Correlation between smoking and neovascularization in biceps tendinopathy: a functional preoperative and immunohistochemical study

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Abstract

Aims: The purpose of this study was to investigate whether smoking is associated with neovascularization in the tendinopathy of the long head of the biceps tendon (LHBT).

Methods: The study included 40 consecutive patients who underwent arthroscopic biceps tenotomy/tenodesis due to chronic biceps tendinopathy and divided into three groups: (1) non-smokers, (2) former smokers, (3) smokers. LHBT tissue samples were stained with H&E, Alcian blue and Trichrome staining. Immunohistochemical examination was performed using anti-CD31 and anti-CD34. The neovessel density score (NDS) was scored by Bonar criteria.

Results: The mean period of smoking was 15.50 years with an average number of 24 cigarettes/day in the former smokers and 21.69 years with an average number of 15 cigarettes/day in the active smokers. The mean NDS was 2.23/3 in non-smokers, whereas it was 1.60/3 in former smokers and 1.31/3 in active smokers. The mean American Shoulder and Elbow Surgeons score equaled 46 in never smoked patients, 43.60 in former smokers, and 41.46 in active smokers. In the patients with smoking history, the disorganized tendinous tissue islands were avascular and composed of compact acidic polysaccharides and mucopolysaccharides. We observed negative correlation between the NDS and the smoking indexes, including cigarettes per day ($p = 0.0150$), smoking years ($p = 0.0140$), pack-years ($p = 0.0088$).

Conclusion: In conclusion, the present study revealed that smoking impairs the vascularization of the biceps tendon in chronic tendinopathy. Clinically, we observed a negative correlation between smoking and neovascularization. Furthermore, there was no correlation between neovascularization and functional preoperative status.

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Introduction

Tobacco smoking has demonstrated a deleterious effect on muscles, bones, and tendons.[1–7] Tobacco smoke constitutes an aerosol of more than 4000 chemicals, for example, nicotine, aromatic hydrocarbons, carbon monoxide, nitrosamines, hydrogen cyanide, and aldehydes.[8] These substances have a negative impact on cells and their metabolism, reduce blood flow to tissues, and disrupt collagen synthesis.[2,9,10] All these effects lead to an impaired regenerative process and then accelerated development of the degenerative process in the tendinous tissue, which clinically manifests as tendinopathy. Histological studies of tendinopathy have demonstrated the disorganization of collagen, an increased number of tenocytes, excessive concentration of glycosaminoglycans in the ground substance, and the pathological expansion of neovessels.[11]

Focused mainly on rotator cuff pathology, studies of tendinopathy proved that tobacco smoking is an important risk factor in the development of this pathology and that it is connected with persistent shoulder pain.[12,13] Moreover, there is a positive relationship between the intensity of smoking and the size of tendon tear.[14] Another important fact is that rotator cuff tendinopathy is strongly associated with the occurrence of the long head of the biceps tendon (LHBT) pathology.[15]

Despite the hypovascular area in the proximal part of the healthy LHBT, near its origin, the degenerative process of tendinous tissue is characterized by an abundant expansion of newly formed capillaries.[15] Numerous studies in ophthalmology and obstetrics revealed that smoking, with the nicotine it delivers, intensifies the neovascularization process, thus exacerbating the pathology.[16–18] The relation seems to be simple – both tendinopathy and smoking increase the formation of neovessels; however, there is a paucity of scientific data about this connection.[10,19,20] Cheema *et al.* noted the correlation between the nicotine exposure, altered healing of the tendon, and decreased neovascularization in an animal study.[10] None of these studies present straight correlation between smoking, tendinous tissue alterations, and neovessels formation in tendinopathy.

The purpose of the study was to investigate the association of smoking with the neovascularization process present in chronic tendinopathy of the biceps tendon. The study was supported with an immunohistochemical examination of the formation of new capillaries in pathologically changed tendinous tissue.

Methods

Bioethics
The study was performed in accordance with the Declaration of Helsinki for experiments involving humans, after receiving permission from the local Bioethics Committee: Bioethics Committee of the Nicolaus Copernicus University in Toruń functioning at Collegium Medicum in Bydgoszcz (approval number KB 598/2016). All patients provided written informed consent before entrance into the study. The authors declare that they obtained written informed consent from both individuals for the publication of Figure 1.

Study cohort
The study included 40 consecutive patients recruited from the Department of Orthopedic Surgery (2016–2018) who underwent arthroscopic tenotomy/tenodesis due to chronic biceps tendinopathy. All patients were diagnosed with symptomatic LHBT tendinopathy based on clinical examination: the tenderness over bicipital groove test (Figure 1A), Speed's test (Figure 1B), Yergason's test (Figure 1C), the Abbott–Saunders test (Figure 1D), and sonographic examination (Figure 1E and F). The patients were also examined with non-contrast magnetic resonance (MRI) and subjected to preoperative evaluation using the American Shoulder and Elbow Surgeons (ASES) score. The patients were selected for the study on the basis of the following inclusion criteria: 3 months minimum duration of symptoms, non-athlete, no history of systemic inflammatory diseases, no previous surgical treatment of the shoulder concerned, and no corticosteroid injections in the past 12 months.

Smoking data
The patients were divided according to their tobacco smoking history. The data on the smoking habits included: cigarette smoking status, cigarette smoking time, the number of cigarettes smoked per day, and pack-years. The smokers group comprised patients who smoked at least 10 cigarettes per day. The non-smokers group was composed of patients who had never used any kind of nicotine or tobacco products. Furthermore, to provide more precise results, former smokers were separated from non-smokers and were included into the smokers group.

