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ORIGINAL PAPER

Metabolomics analysis in pterygium tissue

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Abstract
Purpose The aim of the study was to measure amino acid levels with the metabolomics analysis in pterygium tissue and normal conjunctiva tissue.
Materials and methods In this prospective, randomized, clinical study, a comparison of the amino acid profile of pterygium tissue and normal conjunctiva tissue taken during autograft pterygium surgery was made. After homogenization of the tissues, amino acid levels were measured with chromatography–mass spectrometry (LC–MS/MS) in the biochemistry laboratory. Statistical analysis was made using the Wilcoxon signed-rank test.
Results Evaluation of pterygium and normal conjunctiva tissues of 29 patients, comprising 16 females and 13 males with a mean age of 54.75 ± 11.25 years (range 21–78 years) was made. While a dramatic increase was observed in all the amino acid levels in the pterygium tissue compared to the normal conjunctiva ($p > 0.05$), only the increases in arginine, methionine, glycine and tyrosine amino acids were determined to be statistically significant ($p < 0.01$), ($p = 0.028$), ($p = 0.038$), ($p = 0.046$).
Conclusion Pterygium is known to be degenerative inflammatory fibrovascular tissue. When the aetiology is examined in depth, several metabolic processes are seen to have an effect. Further studies of the amino acid profile with more extensive patient series could confirm the data obtained in the current study and contribute to the clarification of the pathogenesis of pterygium.

Keywords Amino acid · Chromatography–mass spectrometry · Metabolomics · Pterygium

Introduction

Pterygium is a fibrovascular mass that starts from the bulbar conjunctiva and with a wing shape extending to the nasal cornea [1, 2]. It can lead to vision loss because of corneal astigmatism and coverage of the visual axis and may result in poor cosmetic outcomes [3].

Due to high recurrence rates, several studies have researched the pathogenesis of pterygium and have reported that genetic and environmental factors such as ultraviolet (UV) damage and human papillomavirus (HPV) infection play a role. These factors lead to

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chronic inflammation and DNA damage with subsequent uncontrolled cell proliferation, tissue invasion and local angiogenesis [4–8].

In current treatment of pterygium, several different surgical techniques are applied to reduce the recurrence rates [2, 9]. Moreover, in the examination of neoplasia-like genetic characteristics, which are thought to have an effect on the high recurrence rate, p53 tumour suppressor gene and K-ras oncogene mutations are known to have a role in the aetiology [10, 11]. The cell metabolism of pterygium shows differences from normal conjunctiva because of these characteristics.

Previous studies have determined differences in the gene expression, cell growth and vasculogenesis in tissues where there is uncontrolled cell growth and in free amino acid levels such as glutamine, glycine, aspartic acid and serine, which are necessary for the synthesis of growth-promoting hormones or tumour growth factors [12–14]. Therefore, metabolomics analysis has an important place in current cancer and metabolic disease researches. Metabolomics is a new method which is used in the qualitative and quantitative measurement of all the metabolites in tissue or biofluid samples [15]. No study could be found in the literature that has examined the effects of the amino acid profile on pterygium tissue. It was thought that metabolomics analysis, which is often used in research of inflammatory, metabolic diseases, and cancer studies, could be an important milestone in the pathophysiology and treatment of pterygium.

In this metabolomics analysis study, it was planned to examine the amino acid levels in pterygium and normal conjunctiva tissues based on chromatography–mass spectrometry (Shimadzu Corporation, Japan).

Materials and methods

This prospective study included patients diagnosed with pterygium who were planned to undergo pterygium excision with conjunctival autograft transplantation. Informed consent was obtained from all the patients before the procedure. Approval for the study was granted by the Ethics Committee of the Faculty of Medicine at Harran University, Şanlıurfa, Turkey. This study was conducted in accordance with the tenets of the Declaration of Helsinki.

Patients were excluded if they had recently (within 2 weeks) received medical treatment for the eye (topical steroid and cyclosporine eye drops), had a history of ophthalmological surgical procedure (cataract, pterygium, laser surgery, etc.), type 1 pterygium, conjunctivitis, ocular malignancy or inflammatory disease.

Visual acuity and biomicroscopic anterior segment findings were recorded for each patient before and after the procedure. Pterygium was classified as three types: in type 1, the pterygium tissue does not exceed the limbus; in type 2, pterygium tissue is located between the limbus and the optic zone; and in type 3, pterygium tissue reached the optic zone. Type 2 and type 3 pterygium with blurred vision, irregular astigmatism and discomfort feeling in eye were included in the study. In patients with bilateral pterygium, the eye with the more advanced pterygium type was included in the study.

