comparable to previous studies that have shown losses in function associated with a 2.5 kg loss of lean mass. Hughes et al. (53) demonstrated that 11 N·m or ~12% of knee extensor strength is lost per decade in older women. Given the high correlation of low muscle strength to impaired physical function (7; 11; 34; 138) we feel a minimum difference in skeletal muscle strength comparable to that which is lost per decade in older women is a physiologically important difference to expect.

The subject numbers in Table 1 reflects the minimum number of subjects needed for the analysis at 80% power for both mass and strength phenotypes. For FFM differences, the minimum number of subjects required is ~ 47 in each group and a minimum number of ~58 for skeletal muscle strength is required with an alpha level at 0.05 and a power level of 0.80 (Table 1).

**Table 1. Calculation results to determine number of subjects and statistical power for proposed hypotheses.**

| Dependent Variable | Effect Size[1] | Standard Deviation | Effect Size /Standard Deviation | Alpha | Power | Number of subjects required |
|---|---|---|---|---|---|---|
| Muscle Mass[2] | 2.5 | 4.3 | 0.581 | 0.05 | 0.80 | 47 |
| Muscle Strength[3] | 11 | 21 | 0.523 | 0.05 | 0.80 | 58 |

[1]Values in unit difference between groups.
[2] Kg (Total Muscle Mass)
[3] N·m (Knee extensor peak torque)

**Table 2. Calculation results for statistical power from current data.**

| Dependent Variable | Effect Size[1] | Standard Deviation | Alpha | Power |
|---|---|---|---|---|
| Muscle Mass[2] | 1.3 | 4.3 | 0.05 | 0.97 |
| Muscle Strength[3] | 10 | 21 | 0.05 | 1.0 |

[1]Values in unit difference between groups.
[2] Kg (Total Muscle Mass)
[3] N·m (Knee extensor peak torque)

# APPENDIX D- Human Subjects Approval

UNIVERSITY OF MARYLAND
INSTITUTIONAL REVIEW BOARD

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January 3, 2006

**MEMORANDUM**

*Application Approval Notification*

| | |
|---|---|
| To: | Dr. Stephen Roth, Sean Wash, Dongmei Liu<br>Department of Kinesiology |
| From: | Roslyn Edson, M.S., CIP, IRB Manager<br>University of Maryland, College Park |
| Re: | IRB Number: 02-0177<br>Project Title: "Genetic Influence on Aging Skeletal Muscle" |
| **Approval Date:** | December 22, 2005 |
| **Expiration Date:** | December 22, 2006 |
| **Type of Application:** | Renewal |
| **Type of Review For Application:** | Expedited |
| **Type of Research:** | Non-exempt<br>(Please note: This research does not qualify for an exemption because all of the samples were not in existence at the time that the initial IRB application was submitted) |

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 want to continue to collect data from human subjects or analyze data from human subjects after the expiration date for this approval, 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**

*(continued)*

**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.

# APPENDIX E- Tables

**Table A. Concentric knee extensor peak torque values in Follistatin haplotype group 1 homozygous carriers, haplotype group 1 heterozygous carriers, and non-carriers of haplotype group 1 in Caucasian Men and Women.**

| Haplotype Groups | Homozygous Haplotype Group 1 | Heterozygous Haplotype Groups 1 & 2 | Heterozygous Haplotype Groups 1 & 3 | Non-Carriers of Haplotype Group 1 | P-value |
|---|---|---|---|---|---|
| **MEN** | | | | | |
| Concentric (N·m, 30º/sec) (N) | 171.1 ± 3.6 (125) | 168.7 ± 4.7 (73) | 173.7 ± 4.5 (79) | 175.9 ± 6.4 (39) | 0.788 |
| Concentric (N·m, 180º/sec) (N) | 117.8 ± 2.3 (122) | 112.6 ± 3.1 (67) | 117.0 ± 2.9 (78) | 117.3 ± 4.2 (37) | 0.601 |
| **WOMEN** | | | | | |
| Concentric (N·m, 30º/sec) (N) | 111.4 ± 2.8 (94) | 111.5 ± 3.8 (52) | 111.2 ± 3.7 (57) | 110.9 ± 4.0 (46) | 0.999 |
| Concentric (N·m, 180º/sec) (N) | 74.2 ± 1.8 (94) | 72.2 ± 2.5 (50) | 69.9 ± 2.3 (55) | 70.5 ± 2.5 (46) | 0.458 |