Surgery and histopathological evaluation
Surgery was performed under general anesthesia in the beach chair position (Smith&Nephew standard 30° arthroscope) (Figure 2A, B, E and F). At the end of the surgical procedure (tenotomy/tenodesis), biopsies of the intra-articular part of the tendon were harvested for further investigation (Figure 2C and G). About 1–2.5 cm × 0.4–1 cm portions of the intra-articular part of the LHBT were removed with arthroscopic scissors starting from its origin point, at the supraglenoid tubercle, and subsequently stored (for 24 h) in a container filled with 10% fresh buffered formalin.
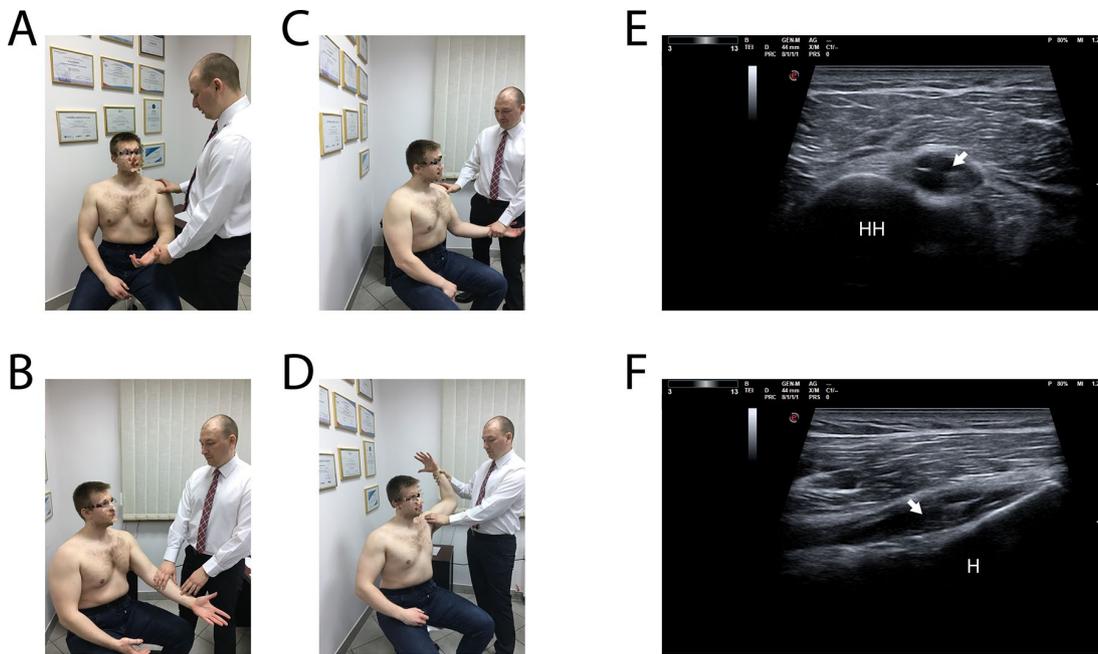
Figure 1. (A–D) Clinical tests performed to diagnose long head of the biceps tendon (LHBT) tendinopathy: (A) tenderness over bicipital groove; (B) Speed's test; (C) Yergason's test; (D) Abbott–Saunders test; (E and F) Sonographic examination scans: (E) short-axis scan of the LHBT with decreased hypoechogenicity (arrow) in the bicipital groove of the humeral head (HH); (F) long-axis scan of the LHBT with decreased hypoechogenicity (arrow), (H, humerus); Esaote My Lab Gamma, 13 MHz linear transducer.
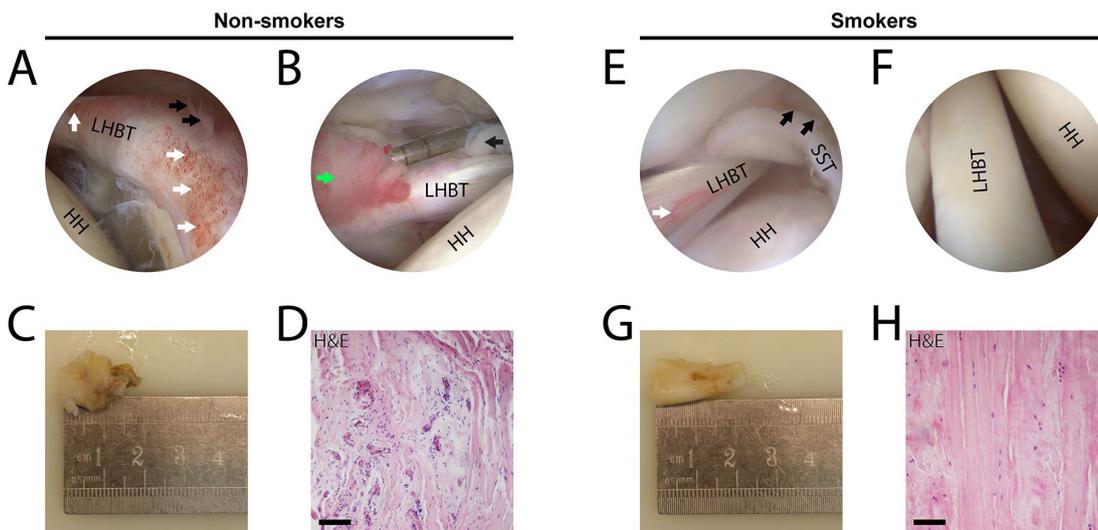
Figure 2. Arthroscopic pictures (posterior portal, Smith&Nephew 30° arthroscope) illustrating: (A) the superior labrum of the glenoid with long head of the biceps tendon (LHBT) anchor, excessive vascularization of the tendon and the superior labrum (white arrows), multiple tears of its structure (black arrows); (B) abundant synovial expansion (green arrow) in the area of biceps anchor and its proximal part, the tear of the intra-articular part of the biceps (black arrow); (E) moderate vascularization of the biceps tendon (white arrow) and concomitant SST tear (black arrows); (F) minimal macroscopic alterations of the LHBT. (C, G) Macroscopic evaluation and measurements of the tendon samples with advanced degeneration of the structure; and (D, H) microscopic evaluation of the tendon samples stained with H&E showed a distinct neovascularization process in non-smokers and abundant non-collagenous extracellular matrix expansion in smokers, neovessel formation absent. H&E, hematoxylin and eosin; HH, humeral head; SST, supraspinatus tendon

