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# Is It Necessary to Add Aprotinin Before Measuring The Level of Irisin in Serum and Plasma Samples?

# Serum ve Plazma Örneklerinde İrisin Seviyesini Ölçmeden Önce Aprotinin İlave Etmek Gerekir mi?

Elif Sahin[1*], Ecem Handiri[1], Diler Us-Altay[2], Esref Edip Keha[3]

[1]*Karadeniz Technical University, Graduate School of Medical Science, Department of Medical Biochemistry, Trabzon, Turkey.*
[2]*Ordu University, Faculty of Health Sciences, Department of Nutrition and Dietetics, Ordu, Turkey.*
[3]*Karadeniz Technical University, Faculty of Medicine, Department of Medical Biochemistry, Trabzon, Turkey.*

**Corresponding author e-mail: elifsahin@ktu.edu.tr*

[1]*https://orcid.org/0000-0001-5864-9548*
[2]*https://orcid.org/0000-0001-2345-6789*
[3]*https://orcid.org/0000-0002-0465-8403*
[4]*https://orcid.org/0000-0002-2271-5811*

## ABSTRACT

Irisin is a myokine with 112 amino acids and its blood concentration is regulated by peroxisome proliferator-activated receptor-γ coactivator1-α (PGC1-α). It is released into circulation from skeletal muscle tissue after a photolytic cleavage of extracellular domain of Fibronectin type III domain-containing protein 5 (FNDC5), a type I integral membrane protein. Aprotinin is a polyvalent serin protease inhibitor. It is added to sample solutions such as serum, plasma or tissue extracts in order to inhibit serine proteases found in the sample medium. Hence, degradation of the proteins to be measured can be prevented. This study has been carried out to obtain a preliminary data if any irisin loss could be seen in the serum samples which are kept at -80°C for a long duration. For this purpose, blood samples of 10 men and 10 women volunteers aged between 25-40 has been used. Aprotinin has been added to the plasma and the serum samples have been kept at -80°C for 3 months. At the end of 3 months, irisin levels of the samples with and without aprotinin have been determined by ELISA. Statistical analysis has shown no difference between the plasma samples with or without aprotinin (p=0.525). However, a significant decrease between the serum samples with and without aprotinin (p=0.009). In conclusion, with the results of this study, no net decision could have been achieved to add aprotinin to the samples for irisin determination with ELISA in plasma and serum kept at -80°C for about 3 months.

**Keywords:** Aprotinin, Irisin, Serine protease

## ÖZET

İrisin, miktarı peroksizom proliferatörü ile aktive edilen reseptör gama koaktivatör1-α (PGC1-α) tarafından düzenlenen, 112 aminoasitten oluşan bir miyokindir. Bir tip I integral membran proteini olan fibronektin tip III domeini içeren protein 5'in (FNDC5) proteolitik parçalanması sonucu iskelet kası dokusundan kan dolaşımına salınır. Aprotinin çok değerlikli bir serin proteaz inhibitörüdür. Serum, plazma veya hücre homojenizatı gibi sulu çözelti örneklerine eklenerek, ortamda bulunan serin proteaz enzimlerini inhibe eder. Bu şekilde, ölçümü yapılacak olan proteinlerin parçalanması önlenir. Çok defa -80°C'de uzun süre saklanma ihtiyacı duyulan örneklerde irisin kaybının olup olmayacağı hakkında bir ön bilgi elde etmek amacı ile bu çalışma yapılmıştır. Bu amaçla, 25-40 yaş arasında 10 erkek ve 10 kadın gönüllüden kan örnekleri alınmış ve bunlardan elde edilen plazma ve serum örneklerine aprotinin eklenerek 3 ay -80°C'de saklanmıştır. 3 ay sonra, ELISA ile aprotininli ve aprotininsiz örneklerde irisin seviyeleri ölçülmüştür. Sonuçların istatistiksel değerlendirilmesiyle, aprotininli ve aprotininsiz plazma örneklerindeki irisin miktarlarında önemli bir farklılık olmadığı gözlemlenirken (p=0.525), aprotininli ve aprotininsiz serum örneklerindeki irisin miktarlarında anlamlı bir azalma bulunmuştur (p=0.009). Sonuç olarak, ELISA ile irisin tayin edilmek üzere 3 ay civarında -80°C'de saklanan plazma ve serum numunelerine aprotinin eklenmesinin gerekip gerekmediği konusunda net bir kanaate varılamamıştır.