Pterygium surgery was applied using the conjunctival autograft transplantation technique under local anaesthetic. Samples were taken from the pterygium tissue and from the superotemporal bulbar conjunctiva of the same patient and were placed in separate Eppendorf tubes, then stored at − 80 °C until measurement of amino acid levels.

Sample preparation

Once the tissues were taken in equal amounts, analyses were performed. Pterygium and normal conjunctiva tissues were washed in cold PBS (Sigma-Aldrich, USA). After the addition of lysis buffer RIPA (10 mM Tris-HC1 pH 8, 1 mM EDTA, 1 mM EGTA, 140 mM NaCl, 1% TritonX-100, 0.1 SDS, 0.1% sodium deoxycholate) to the tissues, homogenization was applied for 1 h at + 4 °C in a homogenizator (Qiagen TissueLyser). With centrifugation at + 4 °C for 10 min at 12,000 × *g*, supernatant of the tissue lysate was removed into new tubes. With the exception of the butylation procedure, the samples were routinely treated and prepared as defined by la Marca et al. [16].

Tandem mass spectrometry

The sample was extracted by dispensing 300 µL of an extraction solution consisting of a mixture of methanol and aqueous solution of 3 mmol/L hydrate hydrazine at an approximate relative volume/volume ratio of

66.6% and 33.3%, respectively. In the extract solution, stable heavy isotope analogues of several amino acids were used for internal standards. Samples obtained from the extract were injected to a LCMS-8040 device (Shimadzu Corporation, Japan). The percentage of each analyte was defined compared to a standard including each analyte. The standard concentrations for amino acids were in the range of 500–2500 μmol/L.

Analysis condition

A run of 2.2 min in FIA flow 0.070 μL/min (A: water + 0.05% of formic acid, B: acetonitrile, A/B: 30%/70%). 40 μL of sample injected (column oven 30 °C, desolvatation line 300 °C, heat 500 °C, nebulizing gas 3 L/min, drying gas 20 L/min). All the collected data were reprocessed using Shimadzu Neonatal Software, which automatically calculated the concentration of each component. Of the 20 amino acids in the study tissues, only alanine, arginine, aspartic acid, citrulline, glutamine, glycine, leucine, methionine, ornithine, phenylalanine, tyrosine and valine amino acids were examined with the amino acid screening method in our biochemistry laboratory.

Statistical analysis

All the data analyses were performed using SPSS 24.0 for Windows software (SPSS Inc., NY, USA). Data were analysed using the Wilcoxon signed-rank test to determine differences between two independent groups. To examine the distributions according to gender, a heat map and Row-Z score were used. A value of $p < 0.05$ was accepted as statistically significant, and $p < 0.01$ as statistically highly significant.

Results

This prospective study included a single eye of 29 patients. The patients were 16 females and 13 males with a mean age of 54.75 ± 11.25 years (range 21–78 years). In all the cases, there was a nasal localization of pterygium, with type 2 pterygium in 24 (83%) eyes and type 3 pterygium in five (17%) eyes (Fig. 1). Before treatment, cataract was determined in five patients and degenerative myopia in one. A statistically significant increase was determined in the corrected distance visual acuity (CDVA) level, 6 months after treatment ($p < 0.01$). The demographic data of the patients and the pre- and post-treatment visual acuity values are given in Table 1. No postoperative complications or recurrence developed in any patient.

The arginine level was found to be significantly high in the pterygium tissue ($p < 0.01$). Methionine, glycine and tyrosine levels also were found to be significantly high in the pterygium tissue ($p = 0.028$), ($p = 0.038$), ($p = 0.046$) (Table 2, Fig. 2). A dramatic increase was determined in the alanine, aspartic acid, glutamine, leucine, ornithine, phenylalanine and valine levels but not of a statistically significant level ($p > 0.05$) (Table 2). All the amino acid levels were higher in the pterygium tissue than in the normal tissue. The distribution of the amino acids is shown in bar plot graph form in Fig. 3.

The Row-Z scores in the heat map system of the amino acid levels in pterygium and normal conjunctiva tissues according to gender are shown in Fig. 4. The colour change from red to green on the map represents an increase in amino acid levels. In males, the amino acid levels in both tissue groups were determined to be higher than those of females, and the amino acid levels, particularly of branched-chain amino acids (BCAAs) (leucine and valine) in pterygium tissue, were low in females.

