

## **Creatine Kinase Clinical Considerations: Ethnicity, Gender and Genetics**

**Patricia A. Deuster, PhD, MPH; Francis G. O'Connor, MD, Kimbra Kenney, MD,  
Yuval Heled, PhD, Sheila Muldoon, MD, Carmen Contreras-Sesvold, MS,  
William W. Campbell, MD**

Uniformed Services University of the Health Sciences  
F. Edward Hébert School of Medicine  
Departments of Military and Emergency Medicine, Neurology, and Anesthesiology  
4301 Jones Bridge Road  
Bethesda, MD 20814-4799  
USA

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[pdeuster@usuhs.mil](mailto:pdeuster@usuhs.mil) / [foconnor@usuhs.mil](mailto:foconnor@usuhs.mil) / [kkenney@usuhs.mil](mailto:kkenney@usuhs.mil) / [Yuval.Heled@sheba.health.gov.il](mailto:Yuval.Heled@sheba.health.gov.il) /  
[smuldoon@usuhs.mil](mailto:smuldoon@usuhs.mil) / [carmen.sesvold@usuhs.mil](mailto:carmen.sesvold@usuhs.mil) / [wcampbell@usuhs.mil](mailto:wcampbell@usuhs.mil)

### **ABSTRACT**

*Exertional rhabdomyolysis (ER), or breakdown of skeletal muscle during exercise, is a potentially life-threatening event. ER can affect Warfighters in multiple settings, yet approaches for identifying persons susceptible to or at risk for ER have not been identified. Despite marked variability in baseline serum creatine kinase (CK), individual CK values are typically used to both diagnose and manage ER. Factors that may affect CD include ethnicity, gender, and genetics. One candidate gene for CK levels is the interleukin-6 (IL-6) gene, which codes for the cytokine, IL6. The purpose of this study was to investigate the contributions of ethnicity, gender, and genetics, in particular the IL-6 polymorphism, to variability in baseline CK.*

*Baseline blood samples were obtained for measuring serum CK and genomic DNA was extracted from peripheral blood leukocytes to genotype participants for the C-174G IL-6 polymorphism. Among the cohort of 841 men and women, 610 were Caucasians/CA; 44 African Americans/AA; 17 Asians/AS; and 36 Hispanics/HI, with a mean age of 22.8±4.9 years. Mean baseline CK for this population was 212±546 (SD) U/L, with a minimum of 20 and maximum of 9,500 U/L. Mean CK was significantly higher in African Americans ( $p < 0.01$ ; 665±1,482 U/L) compared to CA (180±367 U/L), AS (254±870 U/L) and HI (214±115 U/L), and lower in women (133±118 U/L) than men (234±615 U/L). Interestingly, those with the GG genotype had significantly higher baseline CK levels relative to the C+ group (C+: 165±141 U/L vs. GG: 294±874 U/L;  $p < 0.05$ ). Finally, the distribution of the IL-6 polymorphism differed by ethnicity: 77.3% of AA had the GG genotype as compared 34.7%, 60.4%, and 55.6% of CA, AS, and HI, respectively ( $p=0.001$ ).*

*In conclusion, baseline CK levels vary significantly depending on ethnicity, gender, genetics, and body mass index. Laboratory upper limits of normal should be developed to account for ethnic and gender differences. Importantly, ethnic- and gender-specific ranges for CK may assist operational medicine in the diagnosis of ER and safely returning Warfighters to duty. Lastly, the C-174G IL-6 polymorphism influences baseline CK, such that the GG genotype is associated with higher baseline CK values than the CC and CG genotypes. Whether the GG genotype predisposes to ER is unknown. It is also interesting that AA, with a preponderance of the GG genotype, have higher baseline CK levels than other ethnic/racial groups. Further research will be required to address a causal relationship and the implications of these findings.*