The samples collected were dehydrated in increasing concentrations of ethanol, cleared in xylene, embedded in paraffin, and cut into 4 μm thick sections with a microtome (Leica). Histopathological assessment was performed on the sections stained with hematoxylin and eosin (H&E), Alcian blue, and Masson's Trichrome. To visualize the presence of newly formed vessels, the immunohistochemical examination of the immunoreactivity of CD31 and CD34 was performed using mouse monoclonal antibodies against CD31 and CD34 (both from Roche). For the immunohistochemical staining, the sections were deparaffinized, rehydrated, and endogenous peroxidase was blocked with 3% hydrogen peroxide. Staining was carried out using BenchMark GX (Ventana Medical Systems, Roche) in compliance with the manufacturer's instructions. The slides were then analyzed by three independent investigators using a BX46 light microscope (Olympus) at magnification of 2×, 10×, 20×, and 40×. Neovessel density assessment was performed by adapting the vascularity criteria from the Bonar score.[21] Briefly, the neovessel density score (NDS) was calculated on the assumption that zero points (absent neovascularization) refers to normal tendons with an average occurrence of the blood vessels, one point (mild neovascularization) refers to incidental cluster of capillaries less than one per 10 high-power fields (HPFs), two points (moderate neovascularization) refers to 1–2 clusters of capillaries per 10 HPFs, and three points (severe neovascularization) refers to more than two clusters per 10 HPFs. The microscope examiners were blinded to the patients' data.

Statistical analysis

The data were compared with the non-parametric Mann–Whitney U test. Relations between the parameters studied were assessed using the Spearman correlation coefficient and multiple linear regression analysis. All the comparisons between groups and statistical analyses were performed by two independent investigators using Prism software (GraphPad). p-value < 0.05 was considered to be statistically significant. Circular plots were generated by Circos software.[22]

Results

Patient characteristics

The patients' demographic data and characteristics are summarized in Table 1. The study included 40 patients divided into three groups according to their cigarette smoking history: non-smokers (17 patients who had never smoked, 42.50%), former smokers (10 patients with a mean 24.25 pack-year history, 25%), and active smokers (13 patients with 16.89 pack-year history, 32.50%). The overall male to female ratio was 1:1 (non-smokers 1:1.12, former smokers 1:1, active smokers 1:0.86). The mean age at diagnosis was 51.80 years (range 24–75 years). Specifically, at the time of the diagnosis, the mean age was 45.29 years (range 24–65 years) in the non-smokers group, 58.20 years (range 33–75 years) in the former smokers group, and 55.38 years (range 49–62) in the active smokers group. The average number of cigarettes smoked per day was 24 in the former smokers group (range 10–60), whereas in the active smokers group it was 15 (range 10–20). In addition, the mean period of smoking was 15.50 years in the former smokers group (range 5–30 years) and 21.69 years in the active smokers group (range 11–40 years).

Furthermore, the overall mean NDS was 1.77/3 (range 1–3/3). The mean NDS was 2.23/3 (range 1–3) among the patients who had never smoked, 1.60/3 (range 0–3/3) among former smokers, and 1.31/3 (range 0–3/3) among active smokers. The mean ASES score was 46/100 (range 36–60/100) in the group of patients who had never smoked, 43.60 (range 38–52)/100 in the group of former smokers, and 41.46/100 (range 34–50/100) in the group of active smokers.

Macro- and microscopic examination

LHBT tendinopathy was associated with concurrent lesions of the rotator cuff (27 patients, 67.50%). Thirteen patients (32.50%) underwent the tenotomy procedure and 27 patients (67.50%) were subjected to the tenodesis procedure with the use of a dedicated anchor. There were no different anatomic variants of LHBT origins found in the study (Figure 2A, B, E and F). Furthermore, the examination of the LHBT samples revealed a microscopic collagen architecture collapse, altered tenocytes morphology, and glycosaminoglycans accumulation (Figure 2C, D, G and H and Figure 3A–J).

Specifically, we observed an increased number of rounded-shape tenocytes as well as disorganized tendinous tissue islands in all samples

Table 1. Summary of demographic and clinical characteristics.

