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RESEARCH ARTICLE

# Association of Calpastatin (CAST) Gene Polymorphism with Weaning Weight and Ultrasonic Measurements of Loin Eye Muscle in Kıvırcık Lambs [1]

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## Summary

This study was to investigate the association of Calpastatin (CAST) gene with carcass quality characteristics in Kıvırcık lambs, which are important in Turkey in terms of meat production and quality. It was found that allele M of Calpastatin locus was the most common allele. MM, MN and NN genotype frequencies were 72.91%, 22.66% and 4.43%, respectively. This SNP was associated with backfat thickness and skin+backfat thickness values of loin eye muscle (*Musculus longissimus thoracis et lumborum*-MLD) and average daily gain (P<0.05). Live weight, average daily gain, backfat thickness and skin+backfat thickness mean values were found to be lower in animals with NN genotype when compared to those with MM and MN genotype. The results showed that Calpastatin gene affected back fat and skin+backfat and that they had less fatty carcass than those with NN genotype.

***Keywords:*** *Kıvırcık, CAST, Ultrasonic measurement, MLD, Weaning weight*

# Kıvırcık Kuzularda Calpastatin Gen Polimorfizmi ve Sütten Kesim Ağırlığı ve Ultrasonik Göz Kası Ölçümleri İle İlişkisi

## Özet

Bu çalışmada Türkiye'de önemli bir et tipi koyun olan Kıvırcık ırkı kuzularda karkas kalite karakteristikleri ile Calpastatin geninin ilişkisi araştırılmıştır. Kıvırcık kuzularda Calpastatin lokusunun M allelinin en yaygın allel olduğu tespit edilmiştir. MM, MN ve NN genotipleri için genotip frekansları sırasıyla %72.91, %22.66 ve %4.43 olarak bulunmuştur. Bu tek nokta mutasyonunun (SNP) bel gözü kasına (*Musculus longissimus thoracis et lumborum*-MLD) ait yağ kalınlığı ve deri+yağ kalınlığı değerleri ve günlük canlı ağırlık artışı ile ilişkili olduğu ortaya konmuştur (P<0.05). Canlı ağırlık, ortalama günlük canlı ağırlık artışı, yağ kalınlığı ve deri+yağ kalınlığı ortalamaları NN genotipine sahip hayvanlarda MM ve MN genotipine göre daha düşük bulunmuştur. Elde edilen bulgular yağ kalınlığı ve deri+yağ kalınlığı üzerine Calpastatin allelerinin etkili olduğunu ve NN genotipi taşıyanların diğerlerine göre daha yağsız karkasa sahip olduğunu göstermektedir.

***Anahtar sözcükler:*** *Kıvırcık, CAST, Ultrasonik ölçüm, MLD, Sütten kesim ağırlığı*

## INTRODUCTION

Kıvırcık lamb is known for its meat quality in Turkey. Although there is a limited body of research to scientifically support this information, in a study carried out by Ekiz et al.[1], Turkish Merino, Ramlıç, Kıvırcık, Sakız and Imroz breeds were compared in terms of meat quality. The findings of the study revealed that Kıvırcık breed was superior to other breeds in terms of various meat quality characteristics.

Today lean meat is highly demanded in the market. For this reason, ultrasound technology that is developed to identify carcass condition, in other words, carcass composition and quality in animals that will be marketed, allows for the identification of carcass characteristics in live animals in a rapid and economic manner without giving the animal any harm. Ultrasound measurements in live animals have a practical value to provide selection of

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certain carcass characteristics according to measurement criteria for breeding and to estimate the optimum slaughter or marketing period [2-6].