**Anahtar Kelimeler:** Aprotinin, İrisin, Serin proteaz

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## INTRODUCTION

Irisin was first isolated from muscle tissue in 2012 by Boström and colleagues. Irisin is released from the muscles through the proteolytic cleavage of FNDC5 that is regulated by PGC1-α and known as a type of "Type 1" membrane protein. Irisin, consists of 112 amino acid, converts white adipose tissue to brown adipose tissue and mediates energy consumption.[1] PGC1-α is a transcription coactivator that mediates many biological activities related to energy metabolism. This coactivator regulates the expression of "uncoupling" protein 1 (UCP1) and contributes to the formation of thermogenesis in brown adipose tissue. Expression of PGC1-α enables the formation of FNDC5 and secretion of the irisin in the muscles and rapidly increases FNDC5 during muscle contraction.[2] Exercise quickly and strongly increases the expression of PGC1-α, but this increase is temporary. Because both the mRNA level and the level of PGC1-α quickly return to their original state before exercise.[3] Exercise also activates AMPK, the main regulator of cellular and organismal metabolism. AMPK activates PGC1-α by phosphorylation. Activated PGC1-α causes an increase in FNDC5 and irisin is released from muscle cells by leaving the carboxy terminal of FNDC5. While light and short-term exercises create temporary rises in PGC1-α, long and strong exercises cause permanent elevation.[4] The irisin binds to the not yet known receptors in the white adipose tissue and converts it into brown adipose tissue. Cells of brown adipose tissue contain high amounts of lipid droplets and high numbers of mitochondria compared to white adipose tissue.[5,6]

Proteases, proteinases or peptidases are enzymes that are essential in the control of the composition, size, form and cycle of the proteins synthesized in the organism and represent approximately 2% of the total amount of proteins present in any organism. These enzymes play a role in vital biological processes such as blood clotting, controlled cell death, and tissue differentiation.[7] Proteases are divided into four mechanical classes: serine proteases, metalloproteases, aspartic proteases and cysteine proteases. In recent years, other members of these protease classes have been tried to be identified by using various protease inhibitors.[7] Serine proteases play an important role in the modulation of cell and extracellular proteins in many biological pathways.[8] Different types of proteases play a role in different disease states. Identifying the inhibitors of these enzymes is important for the development of new therapeutic agents.[9] Serine proteases are inhibited by a number of alpha antitrypsin-like physiological protease inhibitors, preventing unnecessary breakdown of blood proteins.[10] In this study, aprotinin, a strong serine protease inhibitor, was used to prevent the breakdown of the irisin myokine in the blood.[11] When aprotinin is used for medical purposes in human, the Lys-15 amino acid in its structure binds tightly to the active serine region of the target protein, causing inhibition of many proteases such as plasmin, trypsin, kallikrein, chymotrypsin, activated protein C, thrombin and neutrophil elastase.[12] Aprotinin, a powerful antiprotease, is added to the samples studied experimentally to prevent the proteolysis of proteins in plasma and serum during the hiding process.[13]

Recent years, the number of studies on irisin had been increasing. Since the collection of material samples takes a long time in studies, sometimes samples need to be stored in the freezer for a while. One of the most important problems for researchers is the uncertainty about how long the samples should be stored in which environment. Since proteolytic enzymes will always be present in the studied samples, they are expected to degrade the proteins to different degrees depending on the storage conditions and duration. In this study, it is aimed to determine whether aprotinin added to serum and plasma samples that kept in freezer had an effect on irisin levels.

## METHODS

### *Organising of experimental group*

This study was carried out with the participation of individuals from different sociological and economic groups of society. Blood samples were collected from 10 male and 10 female volunteers aged between 25-40 years. In the selection of volunteer individuals, pregnancy, acute and chronic kidney and liver disorders, digestive-absorption disorder, coronary artery disease, diabetes, hypertension, cancer and endocrine disorder, menopause state, regular medication, smoking and alcohol use were determined as exclusion criteria. Volunteers were given the necessary information about the study. Informed consents of the volunteers included in the study were obtained. In order to conduct the study, approval was obtained from the Scientific Research Assessment Commission of Karadeniz Technical University, Faculty of Medicine with the decision number 2014/159 and number 188.

### *Collecting blood samples and preparing for study*

For this study, 4 tubes (2 with EDTA and 2 without EDTA) of blood were collected from 20 healthy people on an empty stomach. Aprotinin was added to one of these tubes with and without EDTA. The amount of aprotinin added is 0.6 TIU (trypsin unit) for one mL of blood. Accordingly, 45 µL was added to the tube with EDTA and 75 µL was added to the one without EDTA. All tubes were centrifuged at 3000 rpm for 10 minutes. Supernatants consisting of plasma and serum were transferred to 1 mL tubes and kept for 3 months at -80°C for irisin measurement.

### *Determination of irisin level in plasma and serum samples with ELISA method*

In the measurement of plasma and serum irisin concentration, serum and plasma measurements were carried out in accordance with the kit protocol using the ELISA kit produced for the human irisin measurement (Sunred cat no: 201-12-5328).

### *Statistical analysis*

Analysis results obtained after the study was expressed as mean and standard deviation (X ± SD). The test results were uploaded to the SPSS (Statistics Program for Social and Science) 13.0.1 (License number: 9069727) statistics program and their compatibility with normal distribution was checked with the Kolmogorov-Smirnov Test. Fits the normal distribution; Paired t test was used for binary comparison of dependent parameters. The $p < 0.05$ value of the results in statistical tests was considered significant.