Characteristics	Total	Smoking status		
		Non-smokers	Former smokers	Active smokers
No. of patients	40	17	10	13
Gender				
Male	20	8	5	7
Female	20	9	5	6
Mean				
Age	51.80 (24–75)	45.29 (24–65)	58.20 (33–75)	55.38 (49–62)
Cigarettes per day	10.87 (0–60)	0	24 (10–60)	15 (10–20)
Smoking years	10.92 (0–40)	0	15.50 (5–30)	21.69 (11–40)
Pack-years	11.58 (0–90)	0	24.25 (2.5–90)	16.89 (5.5–23)
Neovessel density score Min. 0 pts, max. 3 pts	1.77 (1–3)	2.23 (1–3)	1.60 (0–3)	1.31 (0–3)
Preoperative ASES score Min. 0 pts, max. 100 pts	43.82 (36–60)	46.00 (36–60)	43.60 (38–52)	41.46 (34–50)

The range of values is indicated in parentheses.
ASES, American Shoulder and Elbow Surgeons; pts, points.

studied (Figure 2D and H and Figure 3A and F). As shown in Figure 3, in the non-smokers group, the disorganized tendinous tissue islands were vascularized and the extracellular matrix (ECM) was loose and composed of both collagenous and non-collagenous material confirmed by different histological staining (Figure 3A–E). In contrast, in the active and former smokers groups, which were collectively treated as smokers, the disorganized tendinous tissue islands tended to be avascular and composed of more compact and amorphous non-collagenous material, which indicates a possible mechanism of inhibited neoangiogenesis (Figure 3F–J). Furthermore, in the disorganized tendinous tissue obtained from the patients with smoking history, the cells were more rounded and morphologically similar to chondroid cells, but revealed the membranous expression of CD34, which, on the one hand, is characteristic and typical of hematopoietic stem cells and, on the other hand, may indicate the presence of tissue resident mesenchymal stem cells or a distinct subset of cells with enhanced progenitor activity (Figure 3J).

Correlation between smoking status, neovessel formation, and preoperative ASES score

As shown in Figure 4A–D and in Table 1, we observed the relationship between the smoking status and different NDS distribution. The NDS was statistically significantly lower in active smokers (median, 1) as compared with the patients who had never smoked cigarettes (median, 2) ($p = 0.0258$). There was no statistically significant difference between former smokers and non-smokers (Figure 4A). However, the classification of patients on the basis of the consumption of tobacco products in their lifetime (non-smokers/smokers) helped us notice a statistically significant decrease in smokers (median, 1) as compared with non-smokers (median, 2) ($p = 0.0324$) (Figure 4C). Furthermore, we observed a statistically significant negative correlation between the NDS and the number of cigarettes smoked per day (Spearman $r = -0.3822$; $p = 0.0150$), smoking years (Spearman $r = -0.3858$; $p = 0.0140$), and pack-years (Spearman $r = -0.4087$; $p = 0.0088$) (Figure 4E–G). There was no statistically significant correlation between the NDS and age (Figure 4H).
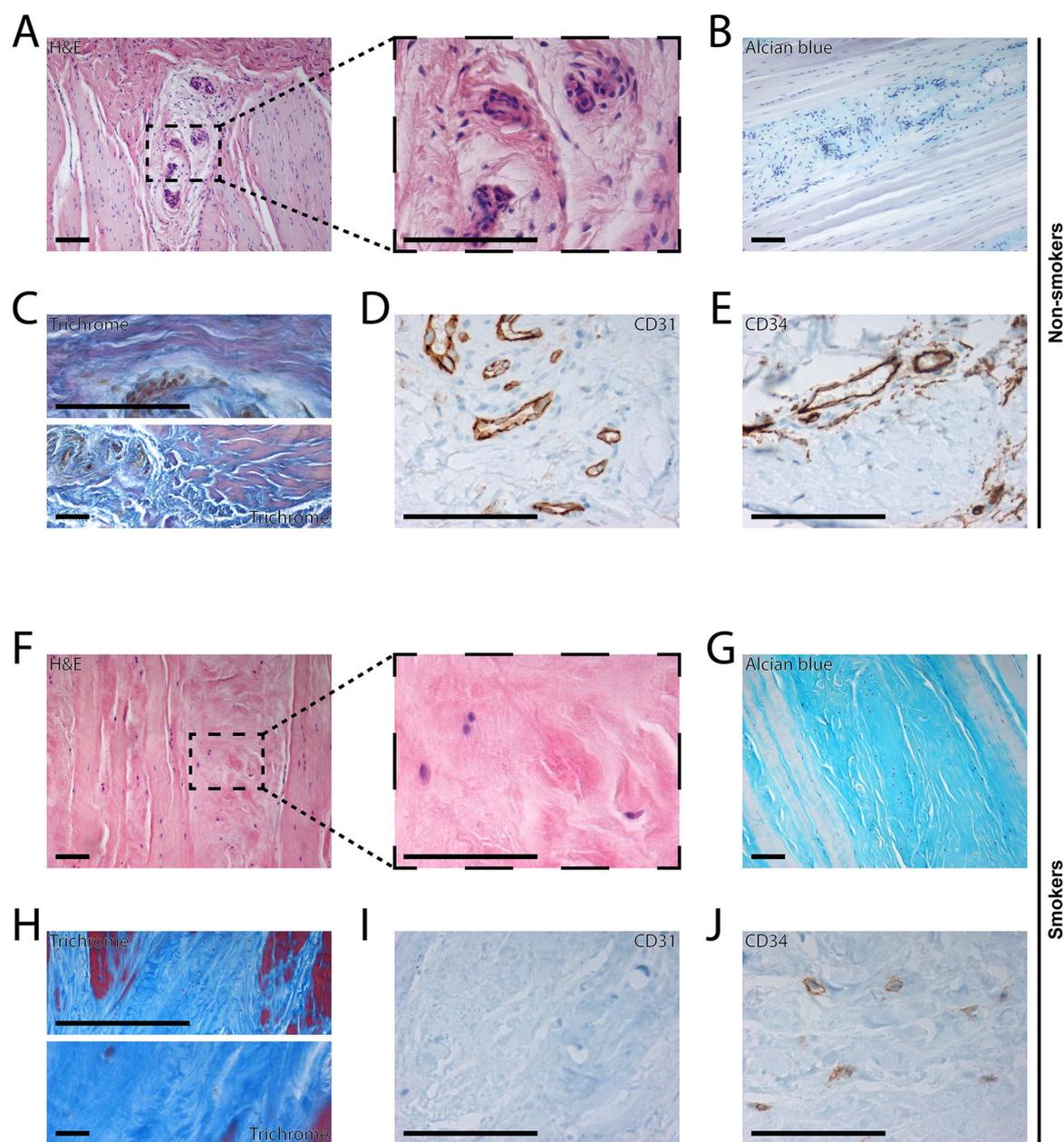
Figure 3. (A–E) Microscopic evaluation of non-smoker tendon samples revealed the formation of neovessels and their positive reaction with anti-CD31 and anti-CD34 antibodies and (F–J) microscopic evaluation of smoker tendon samples revealed disorganized collagen fibers with the expansion of non-collagen extracellular matrix, no visible proliferation of neovessels, no reaction with anti-CD31 antibody and a positive reaction of chondroid-like cells with anti-CD34 antibody. (A, F) H&E; (B, G) Alcian blue; (C, H) Trichrome; (D, I) CD31; (E, J) CD34. Bar = 100 µm.
H&E, hematoxylin and eosin.