Data are least square means ± SE. Age and height were included in the model as significant covariates.

**Table B: Subject characteristics for Follistatin haplotype group 2 carriers and non-carriers of haplotype group 2 in Caucasian Men and Women.**

| **MEN** | | | |
|---|---|---|---|
| Haplotype Group | Carriers of Haplotype Group 2 | Non-Carriers of Haplotype Group 2 | P-value |
| N | 97 | 218 | |
| Age (y) | 62.8 ± 1.7 | 62.2 ± 1.1 | 0.773 |
| Height (cm) | 175.4 ± 0.8 | 176.6 ± 0.5 | 0.195 |
| Weight (kg) | 83.9 ± 1.3 | 84.4 ± 0.9 | 0.777 |
| **WOMEN** | | | |
| N | 88 | 190 | |
| Age (y) | 56.0 ± 1.7 | 57.8 ± 1.2 | 0.388 |
| Height (cm) | 164.0 ± 0.7 | 162.9 ± 0.5 | 0.234 |
| Weight (kg) | 68.7 ± 1.3 | 66.9 ± 0.9 | 0.258 |

Data are means ± SE.

**Table C: Soft tissue FFM variables by carriers and non carrier of haplotype group 2 Caucasian Men and Women.**

| Haplotype Group | Carriers of Haplotype Group 2 | Non-carriers of Haplotype Group 2 | P-value |
|---|---|---|---|
| **MEN** | | | |
| Total Body FFM (kg)<br>(N) | 57.3 ± 0.5<br>(97) | 56.9 ± 0.3<br>(218) | 0.487 |
| Total Leg FFM (kg)<br>(N) | 16.8 ± 0.3<br>(88) | 17.4 ± 0.2<br>(192) | 0.112 |
| **WOMEN** | | | |
| Total Body FFM (kg)<br>(N) | 39.8 ± 0.4<br>(88) | 39.4 ± 0.3<br>(190) | 0.299 |
| Total Leg FFM (kg)<br>(N) | 11.4 ± 0.3<br>(81) | 11.3 ± 0.2<br>(174) | 0.739 |

Data are least square means ± SE. Age and height were included in the model as significant covariates.

**Table D: Concentric knee extensor peak torque values in carriers and non-carriers of haplotype group 2 in Caucasian Men and Women.**

| Haplotype Group | Carriers of Haplotype Group 2 | Non-carriers of Haplotype Group 2 | P-value |
|---|---|---|---|
| **MEN** | | | |
| Concentric (N·m, 30º/sec)<br>(N) | 172.2 ± 3.9<br>(105) | 171.6 ± 2.7<br>(211) | 0.917 |
| Concentric (N·m, 180º/sec)<br>(N) | 115.0 ± 2.6<br>(98) | 117.1 ± 1.8<br>(206) | 0.516 |
| **WOMEN** | | | |
| Concentric (N·m, 30º/sec)<br>(N) | 111.7 ± 3.0<br>(84) | 111.1 ± 2.1<br>(165) | 0.864 |
| Concentric (N·m, 180º/sec)<br>(N) | 71.7 ± 1.9<br>(82) | 72.3 ± 1.4<br>(163) | 0.819 |

Data are least squares means ± SE. Age and height were included in the model as significant covariates.