## RESULTS

Results showed no difference between female and male groups. Hence, all the data were given as a sum all individuals (n=20).

### *Irisin level in serum and plasma samples without aprotinin*

According to the results of the statistical analysis, there is a significant difference between the levels of irisin in serum (12.7±6.30) and plasma (9.56±4.40) samples without aprotinin (p=0.009).

### *Effect of aprotinin addition on the level of the irisin in serum samples*

According to the results of the statistical analysis, it is seen that there is a significant difference between the levels of irisin in the serum samples with and without aprotinin (10.4±4.49 and 12.7±6.30, respectively) (p = 0.008).

### *Effect of aprotinin addition on the level of the irisin in plasma samples*

According to the results of the statistical analysis, there is no significant difference between the levels of irisin in aprotinin and aprotinin-free plasma samples (10.2±4.64 and 9.56±4.40, respectively) (p = 0.525).

## DISCUSSION

To demonstrate the effect of aprotinin, serum and plasma samples were treated with aprotinin. After storing at -80°C for 3 months, irisin measurement was performed in these samples by ELISA method. According to the results, there was no significant difference between male and female groups, both in plasma and serum samples, all were shown in a pool of 20 people. In studies about irisin levels in women and men, different results were found. In a study by Crujeiras et al.,[14] Irisin levels were reported as 353 ± 18.6 ng/mL in women and 267.6 ± 12 ng/mL in men. According to this report, they concluded that "irisin levels are gender-dependent and higher in women than in men". However, in a study by Choi et al.,[15] It was found that serum irisin levels tend to be higher in men than women, but there was no statistically significant difference between both sexes (52.9 ± 32.7, 45.8 ± 30.9, p = 0.063). Therefore, it was concluded that there was no significant difference in serum and plasma samples in terms of gender irisin level without any disease. In this study, serum and plasma samples without aprotinin were evaluated as two dependent groups and a statistically significant difference was found between irisin levels in these groups (p = 0.009). In the literature, no study was found in which the measurement of irisin was measured by ELISA in both serum and plasma samples of the same individuals. Therefore, it was not possible to make a comparison. However, it is thought that this difference may be due to the different cross-interactions with monoclonal antibodies used in ELISA method with different protein types in plasma and serum composition. Plasma and serum samples with and without aprotinin are considered as two dependent groups. The addition of aprotinin to plasma samples did not change the level of irisin and there was no statistically significant difference between the groups (p=0.525). As a preliminary information, it is concluded that the addition of aprotinin to plasma samples is not required for the determination of irisin level by ELISA. It can also be thought that a temperature of -80°C can protect the sample against protease activity within 3

months. Likewise, measurements made on serum samples have yielded different results. According to irisin measurements and statistical evaluation results, the addition of aprotinin to serum samples has been shown to significantly reduce the level of irisin ($p = 0.008$). This decrease may be due to the fact that the aprotinin-protease complex formed by binding of the aprotinin to the serine proteases can bind to the irisin, resulting in incomplete measurement of the irisin by closing the epitope to which the ELISA antibody will bind. In plasma, these aprotinin-protease complexes may be bound to coagulation protein and irisin molecules can be preserved from proteases. However, the results of the present study is not sufficient for a clear conclusion about the necessity of adding aprotinin to serum and plasma samples that will be kept at -80°C for a long time. This is can be discussed within the framework of the properties of ELISA kits used to measure the amount of irisin and number of samples used in the study and the storage time. Also, it is frequently stated by researchers in this field that monoclonal antibodies in ELISA kits produced for the measurement of irisin in serum and plasma samples have different epitopes as well as are marketed without being fully seated.[16] Therefore, methods should be developed to accurately measure the FNDC5/irisin amino acid sequence. Considering all these, the measurement of the irisin by ELISA does not provide a clear accuracy.

## CONCLUSION

In conclusion, with the results of this study, no net decision could have been achieved to add aprotinin to the samples for irisin determination with ELISA in plasma and serum kept at -80°C for about 3 months. However, since the study is planned to derive a preliminary result and the number of samples used in this study is too few, a certain result could not be reached on the necessity of aprotinin. In order to reach a definitive conclusion, the number of samples should be at least 50. Also, for a better judgment, irisin measurements should be made on different times windows such as on the first day of collection of blood samples, after the 3 months and after 1 year preserving at -80°C.

### Authorship contribution statement

Consept and desing: EEK, EH
Acquisition of data: EH, ES, DUA
Analysis and interpretation of data: EEK, EH, DUA
Drafting of the manuscript: ES.
Critical revision of the manuscript for important intellectual content: EEK and ES.
Statistical analysis: ES, DUA.

### Declaration of competing interest

None of the authors have potential conflicts of interest to be disclosed.

### Ethical approval

This study was approved by Karadeniz Technical University, Faculty of Medicine Scientific Research Ethics Committee with the decision number 2014/159 and number 188.

### Availability of data and materials

All data generated or analyzed during this study are included in this published article.


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