Alfredson and Öhberg demonstrated that tendon neovascularization may be accompanied by nerve ingrowth and further release of neurotransmitters, which correlates with pain.[23] In our study, we decided to compare the smoking status and neovessels formation with preoperative ASES score. There were no statistically significant differences between the smoking status and preoperative ASES score (Figure 4I), even after the classification of patients on the basis of the consumption of tobacco products in their lifetime (non-smokers/smokers) (Figure 4J). Moreover, no
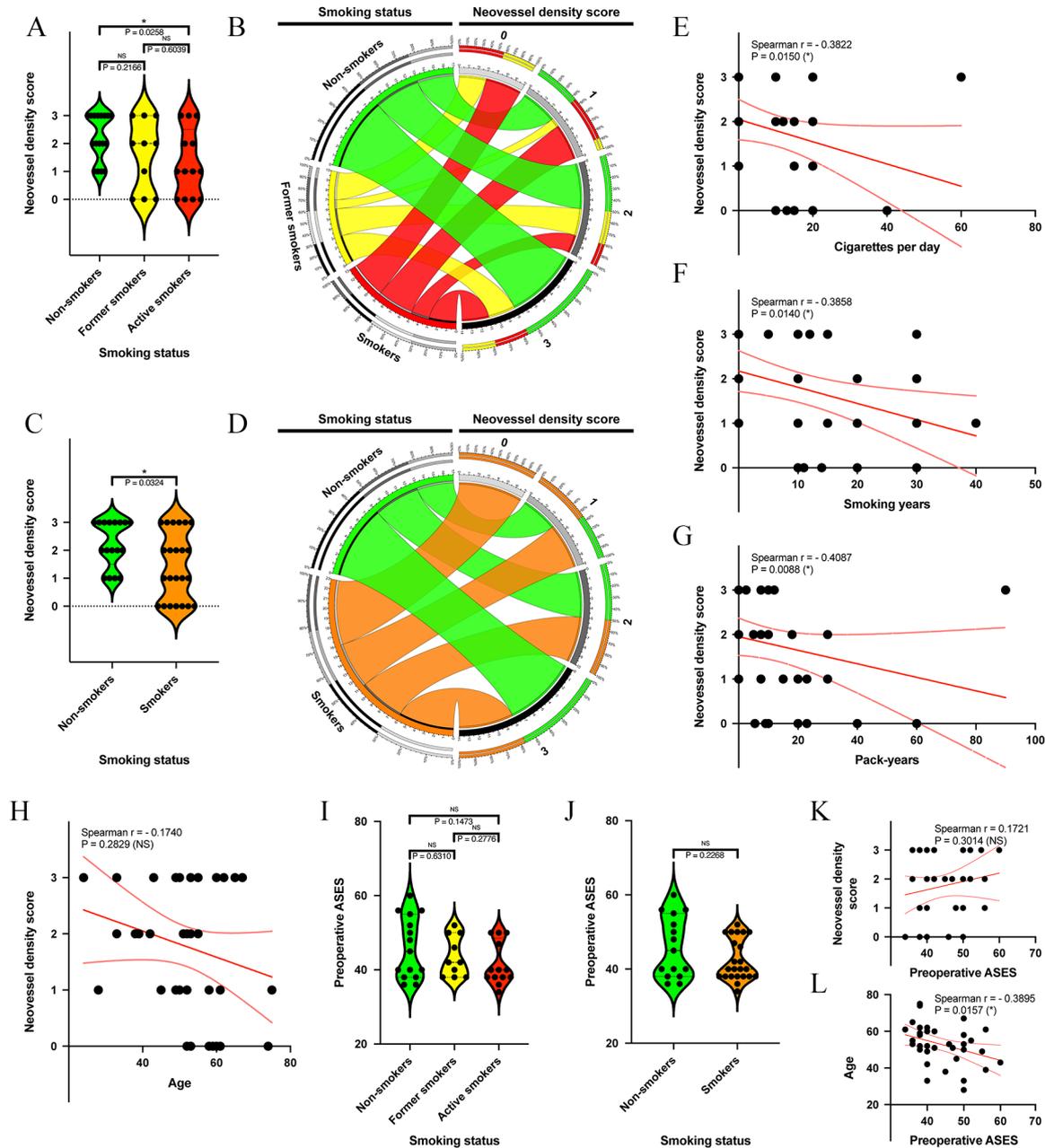
Figure 4. Summarized statistical analysis depending on the smoking status, neovessel density score, age, and preoperative American Shoulder and Elbow Surgeons (ASES) score. (A, B) Comparison and dependence of NDS according to smoking status; (C, D) comparison and dependence of neovessel density score (NDS) according to smoking history; (E–G) correlation between the NDS and the number of cigarettes smoked per day, smoking years, pack-years; (H) correlation between the NDS and age; (I) comparison of preoperative ASES score and smoking status; (J) comparison of preoperative ASES score and smoking history; (K) correlation between the NDS and preoperative ASES score; and (L) correlation between the age and preoperative ASES score.