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**Exertional rhabdomyolysis (ER), or breakdown of skeletal muscle during exercise, is a potentially life-threatening event. ER can affect Warfighters in multiple settings, yet approaches for identifying persons susceptible to or at risk for ER have not been identified. Despite marked variability in baseline serum creatine kinase (CK), individual CK values are typically used to both diagnose and manage ER. Factors that may affect CD include ethnicity, gender, and genetics. One candidate gene for CK levels is the interleukin-6 (IL-6) gene, which codes for the cytokine, IL6. The purpose of this study was to investigate the contributions of ethnicity, gender, and genetics, in particular the IL-6 polymorphism, to variability in baseline CK. Baseline blood samples were obtained for measuring serum CK and genomic DNA was extracted from peripheral blood leukocytes to genotype participants for the C-174G IL-6 polymorphism. Among the cohort of 841 men and women, 610 were Caucasians/CA; 44 African Americans/AA; 17 Asians/AS; and 36 Hispanics/HI, with a mean age of 22.8±4.9 years. Mean baseline CK for this population was 212±546 (SD) U/L, with a minimum of 20 and maximum of 9,500 U/L. Mean CK was significantly higher in African Americans (p<0.01; 665±1,482 U/L) compared to CA (180±367 U/L), AS (254±870 U/L) and HI (214±115 U/L), and lower in women (133±118 U/L) than men (234±615 U/L). Interestingly, those with the GG genotype had significantly higher baseline CK levels relative to the C+ group (C+: 165±141 U/L vs. GG: 294±874 U/L; p < 0.05). Finally, the distribution of the IL-6 polymorphism differed by ethnicity: 77.3% of AA had the GG genotype as compared 34.7%, 60.4%, and 55.6% of CA, AS, and HI, respectively (p=0.001).**

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## **INTRODUCTION**

The enzyme, creatine kinase (CK), a clinical indicator of muscle damage, is essential for maintaining intracellular ATP levels, as it reversibly catalyzes the transfer of phosphate between ATP and creatine phosphate (3). This enzyme and its product/substrate, creatine phosphate, have a rich history dating back to 1909. Thonberg first identified creatine in 1911, and in 1928 Fiske and Subbarow (10) showed that creatine phosphate was critical for permitting skeletal muscle to contract. CK has five functional forms or isoenzymes, three are cytosolic and two are mitochondrial. The cytosolic forms are composed of two subunits, muscle (M) and brain (B), which dimerize to form CK-MM, CK-MB, and CK-BB isoforms (5). The CK enzyme is most abundant in muscle, brain, and heart (26), with the CK-MM isoform specific to striated muscle (36). In 1973, Turner et al. demonstrated that CK-MM is localized to the M-line of skeletal muscle (33). Both mitochondrial isoenzymes, non-sarcomeric and sarcomeric, are octamers consisting of four dimers each (5).

The use of serum CK as a diagnostic tool in human disease was introduced by Okinaka et al (24) in 1959. Henson and Rao (14) reported that serum CK was the most specific biochemical test for muscle damage with confirmation and validation in 2008 by Kumbhare et al. (18). Typical measures of CK in serum include all five isoenzymes. Serum fractionation, or analysis of isoenzyme distribution, provides specific diagnostic information. Elevation in levels of isoenzymes are indicators of diseases and/or injuries in specific tissue: CK-MM for muscle, CK-MB for heart (38) and cerebrospinal fluid, CK-BB for brain injury (13). Total serum CK from normal individuals is primarily the cytosolic skeletal muscle CK-MM isoform, and fractionation is not routinely carried out. Despite the many years of research on serum CK and its close association with muscle damage, most clinical laboratories have a narrow range of normal values, without always making adjustments for gender or ethnicity. Likewise, other factors that may contribute to variation in CK values have received limited attention, despite the widespread use of CK levels clinically.

Within the military, exertional rhabdomyolysis (ER), or breakdown of skeletal muscle during exercise, is a potentially life-threatening event that occurs due to rigorous physical training, particularly in the first two weeks after initiating training. The level of CK is typically used to diagnose ER, but without knowledge of baseline values, this can be problematic. The present study was conducted to explore various factors that may influence baseline CK values. In particular, we were interested in potential ethnic and gender differences, anthropometric factors, and the role of genetics. Specifically, we were interested in the interleukin-6 (IL6) gene, because it is produced by skeletal muscle during physical activity (27) and serves a pivotal role in the acute phase inflammatory response (31). The IL6 gene, which is mapped to chromosome 7p21–24, has a common G>C polymorphism in its upstream promoter at position -174. The GG genotype of this polymorphism has been shown to influence IL-6 release (35).