In recent years certain highly effective genes that affect meat yield and quality were identified. Calpastatin gene is one of these genes. Calpastatin (CAST), is the endogenous and specific inhibitor of Calpains, inhibits the calpain activity in post-mortem tissue and thus regulates the rate and extent of post-mortem meat tenderization. Therefore, CAST might be a potential candidate gene to control the development of farm animals [7]. This gene is located on the fifth chromosome of sheep genome. Calpastatin and Calpain were shown to have significant effects on live weight and meat quality. CAST gene was identified in sheep genome for the first time by Palmer et al.[8]. The study based on PCR-RFLP found that Dorset sheep had two different alleles (M and N) of CAST gene. Previous studies reported that Calpastatin gene affected growth characteristics and meat hardness [9-12].

This study analyzed the effect of CAST gene on live weight, average daily gain and some ultrasonic measurements in loin eye muscle *(Musculus longissimus thoracis et lumborum-MLD)* (backfat thickness, skin+backfat thickness, muscle depth, muscle width). A review of the literature was found no study to associate Calpastatin gene with carcass quality characteristics in Turkish sheep breeds. This study will significantly contribute to genomic selection works.

## MATERIAL and METHODS

All procedures were approved by local ethical committee of Adnan Menderes University (124-HEK/2009/53 Date: 02.09.2009).

The animal population was consisted Kıvırcık lambs (n=203) in 10 integrated flocks within the scope of TÜBİTAK-KAMAG 1007 project (Project No. 109G014). Sampling location and sample size of Kıvırcık lambs are given in *Table 1*.

Blood samples were collected from the animals into vacuumed tubes containing K3-EDTA. The samples were stored at -20°C until utilization. The DNA was isolated with DNA isolation kit (Applied Biological Materials Column-Pure Blood Genomic DNA Kit, Canada) from blood samples according to manufacturer's instructions in the Adnan Menderes University Faculty of Agriculture Department of Animal Science, Genetic Labaratory, Aydın. Quantity and quality of the DNA were checked with NanoDrop 2000 spectro-photometer (Thermo Scientific, USA).

Genotypes were identified in terms of Calpastatin gene using the Restriction Fragment-Length Polymorphism (PCR-RFLP) method and employing primer pair reported by Khederzadeh [13] (CAST F: 5'-CCTTGTCATCAGACTTCACC-3', CAST R: 5'-ACT GAG CTT TTA AAG CCT CT-3'). A PCR mixture containing dNTP (0.2 mM), $MgCl_2$ (2.0 mM), primers (0.25 µM), PCR buffer (1X) and Taq DNA polymerase and 100 genomic DNA and $ddH_2O$ with a total volume of 25 µl was prepared for Polymerase Chain Reaction.

***Table 1.*** *Sampling location and sample sizes for Kıvırcık lambs*
***Tablo 1.*** *Kıvırcık kuzularda örnekleme lokasyonları ve örnek sayıları*

| Farm ID | Location | N |
|---|---|---|
| 1 | Kıran/Eşme/Uşak | 28 |
| 2 | Ahmetler/Eşme/Uşak | 23 |
| 3 | Yeleğen/Eşme/Uşak | 17 |
| 4 | Yeleğen/Eşme/Uşak | 30 |
| 5 | Ahmetler/Eşme/Uşak | 30 |
| 6 | Ahmetler/Eşme/Uşak | 15 |
| 7 | Güllübağ/Eşme/Uşak | 13 |
| 8 | Yeleğen/Eşme/Uşak | 16 |
| 9 | Oymalı/Eşme/Uşak | 16 |
| 10 | Güllübağ/Eşme/Uşak | 15 |
| **Total** | | **203** |

The PCR cycling condition was a preliminary denaturizing at 95°C for 2 min, followed by 1 cycle, denaturing at 95°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 2 min followed by 35 cycles and 10 min at 72°C as a final extension. The PCR reactions were performed on the ABI Veriti thermocyler. The corresponding PCR products were amplified 565 bp fragments.

Amplified DNA regions were digested with *Msp*I restriction enzyme (Fermentas) for genotyping. For restriction digestion, 3 µl of 10X Buffer Tango, 0.50 µl of $ddH_2O$ and 1.50 µl of *Msp*I (Fermantas) enzyme were added to the PCR products (25 µl) and this mix were incubated at 37°C for at least 6 h. DNA fragments were separated in 2% agarose gel. The fragments were imaged and genotypes were identified.