**Table E: Concentric knee extensor peak torque values in carriers and non-carriers of haplotype group 3 in Caucasian Men and Women.**

| Haplotype Group | Carriers of Haplotype Group 3 | Non-carriers of Haplotype Group 3 | P-value |
|---|---|---|---|
| **MEN** | | | |
| Concentric (N·m, 30º/sec) (N) | 173.2 ± 3.9 (107) | 171.1 ± 2.8 (209) | 0.648 |
| Concentric (N·m, 180º/sec) (N) | 116.8 ± 2.5 (104) | 116.2 ± 1.8 (200) | 0.843 |
| **WOMEN** | | | |
| Concentric (N·m, 30º/sec) (N) | 111.4 ± 3.8 (96) | 111.4 ± 2.2 (153) | 0.948 |
| Concentric (N·m, 180º/sec) (N) | 69.9 ± 1.8 (94) | 73.4 ± 1.4 (151) | 0.13 |

Data are least square means ± SE. Age and height were included in the model as significant covariates.

**Table F. Soft tissue FFM variables for Follistatin haplotype group 3 homozygous carriers, haplotype group 3 heterozygous carriers, and non-carriers of haplotype group 3 in Caucasian Men and Women.**

| Haplotype Groups | Homozygous Haplotype Group 3 | Heterozygous Haplotype Groups 3 & 1 | Heterozygous Haplotype Groups 3 & 2 | Non-Carriers of Haplotype Group 3 | P-value |
|---|---|---|---|---|---|
| **MEN** | | | | | |
| Total Body FFM (kg) (N) | 56.5 ± 1.9 (7) | 56.1 ± .5 a (83) | 57.0 ± 1.0 (21) | 57.4 ± .3 b (204) | a vs b 0.045 |
| Total Leg FFM (kg) (N) | 19.1 ± 1.4 a (4) | 16.6 ± 0.7 b (72) | 15.8 ± 0.7 c (17) | 17.5 ± 0.2 d (187) | a vs b 0.10<br>a vs c 0.041<br>b vs d 0.031<br>c vs d 0.018 |
| **WOMEN** | | | | | |
| Total Body FFM (kg) (N) | 39.8 ± 0.9 (17) | 39.8 ± 0.4 (65) | 39.2 ± 0.7 (25) | 39.3 ± 0.3 (171) | 0.738 |
| Total Leg FFM (kg) (N) | 11.8 ± 0.6 (14) | 11.7 ± 0.3 (61) | 10.8 ± 0.5 (22) | 11.2 ± 0.2 (158) | 0.229 |

Data are least square means ± SE. Age and height were included in the model as significant covariates.

**Table G: Subject characteristics by Follistatin (**rs: # 3797297**) genotype for Caucasian Men and Women.**

| Genotype Group | CC | AC | AA | P-value |
|---|---|---|---|---|
| **MEN** | | | | |
| N | 199 | 104 | 12 | |
| Age (y) | 61.8 ± 1.2 | 64.1 ± 1.6 | 57.2 ± 4.8 | 0.28 |
| Height (cm) | 176.2 ± .5 | 176.3 ± .7 | 176.6 ± 2.2 | 0.976 |
| Weight (kg) | 84.7 ± .9 | 83.9 ± 1.3 | 80.3 ± 3.7 | 0.505 |
| **WOMEN** | | | | P-value |
| N | 173 | 91 | 20 | |
| Age (y) | 57.7 ± 1.2 | 55.6 ± 1.7 | 58.3 ± 3.6 | 0.428 |
| Height (cm) | 163.6 ± .5 | 162.9 ± .7 | 161.7 ± 1.5 | 0.99 |
| Weight (kg) | 67.2 ± 1.0 | 67.5 ± 1.3 | 68.3 ± 2.8 | 0.929 |

Data are means ± SE.