correlation between the NDS and preoperative ASES score was noticed (Figure 4K). However, a statistically significant negative correlation was found between age and preoperative ASES score (Spearman $r = -0.3895$; $p = 0.0157$) (Figure 4L).

Multiple linear regression analysis confirmed that smoking indexes are useful to predict NDS (Table 2). These variables statistically significantly predicted NDS in patients with smoking history [$F(5, 17) = 3.747$; $p = 0.0181$; $R^2 = 0.5243$]. Cigarettes

Table 2. Results of multiple linear regression analysis showing relationships between neovessel density score and smoking indexes, age, and preoperative ASES score.

Variable	All patients				Non-smokers			
	Estimate	SD	95% CI	p value	Estimate	SD	95% CI	p value
Intercept	1.531	1.864	−2.266 to 5.328	0.4177	2.115	1.977	−2.192 to 6.422	0.3056
Cigarettes per day	−0.03338	0.04169	−0.1183 to 0.05154	0.4293	N/A	N/A	N/A	N/A
Smoking years	−0.04152	0.02372	−0.08983 to 0.006798	0.0896	N/A	N/A	N/A	N/A
Pack-years	0.02901	0.03062	−0.03336 to 0.09138	0.3506	N/A	N/A	N/A	N/A
Age	0.001817	0.02135	−0.04167 to 0.0453	0.9327	0.007839	0.02423	−0.045 to 0.06064	0.7519
Preoperative ASES score	0.01406	0.02846	−0.0439 to 0.07203	0.6246	−0.006197	0.03031	−0.07224 to 0.05984	0.8414

Variable	Smokers				Former smokers				Active smokers			
	Estimate	SD	95% CI	p value	Estimate	SD	95% CI	p value	Estimate	SD	95% CI	p value
Intercept	−0.1114	1.801	−3.911 to 3.688	0.9514	−5.059	3.378	−14.44 to 4.319	0.2085	19.04	0.2526	18.44 to 19.63	**<0.0001**
Cigarettes per day	−0.2153	0.05515	−0.3317 to −0.09895	**0.0011**	−5.059	3.378	−14.44 to 4.319	0.0731	0.8814	0.03747	0.7928 to 0.9700	**<0.0001**
Smoking years	−0.1325	0.06078	−0.2607 to −0.004224	**0.0437**	−0.3448	0.08255	−0.5740 to −0.1157	**0.0139**	0.7734	0.05576	0.6416 to 0.9053	**<0.0001**
Pack-years	0.1519	0.05449	0.03689 to 0.2668	**0.0127**	0.1966	0.05484	0.04433 to 0.3488	**0.0231**	−1.052	0.06502	−1.205 to −0.8978	**<0.0001**
Age	0.02885	0.03244	−0.03958 to 0.09729	0.3862	0.04284	0.02392	−0.02357 to 0.1093	0.1478	−0.4318	0.008651	−0.4522 to −0.4113	**<0.0001**
Preoperative ASES	0.08325	0.04671	−0.01530 to 0.1818	0.0926	0.2080	0.06123	0.03795 to 0.3780	**0.0274**	−0.1342	0.006112	−0.1487 to −0.1198	**<0.0001**

ASES, American Shoulder and Elbow Surgeons; CI, confidence interval; SD, standard deviation.

per day, smoking years, and pack-years added statistically significantly to the prediction ($p = 0.0011$, $p = 0.0437$, and $p = 0.0127$, respectively).

Discussion

The relation between smoking and tendinopathy seems to be simple: smoking is supposed to enhance the neovascularization process due to subsequent hypoxia and the local impairment of the microvascular perfusion. However, the data presented in our study imply that this relation is simple and, contrariwise, that tobacco smoke leads to decreased neovessel formation in the tendinopathy of LHB. The effect of smoking on tendons has not been well-established, which is the reason why this study was performed.