The lambs were monitored form the time of birth to the time of weaning (mean age 3.5 months). Live weight of the lambs were determined using electronic scale with a sensitivity of 50 g in the time of marketing and average daily gain increase was calculated until the time of birth to the time of weaning. Measurements on the characteristics of MLD were conducted on the area between 12. and 13. ribs using a linear probe (8 MHz) with a scanning area of 6 cm in an ultrasound device (Pie Medical Falco 100). The characteristics of MLD were determined to be backfat thickness (BFT), skin+backfat thickness (S+BFT), muscle depth (MD) and muscle width (MW) *(Fig. 1)*.

Allele and genotype frequency analysis and chi-square ($\chi 2$) test were carried out using GenAlEx [14] and Popgene32 [15]

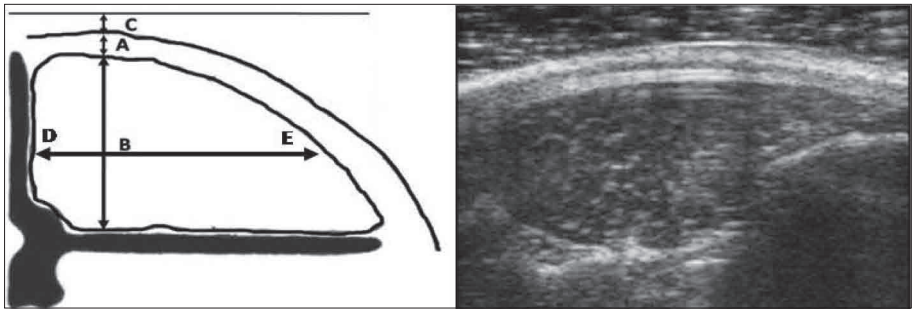


**Fig 1.** Measured properties belong to MLD (A: Backfat thickness, B: Muscle depth, A+C: Skin + Backfat thickness, D-E: Muscle width) and ultrasound imaging of MLD

**Şekil 1.** Ölçülen MLD kasına ait özellikler (A: Yağ Kalınlığı, B: Kas Derinliği, A+C: Deri+Yağ Kalınlığı, D-E: Kas Genişliği) ve ultrason görüntüsü

programs. Analysis of variance was conducted to investigate effects of genotypes on Weining Weight (WW), Avarage Daily Gain (ADG), Backfat Thickness (BFT), Skin with Backfat Thickness (S+BFT), Muscle Depth (MD) and Muscle Width (MW). Least Squares means and standart errors estimated using the GLM (Generalized Linear Models) procedure of SAS [16] according to followning linear models:

*Model for weaning weights of lambs:*

$$y_{ijkl}=\mu+a_i+b_j+c_k+b_1(X_{ijkl}-X)+e_{ijkl}$$

*Model for ultrasonic measurements*

$$y_{ijkl}=\mu+a_i+b_j+c_k+b_1(Q_{ijkl}-\bar{Q})+e_{ijkl}$$

*Where*

$a_i$ = Fixed effect of genotype (i=MM, MN and NN)

$b_j$ = Fixed effect of bith type (j=single, twin and triplets)

$c_k$ = Fixed effect of gender (k=male and female)

$b_1$ = Regression coefficient of lamb age at weaining on weaning weight

$b_2$ = Regression coefficient of weaning weight on ultrasonic measurements

$X_{ijkl}$ = Age of lamb at weaning

$\bar{X}$ = Mean lamb age at weaning

$Q_{ijkl}$ = Weaning weight of lamb

$\bar{Q}$ = Mean weaning weight of lambs

$e_{ijkl}$ = Random errors with the assumption of N (0, $\sigma^2$)