**Table H: Soft tissue FFM variables by Follistatin (**rs: # 3797297**) genotype in Caucasian Men and Women.**

| Genotype Group | CC | AC | AA | P-value |
|---|---|---|---|---|
| **MEN** | | | | |
| Total Body FFM (kg) (N) | 57.4 ± 0.3 a (199) | 56.3 ± 0.5 b (104) | 57.4 ± 1.4 (12) | a vs b 0.067 |
| Total Leg FFM (kg) (N) | 17.6 ± 0.2 a (182) | 16.5 ± 0.3 b (89) | 17.4 ± 1.0 (9) | a vs b 0.004 |
| **WOMEN** | | | | |
| Total Body FFM (kg) (N) | 39.4 ± 0.3 (173) | 39.6 ± 0.4 (91) | 39.7 ± 0.8 (20) | 0.832 |
| Total Leg FFM (kg) (N) | 11.2 ± 0.2 (160) | 11.5 ± 0.2 (84) | 11.8 ± 0.6 (17) | 0.482 |

Data are least square means ± SE. Age and height were included in the model as significant covariates.

**Table I: Concentric knee extensor peak torque values by Follistatin (**rs: # 3797297**) genotype in Caucasian Men and Women.**

| Genotype Group | CC | AC | AA | P-value |
|---|---|---|---|---|
| | | **MEN** | | |
| Concentric (N·m, 30º/sec) (N) | 170.4 ± 2.8 (205) | 173.3 ± 4.0 (101) | 175.4 ± 11.7 (12) | 0.795 |
| Concentric (N·m, 180º/sec (N) | 115.8 ± 1.8 (196) | 117.5 ± 2.6 (98) | 112.9 ± 7.8 (11) | 0.783 |
| | | **WOMEN** | | |
| Concentric (N·m, 30º/sec) (N) | 111.6 ± 2.2 (156) | 111.8 ± 3.0 (84) | 105.7 ± 6.9 (16) | 0.705 |
| Concentric (N·m, 180º/sec) (N) | 73.7 ± 1.4 (154) | 69.7 ± 1.9 (82) | 66.8 ± 4.3 (16) | 0.122 |

Data are least square means ± SE. Age and height were included in the model as significant covariates.

**Table J: Subject characteristics by Follistatin (**rs: #12152850**) genotype for Caucasian Men and Women.**

| Genotype Group | CC | CT | TT | P-value |
|---|---|---|---|---|
| | | **MEN** | | |
| N | 225 | 91 | 4 | |
| Age (y) | 61.6 ± 1.1 a | 63.0 ± 1.8 b | 77.2 ± 8.3 c | a vs c 0.065<br>b vs c 0.098 |
| Height (cm) | 176.6 ± .5 a | 175.7 ± .8 b | 167.7 ± 2.2 c | a vs c 0.019<br>b vs c 0.037 |
| Weight (kg) | 84.7 ± .9 | 83.9 ± 1.3 | 80.3 ± 3.7 | 0.505 |
| | | **WOMEN** | | |
| N | 205 | 84 | 10 | |
| Age (y) | 56.8 ± 1.1 | 56.0 ± 1.8 | 59.7 ± 5.2 | 0.774 |
| Height (cm) | 163.2 ± .5 | 164.2 ± .7 | 163.2 ± 2.1 | 0.513 |
| Weight (kg) | 66.9 ± .9 | 68.7 ± 1.4 | 67.2 ± 3.9 | 0.556 |

Data are means ± SE.

**Table K: Soft tissue FFM variables by Follistatin (**rs: #12152850**) genotype in Caucasian Men and Women.**

| Genotype Group | CC | CT | TT | P-value |
|---|---|---|---|---|
| | | **MEN** | | |
| Total Body FFM (kg)<br>(N) | 57.0 ± 0.3 a<br>(225) | 57.0 ± 0.5 b<br>(91) | 65.2 ± 2.4 c<br>(4) | a & b vs c 0.001 |
| Total Leg FFM (kg)<br>(N) | 17.4 ± 0.2<br>(199) | 16.9 ± 0.3<br>(82) | 17.4 ± 1.4<br>(4) | 0.501 |
| | | **WOMEN** | | |
| Total Body FFM (kg)<br>(N) | 39.5 ± 0.2<br>(205) | 39.9 ± 0.4<br>(84) | 39.2 ± 1.1<br>(10) | 0.668 |
| Total Leg FFM (kg)<br>(N) | 11.8 ± 0.7<br>(186) | 11.4 ± 0.3<br>(76) | 11.3 ± 0.2<br>(10) | 0.82 |

Data are least squares means ± SE. Age and height were included in the model as significant covariates.