## **MATERIALS AND METHODS**

### **Subject Population**

The participants included men and women from three different study populations, all of which were conducted to examine potential determinants of baseline CK levels. The first study population consisted of 141 men and women who underwent testing in a laboratory setting to monitor CK responses to exercise. Most were active duty men and women (75%), but some were civilian who met military entrance standards. Study population two consisted of 191 basic trainees beginning basic military training at Ft Benning, Georgia and population three consisted of 449 men and women starting basic Marine training at Quantico Virginia. All participants were informed of the purposes and procedures and provided written consent prior to participation. All three studies were approved and monitored by the Uniformed Services University of the Health Sciences

Institutional Review Board (IRB), and both the Quantico and Ft. Benning studies were approved by the National Naval Medical Center IRB and the Eisenhower Army Medical Center IRB, respectively. Participant assessments consisted of weight and height, and obtaining fasting blood draws for CK and DNA analyses. Body mass index (BMI) was calculated as weight in kg divided by height<sup>2</sup> in meters.

### Procedures/Measurements

Blood samples were collected between 0700 and 0900 h coagulant free-tubes for CK analysis and EDTA-containing tubes for DNA analyses. EDTA samples were centrifuged at low speed to separate the cells and plasma and DNA was extracted from the buffy coat by using the QIAamp DNA mini kit 250 (Qiagen, Valencia, CA). Serum CK samples were analyzed in CLIA certified laboratories by a Vitros 250 Chemistry System (Ortho-Clinical Diagnostics, Johnson and Johnson Company, Rochester, NY, USA).

### Genotyping Methods for the IL6 C-174G Polymorphism

The IL6 C-174G single nucleotide polymorphism (SNP; rs1800795) in the 5' untranslated promoter region was analyzed. Genomic DNA was prepared and polymerase chain reactions were performed as described by (8) to amplify the region of interest. The primers used were 5'-TGACTTCAGCTTTACTCTTTG T- 3' (forward) and 5'-CTGATTGGAAACCTTATTAAG-3' (reverse). Each reaction contained 50 ng of genomic DNA, 300nM of each primer, 7.5 µl of Biochain Pro QPCR SuperMix, (Hayward, Ca., USA), and PCR Grade Water (Fisher BioReagents, Fairlawn, NJ, USA) to a final volume of 15 µl. Thermocycler conditions included an initial denaturation at 95°C for 3 minutes followed by 35 cycles of 95°C for 1 minute, 55°C for 95 seconds, and 72°C for 1 minute. This concluded with a final extension at 72°C for 4 minutes. This results in an amplicon of 198 base pairs (bp) in length.

The PCR products were subjected to SfaN1 restriction enzyme digestion. The 25 µl digestion reaction, which contained 5 µl of PCR product, 0.5 µl (1 unit) SfaN1, 2.5 µl NEB buffer #3 (New England Biolabs, Ipswich, MA, USA), and 17 µl PCR Grade Water (Fisher BioReagents, Fairlawn, NJ, USA), was incubated overnight in a 37°C water bath. Polymorphisms were identified by the presence or absence of the restriction site as displayed in Figure 1. The C allele lacks the digestion site and is 198 bp in length, whereas the G allele is recognized by the enzyme and is cut to produce two bands 140 and 58 bp in length. Amplicons and their digestion products were detected by 2% agarose gel electrophoresis. Subsequent documentation was performed with the Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA, USA).

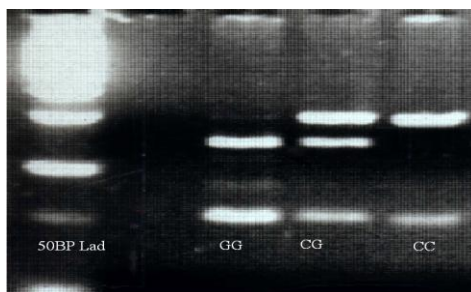


Figure 1: Photograph of the digestion products for the IL6 C-174 G polymorphism: GG, CG, and CC.

**Statistical Analyses**

Frequency distributions and descriptive analyses were conducted first, and selected variables were dichotomized for bivariate analyses: BMI was dichotomized as <25 or ≥ 25 and CK values were dichotomized as ≤ 150 or > 150 U/L based on the upper limit of normal for the laboratory. Likewise, the IL6 genotypes were dichotomized by combining the CC and CG as C+ versus the GG. Analysis of variance (ANOVA) techniques were used to examine differences by ethnicity, gender, and genotype and Chi Square analyses were used to examine bivariate associations among genotypes, CK, and BMI. The statistical significance level was set at p< 0.05, and data are presented as mean ± standard deviation. Data were analyzed using SPSS statistical package (SPSS, version 16.0.1; SPSS, Inc., Chicago, IL).