Discussion

It was aimed to investigate the effect of the amino acid profile on pterygium at the metabolomics level, and a scan of the literature was made on this subject. However, no study could be found in the literature that had examined the relationship between pterygium and amino acid levels.

In the process of fibrovascular proliferation, cell metabolism in pterygium tissue is seen to be different from that of normal tissue and there is a change in the amino acid requirements of cells in this process. In this study, an evaluation was made with metabolomics analysis of the amino acid profile in pterygium tissue. An increase was determined in all the amino acid levels in pterygium tissue and this increase was statistically significant in methionine, tyrosine, arginine and glycine amino acids.

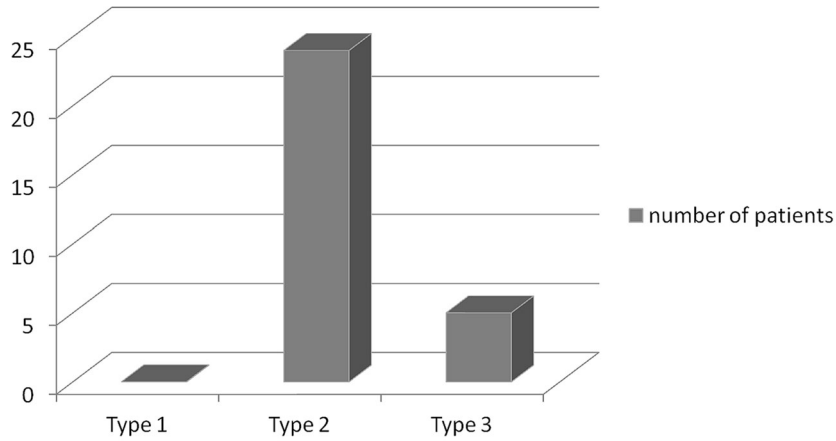

Fig. 1 Distribution of cases according to pterygium types

Table 1 Demographics and ocular parameters before and after pterygium surgery

	Age (years)	Preop ocular pathologies	CDVA preop (logMAR)	CDVA 6th month (logMAR)	*p* value
Mean ± SD	54.75 ± 11.25	– Cataract (5)	0.36 ± 0.49	0.28 ± 0.44	0.01<
Range	(21–78)	– Deg. Myopia (1)			

CDVA corrected distance visual acuity

Amino acid level distribution was also examined according to gender (Fig. 4). The amino acid levels in both pterygium tissue and normal conjunctiva tissue were determined to be higher in males than in females. Although individual differences were shown, when this was examined on a patient basis, the differences can be attributed to the differences between males and females in amino acid metabolism.

Although several current treatment methods have been developed to reduce pterygium recurrence, the controversy about pathogenesis continues. Despite the malignant characteristics of uncontrolled cell proliferation, corneal invasion, angiogenesis and recurrence after treatment, it is differentiated from other tumours as it does not undergo metastasis [17–19]. Inflammatory processes associated with UV damage underlie these characteristics. Fibrovascular proliferation develops as a result of reduced matrix metalloproteinase (MMP) associated with inflammation and the triggering of some inflammatory processes and is thought to have a role in the forming of pterygium [3, 6].

Proliferative characteristics are accepted in the development of pterygium tissue because of the role of K-ras oncogene and p53 tumour suppressor genes [11, 20]. These genes are known to have important roles in the metabolism of glucose and amino acids. In cells with K-ras mutation, glutamine amino acid is a major carbon source for adenosine triphosphate (ATP) production, nucleotide and protein biosynthesis and regulation of the redox balance [21].

In a study which investigated the effect of inflammation on amino acid metabolism in visceral fat tissue, a reduction was determined in TNFα and other proinflammatory cytokines, BCAAs (leucine, valine) and tricarboxylic acid (TCA) cycle metabolism [22]. In the current study, an increase was observed in BCAAs levels in pterygium tissue (Table 2, Fig. 3).