**Table L: Concentric knee extensor peak torque values by Follistatin (**rs: #12152850**) genotype in Caucasian Men and Women.**

| Genotype Group | CC | CT | TT | P-value |
|---|---|---|---|---|
| | | **MEN** | | |
| Concentric (N·m, 30º/sec)<br>(N) | 172.1 ± 2.7<br>(223) | 171.4 ± 4.1<br>(96) | 175.8 ± 16.5<br>(6) | 0.963 |
| Concentric (N·m, 180º/sec<br>(N) | 117.6 ± 1.7<br>(218) | 114.5 ± 2.7<br>(89) | 115.7 ± 10.5<br>(6) | 0.632 |
| | | **WOMEN** | | |
| Concentric (N·m, 30º/sec)<br>(N) | 110.6 ± 2.1<br>(182) | 113.0 ± 3.1<br>(82) | 110.6 ± 2.1<br>(7) | 0.736 |
| Concentric (N·m, 180º/sec)<br>(N) | 71.6 ± 1.4<br>(180) | 72.3 ± 2.0<br>(80) | 73.1 ± 6.9<br>(7) | 0.95 |

Data are least squares means ± SE. Age and height were included in the model as significant covariates.

**Table M: Subject characteristics by Follistatin haplotype groups in Caucasian Men and Women.**

| Haplotype Groups | Homo Haplotype Group 1 | Homo Haplotype Group 2 | Homo Haplotype Group 3 | Hetero Haplotype Group 1 & 2 | Hetero Haplotype Group 1 & 3 | Hetero Haplotype Group 2 & 3 | P-value |
|---|---|---|---|---|---|---|---|
| **MEN** | | | | | | | |
| N | 128 | 4 | 12 | 67 | 83 | 21 | |
| Age (y) | 59.4 ± 1.4 a | 77.2 ± 8.1 b | 57.2 ± 4.7 c | 65.4 ± 2.0 d | 66.1 ± 1.8 e | 56.3 ± 3.6 f | a vs b, d, & e <0.05<br>b vs c & f <0.05<br>d vs f <0.05<br>e vs f <0.05 |
| Height (cm) | 176.9 ± 0.6 | 167.7 ±3.7 | 176.6 ± 2.2 | 175.3 ± 0.9 | 176.1 ± 0.8 | 177.0 ± 1.6 | b vs a, c, d, e, & f <0.05 |
| Weight (kg) | 85.4 ± 1.1 | 91.8 ± 6.4 | 80.4 ± 3.7 | 82.8 ± 1.6 | 83.1 ± 1.4 | 87.5 ± 2.8 | 0.271 |
| **WOMEN** | | | | | | | |
| N | 108 | 10 | 17 | 53 | 65 | 25 | |
| Age (y) | 58.8 ± 1.6 | 59.7 ± 5.1 | 59.0 ± 4.0 | 55.7 ± 2.2 | 55.9 ± 2.0 | 55.1 ± 3.3 | 0.732 |
| Height (cm) | 163.2 ± 0.7 (99) | 163.2 ± 2.2 (10) | 161.2 ± 1.7 (14) | 164.9 ± 0.9 (49) | 163.1 ± 0.8 (61) | 162.6 ± 1.4 (22) | 0.460 |
| Weight (kg) | 65.4 ± 1.2 a | 67.3 ± 3.9 | 69.2 ± 3.0 | 71.3 ± 1.7 b | 69.0 ± 1.5 | 64.1 ± 2.5 c | b vs a 0.005<br>b vs c 0.018 |

Data are means ± SE.