**RESULTS**

**CK in the General Population**

Overall, 781 persons (695 men and 86 women) participated in the study to include 610 Caucasians (CA), 44 African Americans (AA), 91 Asians (AS), and 36 Hispanics (HI). The mean age of the group was 22.8 ± 4.9 years, with average weight, height, and BMI of 77.6 ± 10.3 kg, 1.79 ± 0.18 m, and 24.7 ± 3.1 kg/ht<sup>2</sup>, respectively. Over 40% of the total sample had BMI values ≥ 25. The mean CK for the entire sample was 212 ± 546 U/L (Range: 20 to 9500) and the median was 137 U/L: CK was positively skewed (skewness = 13.2) with large positive kurtosis (kurtosis = 197.3). Natural logarithmic transformation of CK resulted in a normal distribution with a mean of 4.89 ± 0.8, a skewness of 0.36 and a kurtosis of 3.4. Untransformed data are provided in all graphs and tables.

**CK by Ethnicity and Gender**

Table 1 presents the general characteristics of the sample by ethnicity. Overall, AA were older and AS were shorter than the other ethnicities. The sample of HI had a significantly higher BMI than CA, AA, or AS. Importantly, CK levels were significantly higher in AA as compared to CA, HI and AS, as shown in Table 1. Because CK values are non-normally distributed, the median values for CA, AA, AS and HI were 127, 222, 143, and 178 U/L, respectively.

**Table 1: General Characteristic and Creatine Kinase Levels by Ethnicity (Mean ± SD).**

	CA (n=610)	AA (n=44)	AS (n=91)	HI (n=36)
<b>Age (yrs)</b>	22.6 ± 4.8	25.1 ± 6.2*	22.6 ± 3.6	23.6 ± 6.6
<b>Weight (kg)</b>	78.0 ± 10.2	78.1 ± 11.0	75.6 ± 9.2	78.1 ± 10.4
<b>Height (m)</b>	1.81 ± 0.18	1.79 ± 0.27	1.73 ± 0.12*	1.80 ± 0.31
<b>BMI (wt/ht<sup>2</sup>)</b>	24.6 ± 3.1	24.9 ± 3.2	24.5 ± 2.4	26.1 ± 4.3*
<b>CK (U/L)</b>	180 ± 367	665 ± 1,482**	254 ± 870	214 ± 115

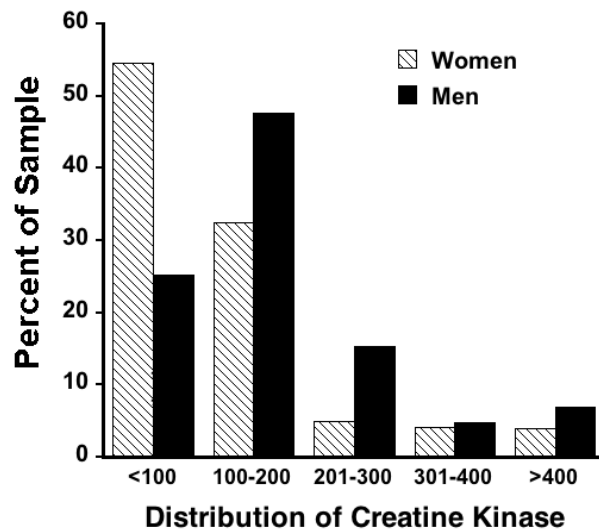
\*p < 0.05

In addition to ethnicity, a gender effect was noted for CK. Table 2 presents the population characteristics by gender. Because of the small number of AA (n=9) and HI (n=2) women, the sample could not be analyzed by both gender and ethnicity. However, CK levels were significantly lower in women than men (P<0.01) with median values of 140 and 90 U/L, for men and women respectively.