# RESULTS

The amplified region of calpastatin gene produced a 565 bp DNA fragment using MspI enzyme in RFLP method. DNA bands obtained from PCR-RFLP were imaged by separation in 2% agarose gel *(Fig. 2)*. Two alleles (M and N) and subsequently three genotypes observed. Bands with different lengths produced as a result of a single point mutation (CCGG → CCAG) in the calpastatin gene, which removes the MspI restriction cut site (...C▼CGG...). The cut area disappeared and no cut took place due to point mutation that occurred in cut region of the enzyme. Thus, in individuals with this situation, only a single band at 565 base pairs was observed and these individuals were genotyped as NN. Due to a mutation only in one of the alleles the individuals showing three bands at the length of 565, 306 and 259 base pairs were genotypes as MN, while the individuals showing two bands at the length of 306 and 259 were genotypes as MM.

Allele frequencies, genotype frequencies, observed (Ho) and expected heterozygosity (He) values obtained from the study and the results of Chi-square test performed for Hardy-Weinberg equilibrium are presented in *Table 2*.

The frequency of alleles M and N in Kıvırcık lambs were found to be 84.24% and 15.76% respectively. This result indicated that allele M was more common in the populations. Although all three of MM, MN and NN genotypes were observed in Kıvırcık breed. It was found that MM genotypes had the highest genotype frequency (72.91%). Observed heterozygosity (Ho) and expected heterozygosity (He) values of Calpastatin gene were found to be 0.227 and 0.266 respectively. Furthermore, it

**Fig 2.** Genotyping for CAST gene by the PCR-RFLP analysis

**Şekil 2.** PCR-RFLP yöntemi ile CAST geninin genotiplenmesi

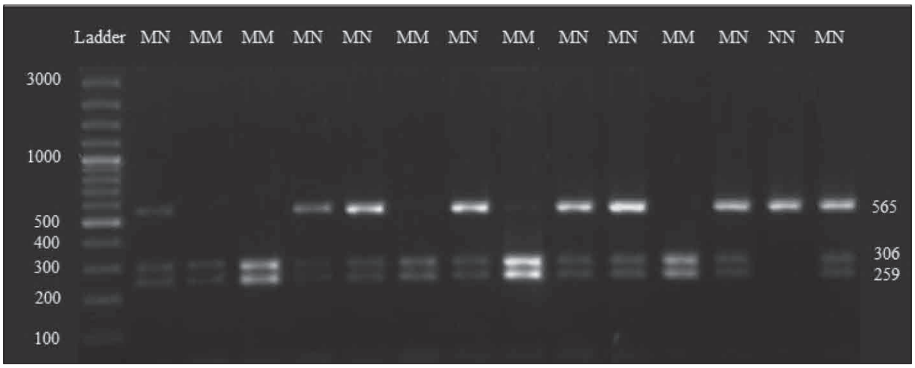

***Table 2.*** *Allele and genotype frequencies, observed (Ho) and expected heterozygosity (He) and Chi-Square test values for the Hardy Weinberg equilibrium belong to Calpastatin locus*

***Tablo 2.*** *Calpastatin lokusuna ait allel ve genotip frekansları, gözlenen (Ho) ve beklenen heterozigotluk (He) ve Hardy Weinberg dengesi için Ki-kare test değerleri*

| Locus | N | Allele Freq (%) | | Genotype Freq (%) | | | Heterozygosity | | |
|---|---|---|---|---|---|---|---|---|---|
| | | M | N | MM | MN | NN | Ho | He | $\chi^2$ |
| CAST | 203 | 84.24 | 15.76 | 72.91 | 22.66 | 4.43 | 0.227 | 0.266 | 4.372* |