**Table N: Soft tissue FFM variables for Follistatin haplotype groups in Caucasian Men and Women.**

| Haplotype Groups | Homo Haplotype Group 1 | Homo Haplotype Group 2 | Homo Haplotype Group 3 | Hetero Haplotype Group 1 & 2 | Hetero Haplotype Group 1 & 3 | Hetero Haplotype Group 2 & 3 | P-value |
|---|---|---|---|---|---|---|---|
| **MEN** | | | | | | | |
| Total Body FFM (kg) (N) | 57.4 ± 0.4 a (128) | 65.1 ± 2.4 b (4) | 57.4 ± 1.3 c (12) | 56.9 ± 0.6 d (67) | 56.1 ± 0.5 e (83) | 56.9 ± 1.0 f (21) | b vs a, c, d, e, f < 0.008 |
| Total Leg FFM (kg) (N) | 17.8 ± 0.3 a (116) | 17.4 ± 1.4 (4) | 17.4 ± 1.0 (9) | 17.1 ± 0.4 (62) | 16.7 ± 0.3 b (72) | 15.8 ± 0.7 c (17) | a vs b & c 0.007 |
| **WOMEN** | | | | | | | |
| Total Body FFM (kg) (N) | 39.0 ± 0.3 a (108) | 39.2 ± 1.1 (10) | 39.8 ± 0.8 (17) | 40.3 ± 0.5 b (53) | 39.9 ± 0.4 (65) | 39.2 ± 0.7 (25) | a vs b 0.029 |
| Total Leg FFM (kg) (N) | 11.0 ± 0.2 (99) | 11.8 ± 0.7 (10) | 11.8 ± 0.6 (14) | 11.6 ± 0.3 (49) | 11.8 ± 0.3 (61) | 10.8 ± 0.5 (22) | 0.176 |

Data are least squares means ± SE. Age and height were included in the model as significant covariates.

**Table O: Concentric knee extensor peak torque values for Follistatin haplotype groups in Caucasian Men and Women.**

| Haplotype Groups | Homo Haplotype Group 1 | Homo Haplotype Group 2 | Homo Haplotype Group 3 | Hetero Haplotype Group 1 & 2 | Hetero Haplotype Group 1 & 3 | Hetero Haplotype Group 2 & 3 | P-value |
|---|---|---|---|---|---|---|---|
| **MEN** | | | | | | | |
| Concentric (N·m, 30º/sec) (N) | 171.1 ± 3.6 (125) | 175.4 ± 16.5 (6) | 175.1 ± 11.6 (12) | 168.7 ± 4.7 (73) | 173.7 ± 4.5 (79) | 176.6 ± 8.8 (21) | 0.957 |
| Concentric (N·m, 180º/sec (N) | 117.8 ± 2.3 (122) | 115.4 ± 10.6 (6) | 113.2 ± 7.8 (11) | 112.6 ± 3.2 (67) | 117.0 ± 2.9 (78) | 120.2 ± 5.8 (20) | 0.786 |
| **WOMEN** | | | | | | | |
| Concentric (N·m, 30º/sec) (N) | 111.4 ± 2.9 (94) | 105.8 ± 10.5 (7) | 108.3 ± 7.4 (14) | 111.6 ± 3.9 (52) | 111.2 ± 3.7 (57) | 113.7 ± 5.5 (25) | 0.986 |
| Concentric (N·m, 180º/sec (N) | 74.2 ± 1.8 (94) | 72.8 ± 6.5 (7) | 68.8 ± 4.7 (14) | 72.2 ± 2.5 (50) | 70.0 ± 2.4 (55) | 70.8 ± 3.5 (25) | 0.724 |

Data are least square means ± SE. Age and height were included in the model as significant covariates

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