**Table 2: General Characteristic and Creatine Kinase Levels by Gender (Mean ± SD).**

Measures	Women (n=86)	Men (n=695)
Age (yrs)	24.7 ± 4.9	22.8 ± 5.1
Weight (kg)	68.8 ± 7.6	79.1 ± 10.1
Height (m)	1.60 ± 0.16	1.82 ± 0.18
BMI (wt/ht <sup>2</sup> )	22.9 ± 2.4	25.0 ± 3.2
CK (U/L)	122 ± 118	234 ± 615

Figure 2 presents the distribution of CK as a function of gender: over 50% of women had CK values less than 100 as compared to 25% of men. For men and women, the percent with CK values over 400 U/L was less than 8%.

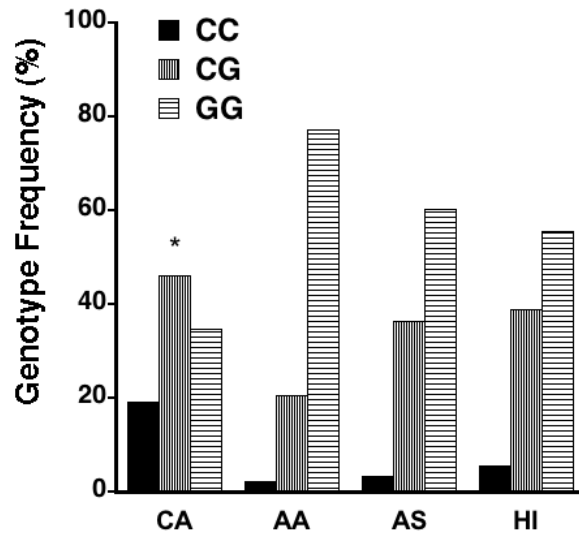


**Figure 2: Distribution of baseline CK by gender.**

Levels of CK were also associated with BMI, such that 48.3% (168/320) persons with a BMI ≥ 25 (overweight) had CK >150 U/L compared to 34.5% with a BMI < 25 (Chi Square = 1.54; p < 0.001). Thus, persons with a BMI ≥ 25 were 1.4 times more likely to have a CK value above the upper limit of normal in our laboratory.

**CK and Genetics: IL6**

All samples were genotyped for the IL6 C-174G polymorphism. Overall, the genotypic frequency was 41.0% GG, 43.3% GC, and 15.7% CC; the frequency of the C allele was 0.37. The genetic distributions for the entire sample population and each of the four ethnic groups were in Hardy-Weinberg equilibrium. Figure 3 presents the genotype distributions by ethnicity: the percentage of CC genotypes was significantly higher in CA than AA, AS, and HI ( $p < 0.001$ ). Likewise, the frequency of the C allele differed, with frequencies of 0.42 for CA, 0.12 for AA, 0.21 for AS, and 0.25 for HI.



**Figure 3: Genotype frequency for the IL6 C-174G polymorphism for Caucasians (CA), African Americans (AA), Asians (AS) and Hispanics (HI).**

Concentrations of CK differed by genotype. Figure 4, presents CK values by genotype: when the entire population was examined CK levels were significantly higher in GG as compared to CC and CG genotypes. When the analyses were conducted for CA alone, given the larger sample size, and the finding was maintained. CK levels were  $159 \pm 133$  U/L for C+ genotypes and  $220 \pm 598$  U/L for the GG genotype ( $p < 0.05$ ). In contrast, when the data for AA, AS, and HI were combined, CK differences by genotype were not significant, in part because of the small number of CC genotypes. Although combining the CC and CG resulted in higher CK values for the GG genotype ( $435 \pm 1,236$  U/L; median 171) than the C+ genotype ( $206 \pm 182$  U/L; median = 158), the results were not significant ( $p=0.15$ ). However, our sample sizes for the AA and HI are small, and an ethnic difference in genotype frequency may exist that we were not able to detect.



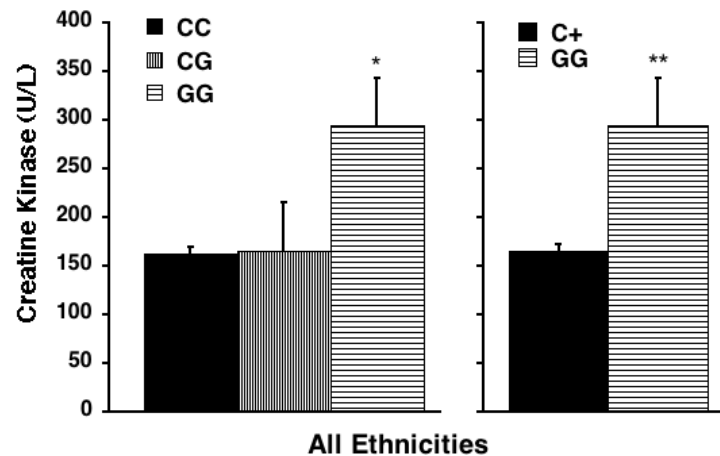


Figure 4: Serum CK levels by IL6 genotype for all ethnicities (\* $p < 0.01$  and \*\* $p < 0.01$ ).