Muima et al. [23] determined that apoptosis was induced in liver cells with mechanisms mediated by mammalian target of rapamycin complex 1—(mTORC1)—and complex 2—(mTORC2)—of BCAAs. As the inflammatory processes in the pterygium tissue indirectly reduce BCAAs use of cells, they could be effective in the reduction of apoptosis, suggesting that the role of BCAAs is important in this process. From the data of previous research and the current study, it can be concluded that both the inflammatory processes in pterygium tissue and the

Table 2 Tissue concentrations of amino acids in pterygium and control groups

Amino acids	Mean (μmol/L)	Median (μmol/L)	IQR	*p* values
Arg				
Pterygium	3058.64	2728	2506	< 0.01
Normal tissue	1670.44	612	425	
Gly				
Pterygium	4996.68	3112	2136	0.038
Normal tissue	1720.45	1537	1802	
Met				
Pterygium	756.14	335.60	230.60	0.028
Normal tissue	299.77	87.20	78.20	
Tyr				
Pterygium	1588.47	1512	1227	0.046
Normal tissue	497.22	511	775	
Phe				
Pterygium	2291.97	854	980	0.972
Normal tissue	1140.51	804	1625	
Val				
Pterygium	4517.17	2324	1438	0.929
Normal tissue	2109.26	1994	2113	
Leu				
Pterygium	3162.69	1569	1766	0.583
Normal tissue	1402.12	1045	1685	
Gln				
Pterygium	5215.25	2603	3428	0.484
Normal tissue	2124.05	1739	1436	
Asp				
Pterygium	4064.11	1650	1779	0.678
Normal tissue	1834.89	1308	2070	
Orn				
Pterygium	2585.35	1331	726	0.463
Normal tissue	978.79	1029	947	
Ala				
Pterygium	5883.50	2408	1601	0.859
Normal tissue	2298.19	2328	480	
Cit				
Pterygium	219.94	253	183	0.441
Normal tissue	184.99	201	198.50	

IQR interquartile range, *p* by Wilcoxon signed-rank test

Arg arginine, *Gly* glycine, *Met* methionine, *Tyr* tyrosine, *Phe* phenylalanine, *Val* valine, *Leu* leucine, *Gln* glutamine, *Asp* aspartic acid, *Orn* ornithine, *Ala* alanine, *Cit* citrulline

proliferative characteristics could be related to amino acid metabolism.

Neoplastic cells require some amino acids such as glutamine, glycine, aspartic acid and serine for DNA synthesis, new vessel formation, duplication of proteins and for the synthesis of hormones such as growth-promoting hormones or tumour growth factors. Glutamine, which is a significant source of nitrogen for cell proliferation, is used by cells at a high rate [21]. In the current study, glutamine was determined at a higher rate in pterygium tissue than in the normal conjunctiva tissue (Table 2, Fig. 3).

In the current study, arginine levels were determined at a significantly high rate in pterygium tissue (Table 2, Fig. 2). In addition, ornithine levels were determined to be high in pterygium tissue. Several

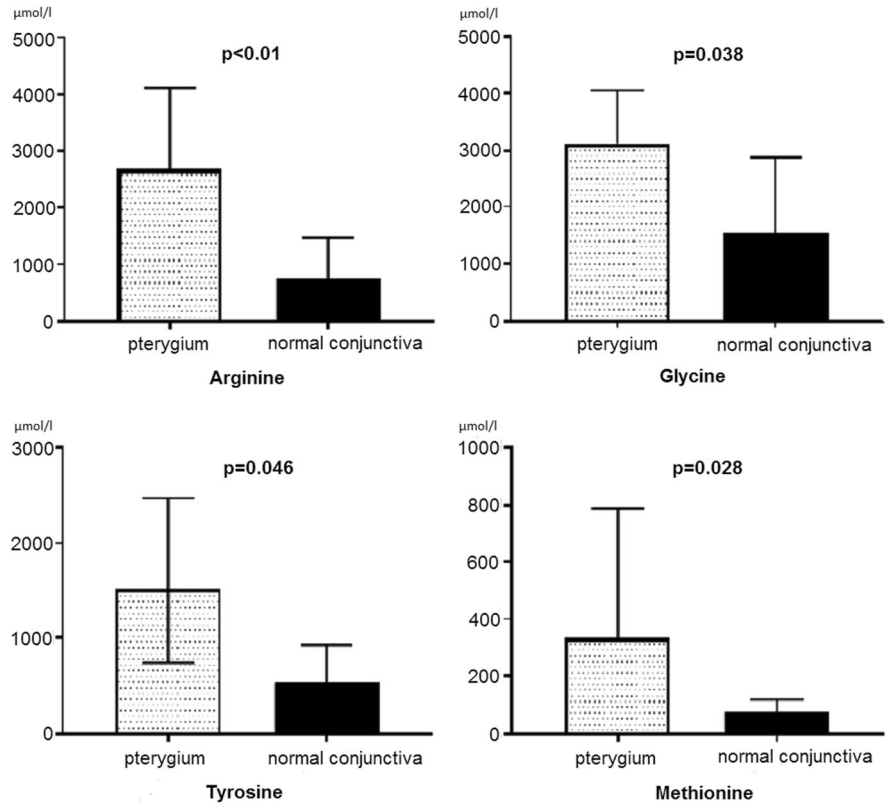

Fig. 2 Metabolomics analysis results of the pterygium and normal conjunctiva samples. *Arg* arginine, *Gly* glycine, *Met* methionine, *Tyr* tyrosine