** P<0.05*

***Table 3.*** *Least square means and standard errors for ultrasound measurements of MLD*

***Tablo 3.*** *MLD kasının ultrasonik ölçümlerine ait en küçük kareler ortalamaları ve standart hataları*

| Genotype | N | WW (kg) | ADG (kg) | BFT (cm) | S+BFT (cm) | MD (cm) | MW (cm) |
|---|---|---|---|---|---|---|---|
| | | P=0.595 | P=0.036 | P=0.022 | P=0.031 | P=0.270 | P=0.266 |
| MM | 148 | 25.52±0.502 | 0.22±0.002 | 0.21±0.007 | 0.54±0.010 | 1.75±0.020 | 3.63±0.031 |
| MN | 46 | 25.94±0.765 | 0.22±0.004 | 0.22±0.011 | 0.56±0.015 | 1.80±0.030 | 3.71±0.047 |
| NN | 9 | 24.13±1.673 | 0.20±0.008 | 0.15±0.024 | 0.46±0.032 | 1.75±0.065 | 3.66±0.101 |
| **BirthType** | | P=0.032 | P=0.008 | P=0.487 | P=0.270 | P=0.080 | P=0.760 |
| 1 | 84 | 26.51±0.747 | 0.21±0.004 | 0.18±0.011 | 0.51±0.014 | 1.79±0.029 | 3.65±0.046 |
| 2 | 100 | 24.69±0.716 | 0.20±0.003 | 0.19±0.01 | 0.51±0.013 | 1.73±0.027 | 3.68±0.042 |
| 3> | 19 | 24.40±1.214 | 0.22±0.006 | 0.21±0.018 | 0.54±0.023 | 1.78±0.048 | 3.67±0.074 |
| **Gender** | | P=0.000 | P=0.199 | P=0.838 | P=0.108 | P=0.014 | P=0.403 |
| Male | 112 | 26.43±0.740 | 0.21±0.004 | 0.19±0.011 | 0.51±0.014 | 1.73±0.029 | 3.65±0.045 |
| Female | 91 | 23.97±0.766 | 0.21±0.004 | 0.20±0.011 | 0.53±0.015 | 1.80±0.030 | 3.68±0.047 |
| **Reg. Linear** | | | P=0.000 | P=0.000 | P=0.000 | P=0.000 | P=0.000 |
| WW | | - | 0.007±0.000 | 0.008±0.001 | 0.011±0.001 | 0.024±0.002 | 0.025±0.004 |
| | | P=0.000 | | | | | |
| Age | | 0.204±0.025 | | | | | |
| **General** | 203 | 25.20±0.669 | 0.21±0.003 | 0.19±0.01 | 0.52±0.013 | 1.77±0.026 | 3.67±0.041 |

***WW:*** *Weaning weight,* ***ADG:*** *The average daily gain,* ***BFT:*** *backfat thickness,* ***S+BFT:*** *Skin with backfat thickness,* ***MD:*** *Muscle depth:* ***MW:*** *Muscle width*

was observed that the population which was analyzed in terms of this gene was not at Hardy-Weinberg equilibrium. Least squares mean and standard errors obtained from marketing and weaning live weight, average daily gain and ultrasonic measurements of MLD are presented in *Table 3*.

It was found that there was a statistically significant difference (P<0.05) between the genotypes in terms of average daily gain (ADG) and backfat thickness (BFT), skin+backfat thickness (S+BFT) values among ultrasound criteria. There was no statistically significant difference between the genotypes for WW, MD and MW.

There was a statistically significant difference between types of birth in terms of weaning weight (WW) (P<0.05) and ADG (P<0.01). However, there was no statistically significant difference in terms of other characteristics (P>0.05). It was found that lamb age, which is considered as a covariate had a significant effect on lamb weaning weight (P<0.01). Lamb weaning weight had a significant effect on ultrasound measurement parameters.

# DISCUSSION

It was found that birth type and sex had a significant effect on weaning weight. Only birth type was found to have a significant effect on live weight gain. Gender had a statistically significant effect on muscle depth. These results are consistent with literature data [3,17].