Interestingly, the IL6 -174 GG genotype was also associated with higher BMI, such that 46.3% (99/214) of persons with the IL6 GG genotype had a BMI  $\geq 25$  compared with 34.7% and 35.1% of persons with the CC and CG genotypes, respectively ( $p = 0.02$ ). When the CC and CG genotypes were combined, similar findings were noted: 46.3% of those with the GG genotype had BMI  $\geq 25$  compared with only 35% (141/403) of the other genotypes ( $p = 0.006$ ). Thus, persons with the IL6 GG genotype are 32% more likely to have a BMI  $\geq 25$  than persons with other genotypes. When ANOVA was used to analyze differences in BMI by genotype, the BMI means ( $\pm$  SD) were  $24.4 \pm 2.6$ ,  $24.6 \pm 3.2$ , and  $25.0 \pm 3.4$  for CC, CG, and GG, respectively ( $p=0.235$ ). However, our range of BMI was limited and the 90<sup>th</sup> percentile was 28.5. Thus, the distribution of BMI was weighted to BMI  $< 25$ .

## DISCUSSION

Serum levels of CK have been and continue to be used widely by physicians to assess various aspects of health and disease (4, 7, 14, 15, 18, 21, 23). In particular it is used in the military setting to diagnose and manage muscle damage, exertional rhabdomyolysis, and to detect various myopathies. However, the large variability in baseline levels may reflect prior exercise (23), muscle damage (7, 18), a metabolic myopathy (34), or any number of other factors. The present study provides support for differences in CK levels at baseline to reflect ethnicity, gender, BMI, and genetics, in particular, the IL6 GG genotype of the C-174G polymorphism. These findings indicate that the military might consider developing different normal ranges based on ethnicity, gender, and BMI. Further when CK is used for a clinical diagnosis, these factors need to be considered.

Although it has been known for years that CK levels vary among the population, the clear recognition of ethnic and gender differences has only been recent (6, 21). Interestingly Olerud et al. (25) investigated Marine recruits and reported higher CK levels among AA, but the findings had minimal impact on the range of normal values. Recently Brewster et al. (6) noted that an upward adjustment of the reference interval was necessary for the entire population, with median values of 110 and 72 U/L for CA men and women, respectively and medians of 213 and 124 U/L for AA men and women, respectively. Interestingly, their medians for South Asian men and women were 143 and 87 U/L; our values were 154 and 96 U/L. Thus our values are consistent with their data. Neal et al. (21) also reported significant ethnic and gender differences in

CK levels and that levels for young AA men exceeded the upper limit of normal. Again our findings in a young military population are consistent with theirs and indicate that medical providers must use caution when interpreting elevated CK levels, particularly in AA men.

In addition to ethnicity and gender, we found that persons with a BMI of 25 and over were more likely to have high baseline CK levels. Although BMI is used to classify persons as normal weight, overweight, and obese, having a BMI of 25 or more does not imply excess body fat, and in fact may be associated with muscle bulk and more lean body mass. Swaminathan et al. (32) had earlier reported a significant association between lean body mass and CK levels in health men and women, but since measures of lean body mass were not available, we cannot assume our BMI association with baseline CK reflects more lean muscle mass. It is, however, a reasonable explanation for the finding. In addition to body mass, others have noted that CK levels are influenced by age (5, 32), but our limited age range prohibited any such analyses.