The theory on the gradual degeneration of tendinous tissue in hypovascular regions is important and such an area does exist in the intra-articular part of the LHBT, 1–3 cm from its origin.[15,24] On the other hand, hypoxia caused by tobacco smoke triggers vascular endothelial growth factor (VEGF) synthesis, responsible for capillaries expansion, and stimulates the angiogenesis process.[1] Nicotine is an important vasoconstrictor, limiting oxygen supply to tissues.[2] Moreover, tobacco smoking alternates collagen synthesis, simultaneously impairing the healing of tendons.[16] Studies revealed that in the smokers group, the collagen was less mature, with abnormal architecture and additional disorders of the ECM composition.[9] In our cohort, collagen architecture was abnormal. Furthermore, in the non-smokers, the disorganized tendinous tissue was loose and composed of both collagenous and non-collagenous material. On the other hand, in the smokers group, the disorganized tendinous tissue islands were avascular and composed of more compact and amorphous non-collagenous material.

The neovascularization process is typical of osteoarthritis, retinopathy, inflammation, tumors, and advanced tendinopathy.[25] Nicotine increases the severity and the size of the neovascularization process in macular degeneration.[16–18] Macular degeneration is the major cause of blindness in the elderly in the developed world, strongly associated with increasing age and cigarette smoking.[16] Smoking during pregnancy is associated with obstetric complications, spontaneous pregnancy loss, and placental abruption.[26] Kawashima *et al.*

revealed that maternal cigarette smoking seems to increase the expression of the placental growth factor, which promotes angiogenesis and has an impact on spiral artery remodeling during the first trimester. However, some by-products of tobacco smoke may alter the placental gene expression profile and contribute to a reduced incidence of preeclampsia.[26] Solid tumors require neovascularization for their growth and nicotine promotes pathological processes.[27] Nicotine accelerates tumor growth by stimulating VEGF expression in tumor cells and subsequent new capillaries expansion.[27] All of these studies discussed the importance of nicotine as an angiogenesis process promoter. Nicotine stimulates the nicotinic cholinergic receptors on endothelial cells and makes them proliferate, migrate, and form capillaries.[28]

Nevertheless, some studies suggest that tobacco smoking has a negative impact on the neovascularization process, as revealed in this study, and that this impact may result from the final effect of all substances included in smoke. Michaud *et al.* demonstrated that smoking has a negative effect on neovascularization and is associated with an important reduction of capillary density in ischemic muscles.[29] They noted reduced expression of the hypoxia-inducible factor 1-alpha and VEGF, which are responsible for hypoxia-induced neovascularization. However, the specific element of tobacco smoke responsible for this action is unknown and it is suggested that carbon monoxide may play a main role in this respect.[29]

The relationship between rotator cuff (RC) injuries and LHBT disorders is very strong and these pathologies may exacerbate each other. Numerous studies about the influence of tobacco smoking on the progression of the RC tendinopathy revealed difficulties with recovery, poor postsurgical outcomes, more frequent surgical revisions, and an increased apoptosis process with early-onset tendon degeneration.[2,4,9,14,19] Lastly, tobacco smoking reduces RC tendon healing capacity and accelerates the degenerative process.[4,6,12] In our group, RC tears appeared simultaneously with LHBT pathology in 67.5% of cases, a relationship emphasized by many authors.[30,31]

On the opposite side of the LHBT, the distal biceps tendon is attached to the radial tuberosity, and Safran *et al.* showed that 43% patients with

distal biceps tendon rupture were active smokers and had a 7.5 times higher risk of complete distal biceps tendon ruptures when compared with the non-smokers group.[7] They concluded that smoking may cause repetitive anoxia in the hypovascular area of the distal biceps enthesis. Our study showed that chronic tobacco smoking causes poor formation of new vessels due to highly packed non-collagenous ECM and may be indirectly responsible for further impaired tendon regeneration.

The Bonar score is used commonly to assess the histopathologic alterations in tendinous tissue. However, this well-established system may be biased by a few factors, which was described by Fearon *et al.*[32] The authors assessed the possible implications of evaluated area in pathological tendon and outcomes measured in Bonar score. The inter-tester reliability of the Bonar score was good; however, authors advised to update the scale with some amendments concerned with vascularity, hypo- and hypercellularity features. The complete lack of vascularity, assumed by Fearon *et al.* as a pathology, should also be graded with three points, as well as the greater than three clusters of vessels per 10 HPFs. Moreover, the authors advised the microscopic evaluation of vascularity under 400× magnification in 10 HPFs or less. In the present study we analyzed 10 HPFs of the most pathological tendinous tissue, which demonstrated presence of the densest vascular network. However, we supported observation with various magnifications: 100–400×. Furthermore, NDS was calculated on the assumption of the classical Bonar system. The avascular tissue in pathological samples was considered as the lowest grade of NDS (zero points), but not the highest (three points), as was suggested by Fearon *et al.* In fact, the scores in active smokers were the lowest due to paucity of neovascularization, which may indicate the need for an update of the classical scoring system.