Our findings showed that allele N had rather low frequencies while allele M had high frequencies. It was observed that 15.76% value we obtained for allele N was slightly lower than the values obtained in Atabi (19%), Kajli (19%), Mutton (19%), Dalagh (20%), Karakul (21%), Polish Merino (24%), Lori (36%) and Zel (25%) breeds [13,18-23]. However, this value was higher than the values obtained from Valachian (3%), Ile de France (5%), Berrichondu Cher (7%), Tsigai (9%), Tsigai x Lacaune (10%), Thalli (10%), Balkhi (12%), Lohi (13%), Kajli (14%) and Arabic (15%) sheep [9,22-25]. On the other hand, in a study carried out on Lacaune and Eastern Friz breed, no allele N was found [24].

Analysis of the results on genotypes showed that NN genotypes had rather low frequencies while MM genotype was more common in populations. In a study carried by Ata and Cemal [26] which analyzed Calpastatin gene polymorphism in Çine Çaparı and Karya breeds, the frequency of MM, MN and NN genotypes were found to be 0.543, 0.388 and 0.069 in Çine Çaparı and 0.296, 0.496 and 0.208 in Karya sheep respectively. Allele and genotype frequencies we obtained showed slight variations when compared to other studies. These variations can be attributed to the use of different breeds in the studies.

Our findings raise suspicion that there is a selection process against NN genotype. Considering this situation, the fact that this population is not at Hardy-Weinberg equilibrium in terms of Calpastatin gene appears as a natural result. Least squares means of ADG, BFT and S+BFT values revealed that the animals with NN genotype had lower values than other genotypes. This appears as a concrete indicator of the process against NN genotype.

Research in this subject mainly concentrated on identification of Calpastatin gene in populations. A review of the literature also found studies associating the gene with phenotypic characteristics [25]. Those studies reported that Calpastatin gene affected live weight and average daily gain values [25,27]. The results of previous studies on live weight and average daily gain showed that the distinction between the genotypes was statistically different and that the animals with MM and MN genotype showed a better performance than those having NN genotype. Studies on other breeds reported that there was a statistically significant difference between Calpastatin genotypes in terms of live weight, weaning weight and average daily gain [25,27,28]. Similarly, our results are consistent with the findings in the study of Sutikno et al.[28] on Indonesia local sheep breed. However, in a study carried out on Romney sheep, it was reported that Calpastatin gene did not affect average daily gain [7].

At physiologic level, Calpastatin gene is an endogenous inhibitor of calpains. Page et al.[29] reported that calpains played an initial role on hardness degree of meat during rigor mortis after the slaughter by destructing myofibrillary proteins. Various researchers who studied meat hardness quality especially in cattle, analyzed physiologic role of Calpastatin on meat hardness together with Calpastatin gene and reported that Calpastatin gene affected the tenderness of meat [11,30-34].

According to the results from this study the investigated population showed a low degree of genetic variability in terms of Calpastatin alleles. This might be explained by only a few rams used as sires in the flock.

A review of literature found no study on the association between MLD characteristics and CAST gene in evaluated lambs. The results showed that Calpastatin alleles affected back fat and skin+backfat and that they had less fatty carcass than those with NN genotype.

There has no study that associated Calpastatin gene and yield characteristics in domestic sheep populations in Turkey. Therefore, this study will make a significant contribution to the literature. The study analyzed the effects of Calpastatin gene on weaning weight, average daily gain and MLD characteristics in Kıvırcık lambs which are important in Turkey in terms of meat quality.

Studies on Calpastatin gene using ultrasonic measurements, that allow for doing selection according to measurement criteria to breed carcass characteristics in live animals, will enhance accuracy of genetic parameters. In this context, the findings reveal that this gene can be an important major gene that can be used in selection programs for meat quality and that they can be reliably used in selection indexes.

Investigation of the region of this gene in a large material using DNA sequence analysis and association of the phenotype data of meat yield and quality characteristics of the emerging polymorphism will significantly contribute to future genomic selection studies.

Further studies on development characteristics, meat quality and genetic analyses in Kıvırcık breed, which are of great importance in terms of meat quality, will provide more concrete data on the functioning mechanism of this gene.

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