Of great interest was the finding of an association between the IL6 C-174G polymorphism and baseline CK. In particular, we found that persons with the GG genotype had significantly higher baseline CK values than either the CC or the CG genotypes. However, this finding was strongest among CA. A significant ethnic difference in genotype distribution was noted, with CA having significantly more CC alleles than either AA, AS or HI. Our frequency data are consistent with the literature: our C allele frequency of 0.42 is comparable to others for CA 0.36 (1), 0.41 (22), 0.40 (9), 0.42 (35) and 0.40, (30). Likewise, our allele frequency for AA of 0.12 is consistent with the literature, despite our small (n=44) sample size. Others have reported frequencies of the C allele as 0.09 (22), 0.05 (9), and 0.09 (35). Importantly all of our genotyping data were in Hardy-Weinberg equilibrium. Although Yamin et al. recently reported that the CC genotype was associated with exaggerated CK responses to exercise (37), inspection of their data and a letter to the editor (19) suggest potential technical problems with their genotyping of the IL6 C-174G polymorphism: the C allele frequency in their Israeli Caucasian sample was only 0.20 and the percentage of GG was 63%. Their frequency data are not consistent with either our data or the published literature (1, 9, 22, 30, (35)).

Our finding that the association between this IL6 SNP and baseline CK was significant for the entire population and for CA, but not for AA, AS, and HI is of interest. The total sample for the non-Caucasians was certainly smaller (n=171) than the total sample of CA (n=610), so the lack of significance may reflect sample size limitations. The findings may also reflect true ethnic differences, but AA do have significantly higher baseline CK levels than the other ethnic groups, and the frequency of the G allele and GG genotype was highest among this sample. A larger sample size would be required to address whether or not the GG genotype can account for a certain percentage of variability in CK levels.

The IL6 C-174G polymorphism was of particular interest because this inflammatory cytokine may have multiple biologic implications in diverse conditions, including cardiovascular disease, glucose intolerance, and transcription of inflammatory mediators (35). Additionally, IL6 levels increase significantly in response to strenuous exercise and have been associated with muscle soreness and swelling (20). Thus, it was a plausible candidate gene to examine with respect to CK. Although several studies have found that the GG genotype is associated with higher IL6 levels, (9, 11, (16)) and appears to modulate the inflammatory response to surgeries (11), this finding is not consistent. Sie et al. (30) in a study of over 3,800 persons did not find any difference in baseline IL6 levels. Thus, the complex interactions between the promoter region polymorphism of the IL6 gene, ethnicity, gender, age, body composition, other genes, as well as other potential mediators remain to be uncovered.

Our finding of an association between BMI and IL6 genotype was significant, but the strength of the association is unclear. A number of studies have shown that the IL6 C-174G polymorphism is related to

obesity (2, 12, 17, 28), but the data are inconsistent. Riikola *et al.* (29) recently reported that men, but not women with the CC genotype have the highest BMIs, whereas Klipstein-Grobusch *et al.* noted the association was stronger in women (17). Recently, a large study conducted by Huth *et al.* (16) concluded the association between IL6 C-174G and BMI or interleukin-6 levels were only evident in some subgroups. Finally, Qi *et al.* (28) reported that although variability of the IL6 gene was related to adiposity, it was unlikely the associations were a result of the IL6 C-174G polymorphism.

Several limitations to this study must be addressed. First, the data were derived from three independent study populations. However, this should not have affected any of the variables of interest. Secondly, exercise levels were not carefully controlled because most of the participants were beginning military training, which is intense physically. Thus the baseline values may not be true “baseline” samples. However, all were taken early in the morning, prior to any exercise. Another limitation mentioned earlier is our sample size for AA and HI; both were less than 50. However, our data for the AA are consistent with the literature, both with respect to CK levels and allele/genotype frequency. Thus, we are comfortable with our conclusions.

In summary, although CK is used for various clinical diagnoses, interpretation of the results requires careful consideration of a number of factors: gender, ethnicity, body mass index, prior physical activity, and perhaps in the future, genetics. It is very clear that CK levels in AA are significantly higher than those found in CA, AS, or HI, and that women have lower values than men. Thus, ethnic- and gender-specific ranges for CK may assist operational medicine in the diagnosis of ER and potentially susceptibility to ER. Although routine genotyping for the IL6 C-174 G polymorphism is not currently conducted, our data indicate this polymorphism influences baseline CK, such that persons with the GG genotype have higher baseline CK values than those with CC or CG genotypes. It is also interesting that AA, with a preponderance of the GG genotype, have higher baseline CK levels than other ethnic/racial groups. Whether the GG genotype predisposes to ER is unknown. Further research will be required to address a causal relationship and the implications of these findings. Lastly, our data suggest that a combination of factors interact to account for the variability in baseline CK values.

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