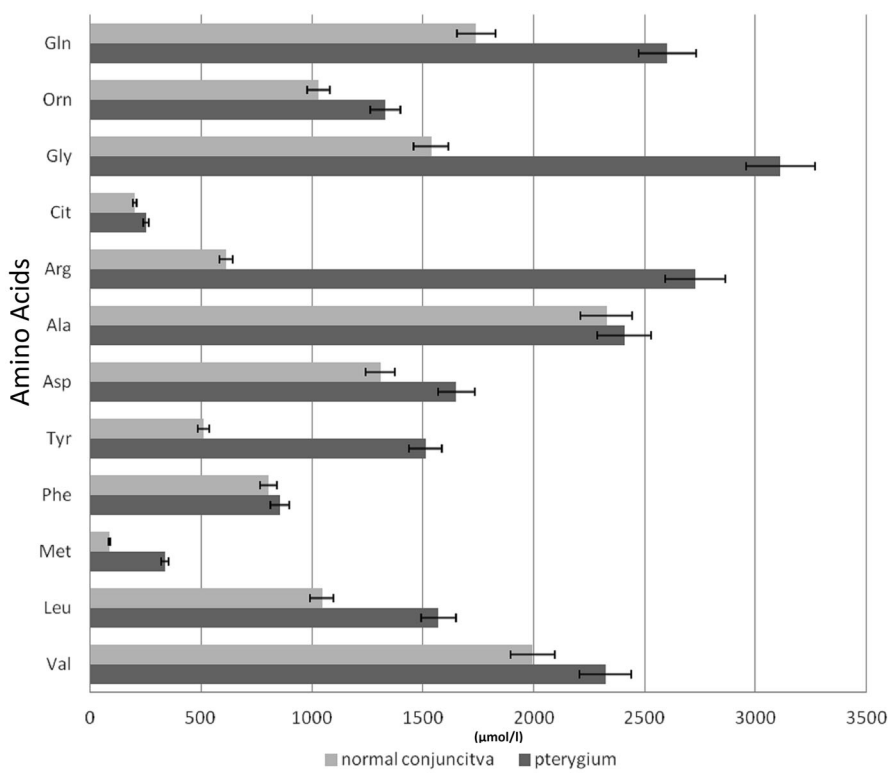

Fig. 3 Mean tissue concentrations of amino acids in pterygium and control groups (with s.e. bars). *Arg* arginine, *Gly* glycine, *Met* methionine, *Tyr* tyrosine, *Phe* phenylalanine, *Val* valine, *Leu* leucine, *Gln* glutamine, *Asp* aspartic acid, *Orn* ornithine, *Ala* alanine, *Cit* citrulline