Some recent studies revealed that the concept of neovascularization in tendinopathy seems to have gained nearly a mythological status.[33] It used to be considered as an important diagnostic and prognostic value, strongly related to the clinical outcome.[33] The most common tendon pathology, Achilles tendinopathy, is often linked with neovascularization and this process, accompanied by nerve ingrowth and neurotransmitters release, was hypothetically responsible for pain.[23] However,

other studies showed that there is no correlation between excessive neovascularization and pain intensity.[34] Moreover, Lian *et al.* examined patellar tendon pathology and revealed an increased number of non-vascular sensory, substance P-positive nerve fibers, and a decreased occurrence of vascular sympathetic nerve fibers.[35] Neovessel formation was also demonstrated by Sengkerij *et al.* in asymptomatic athletes.[36] The results of our study also confirm that the roles are changing and the presence of neovessels is less important than the mediators and cytokines released in the pathologic tendons. New treatment methods such as sclerosant injections, which have been developed in recent years and designed to eliminate vascular ingrowth in the tendinous structure, have played a less important role than expected and their results are not well-established.[37] Platelet-rich plasma is increasingly used in the management of tendon disorders, thus accelerating the process of healing and tissue regeneration, and may bring new hope in the regulation of the neoangiogenesis in tendinopathy.

The assessment of the neovascularization process was based on the immunohistochemical examination of endothelial cells. CD31 is a 130kDa trans-membrane glycoprotein and CD34 is a 110 kDa trans-membrane glycoprotein, both of which play an important role in the angiogenesis process.[1] CD31 and CD34 can be successfully used to visualize the presence of newly-formed vessels in various pathologies.[27] In our study, CD31 and CD34 allowed us to clearly visualize this process and conduct the morphology assessment and quantification. There are other immunohistochemical markers to visualize the neovascularization process, for example, factor VIII-related antigen, CD105, or smooth muscle actin; however, CD31and CD34 were chosen due to the fact that the authors had rich experience with this immunohistochemical marker.

After tendon injury there is a requirement for cell infiltration from the blood system to provide the abundant group of necessary growth factors and cytokines for tissue healing.[38] However, the presence of angiogenic factors was clearly shown in the tendon healing process; the exact amounts of the local mediators are still unknown.[38] In the present study we were also unable to present the possible implication of the growth factors and cytokines in tendinopathy, which could possibly alter the new vessels' formation. The angiogenesis process is

highly regulated by the local microenvironment.[34] Studies demonstrated that there is a mechanical interaction between neovessel formation and ECM, which regulates the topology and elongation of vessels.[39] It depends on the properties of the matrix, such as fibril orientation and density, which are deeply altered in tendinopathy. Edgar *et al.* observed that angiogenesis and network formation are significantly reduced with the increase of the ECM density.[40] This corresponds with our results and the suggestion that lack of neovessel formation is related to intensified production and aggregation of non-collagenous ECM. Furthermore, Cheema *et al.* demonstrated that nicotine leads to worse functional outcomes and biomechanical properties in tendons after injury. They also noted the correlation between altered healing of the tendon and decreased neovascularization.[10] The structure of the ECM is changing throughout the entire neovascularization process; however, it is still unknown how this interacts with chronic tendinopathy.[41] We observed a possible implication of ECM components in inhibition of neoangiogenesis in chronic tendinopathy, in the smoking population, but this finding needs further studies.

Interestingly, we observed CD34+ cells which were morphologically similar to chondroid cells in the pathologically changed tendinous tissue of the patients with smoking history. What is more, due to their morphology, which is not typical of hematopoietic stem cells, these CD34+ cells may be tissue resident mesenchymal stem cells or a distinct subset of cells with enhanced progenitor activity.[42] We suggest here that these cells are responsible for the production and accumulation of non-collagenous ECM in altered tendinous tissue and related to impaired neovessel expansion. Similarly, using a 3D angiogenesis model, Koehler *et al.* demonstrated that high-sulfated glycosaminoglycans derivatives impair the biological activity of VEGF by hindering receptor activation and subsequent signaling, which might be due to impaired receptor.[43] Furthermore, Cheng *et al.* revealed that polysaccharides inhibit cyclin D1 expression through the inhibition of VEGF receptor signaling, leading to the suppression of angiogenesis.[44]

Just as previous reports regarding tendinopathies in humans, our study is limited in several ways. The main limitation was the investigation of patients without isolated LHB tendinopathy, which is frequently connected with RC pathology. However, since isolated cases of LHBT pathology are rare, most studies regarding this tendon included a group of patients similar to ours. Furthermore, patients underwent two different surgical procedures, tenotomy/tenodesis, which produced comparable results.[36] Another weakness comes from the fact that the group of patients included in this study was relatively small and non-homogenous. Moreover, we could not obtain reliable information about nicotine concentration and other harmful substances that all subjects had consumed, and the dose-dependency rate cannot be estimated from this study. Finally, our study was based on a quantitative scoring system, which can be affected by subjective differences between observers. In order to reduce this bias, samples were examined by three professionals and the comparison between groups and statistical analyses were blinded and conducted by two independent investigators.

Conclusion

In conclusion, the present study revealed that smoking impairs the vascularization of the biceps tendon in chronic tendinopathy cases. The new vessel expansion was visualized supported by immunohistochemical staining and quantified using NDS, adapting the criteria of the Bonar score. Clinically, we observed a negative correlation between the smoking history and extent of neovascularization. Although neovascularization is critical to tissue repair, we did not observe its effect on functional preoperative status. The intensity of the neovascularization process did not correlate with the preoperative functional outcomes of patients, which was, rather, related to patients' age. Our studies revealed also a possible implication of ECM in inhibition of neoangiogenesis in chronic tendinopathy. However, further studies on larger sample sizes are required to confirm these findings.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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