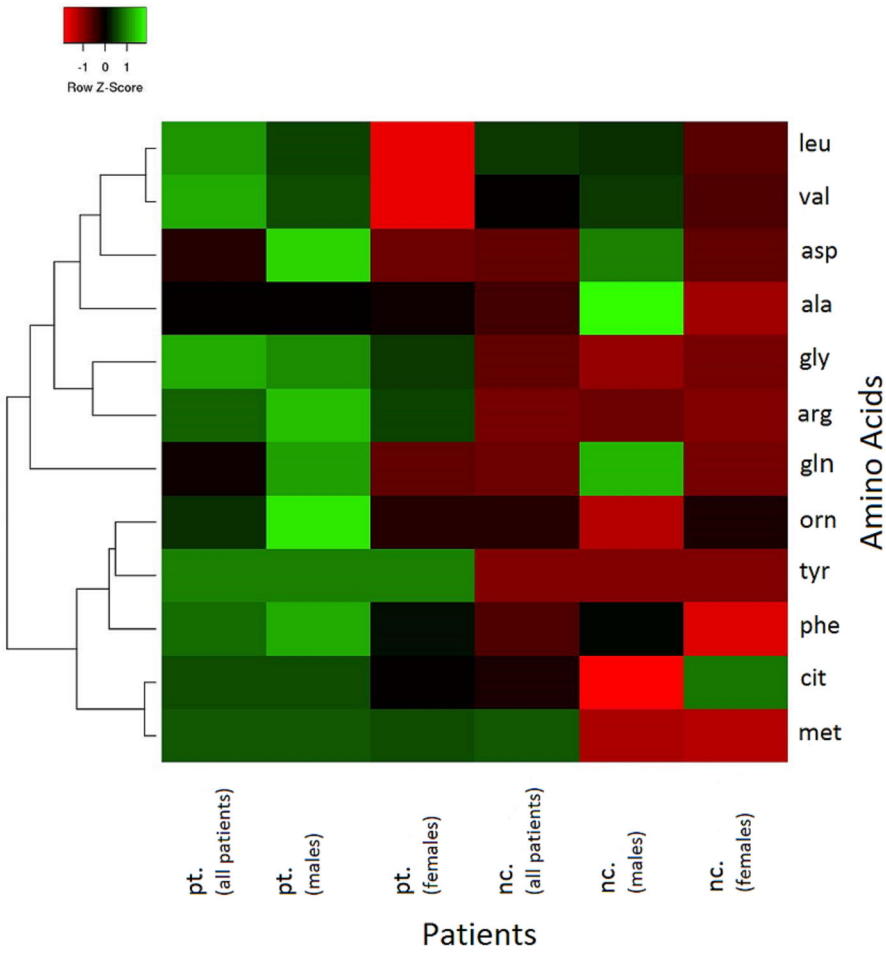

Fig. 4 Heat map analyses showing the distribution of the amino acid values according to gender in all the groups. pt: pterygium, nc: normal conjunctiva. *Arg* arginine, *Gly* glycine, *Met* methionine; *Tyr* tyrosine, *Phe* phenylalanine; *Val* valine, *Leu* leucine, *Gln* glutamine, *Asp* aspartic acid, *Orn* ornithine, *Ala* alanine, *Cit* citrulline

tumour tissues convert arginine to ornithine and urea with the arginase enzyme [24]. The effect of arginase enzyme in pterygium tissue in this situation can be researched in future studies.

Building on the Warburg effect, neoplastic cells use glycine for ATP production and tumour proliferation [14]. In the current study, the glycine level in pterygium tissue was determined to be significantly high, which could be related to the increase in cell turnover in pterygium tissue (Table 2, Fig. 2).

Methionine has a role in cellular reversible oxidation and reduction reactions. In the current study, methionine was determined at a significantly higher rate in pterygium tissue compared to the normal conjunctiva tissue (Table 2, Fig. 2).

Tyrosine is a non-essential, aromatic amino acid (AAA) with a role in protein synthesis and the production of catecholamines, thyroxine and melanin. In the current study, a significant increase was determined in tyrosine levels (Table 2, Fig. 2).

To the best of our knowledge, there is no other study in the literature that has examined amino acid metabolism in pterygium tissue. The majority of previous studies have been made in the form of comparisons of plasma amino acid levels in malignancy, diabetic and control patients [12–14]. Cancer studies have measured free amino acid levels in plasma and have reported that some amino acids could be specific to various cancer types. The current study was conducted on homogenates of pterygium and normal conjunctiva tissues. Therefore, the data obtained in the study are the first to have been obtained in the examination of pterygium tissue in respect of amino acid metabolism.

As in several previous pterygium studies, the normal conjunctiva tissues were obtained from the autograft tissue during the pterygium with autograft

operation [11, 17, 19]. Taking the pterygium tissue and normal conjunctiva tissue from the same individual was an important factor in eliminating the effects of metabolic differences between individuals. Moreover, taking healthy conjunctiva tissue from other individuals would lead to ethical problems.

In conclusion, uncontrolled cell proliferation, normal tissue invasion, tumour suppressor gene and oncogene mutations are known to have a role in pterygium pathogenesis. As the results of the current study showed that amino acid levels were higher in pterygium tissue, this suggests that the metabolism in the pterygium tissue was accelerated by proliferative cells and the effect of inflammation.

Limitations of the study can be said to be that the number of eyes included in the study was limited and that because there are no other studies in the literature that have examined the amino acid profile in pterygium tissue, the data could not be compared with other pterygium studies.

With the results that have emerged from this study, the amino acid profile could open a new window in pterygium pathophysiology. Nevertheless, there is a need for further studies with greater patient numbers to examine the amino acid profile in pterygium with respect to halting proliferation and reducing recurrence after surgery.

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Compliance with ethical standards

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership or other equity interest; and expert testimony or patent-licensing arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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