DOI: https://doi.org/10.2298/SARH241227030A
UDC: 616.711-056.83

ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Trabecular microcalli in lumbar vertebrae of adult men with alcohol-associated liver disease: postmortem micro-computed tomography assessment

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SUMMARY

Introduction/Objective Increased fracture risk was previously associated with alcohol-associated liver disease (AALD), but contemporary literature lacks the assessment of the micro-fracture healing events (microcalli) in these individuals. We aimed to quantify microcalli in a trabecular compartment of lumbar vertebrae obtained from individuals with pathohistological confirmation of AALD.

Methods We used high-resolution micro-computed tomography scanning to evaluate the density of trabecular microcalli in the anterior mid-transverse portion of lumbar vertebral bodies collected from 32 male adult cadaveric donors (age range: 33–75 years), divided into the AALD group (n = 13) and the control group (n = 19). Pathohistological analysis indicated that seven individuals had the initial AALD stage (fatty liver disease), while six individuals had end-stage AALD (alcoholic liver cirrhosis).

Results A declining trend in the density of trabecular microcalli was noted in the AALD group ($1.8 \pm 1.7/mm^3$) compared to the control ($3.3 \pm 2.6/mm^3$), but without reaching statistical significance ($p = 0.080$, Student's t-test). The density of trabecular microcalli was not significantly different between initial and end-stage AALD ($p > 0.05$; ANOVA with Bonferroni correction). Pearson correlation indicated that a decreasing trend in the density of trabecular microcalli was associated with the deteriorated trabecular microarchitecture of the AALD group.

Conclusions The density of trabecular microcalli was not significantly altered in the lumbar vertebrae of men with different stages of AALD, suggesting that AALD does not have a substantial impact on the healing process of trabecular micro-fractures and the formation of trabecular microcalli in the lumbar vertebrae. However, future studies are required to confirm our findings.

Keywords: alcohol-associated liver disease; micro-fracture healing event; microcallus; micro-CT; lumbar vertebrae; men

INTRODUCTION

The adverse impacts of heavy alcohol consumption on population health led the World Health Organization to define standard alcoholic units (i.e., standard drinks) as 10 g of pure ethanol, with both men and women advised not to exceed two standard drinks per day [1]. This definition has not yet been universally adopted, resulting in varying governmental standards and guidelines across different countries [1]. Continuous consumption of more than three standard drinks per day in men and more than two drinks per day in women for more than five years increases the risk of developing alcohol-associated liver disease (AALD) [2]. Chronic alcohol consumption, regardless of the type of alcoholic beverage, disrupts liver metabolic functions, which results in hepatocytic lipid accumulation, causing the appearance of the initial pathohistological AALD manifestation – fatty liver disease (FLD) i.e., steatosis [3]. With further AALD development, acute and/or chronic alcoholic steatohepatitis may occur, which sets a structural base for progressive liver fibrosis [2]. Finally, irreversible end-stage AALD, known as alcoholic liver cirrhosis (ALC), could be developed by fibrous tissue accumulation and nodular organization of liver parenchyma, making these individuals more prone to hepatocellular carcinoma [3].

While contemporary research in AALD focuses mainly on liver-related clinical manifestations, extrahepatic complications are often neglected [4]. Due to damage of the liver-bone bidirectional crosstalk, frequently neglected AALD-induced extrahepatic complications are skeletal alterations, initially classified as hepatic osteodystrophy [4, 5]. Hepatic osteodystrophy is a clinical term that refers to a spectrum of skeletal abnormalities, including osteopenia, osteoporosis, and osteomalacia, depending on the severity and underlying pathophysiological mechanism [4, 5]. However, more recent studies suggest that osteomalacia, a condition characterized by poor bone mineralization and accumulation of unmineralized osteoid, rarely exists in AALD [6, 7]. On the other hand, osteopenia and osteoporosis are reported to affect up to one-half of patients with AALD, which was demonstrated with an increased fracture risk, primarily affecting the lumbar vertebrae [8, 9].

Received • Примљено: December 27, 2024
Revised • Ревизија: April 3, 2025
Accepted • Прихваћено: April 4, 2025
Online first: April 7, 2025

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A major problem in the clinical management of increased bone fragility is its asymptomatic nature, which causes delays in therapeutic and preventive measures [8]. Since osteoporosis and bone fragility are more frequently observed in postmenopausal women, and AALD is more frequent in men, it is essential to note that AALD may cause a change in the sex-specificity of fracture risk [10]. Additionally, a particularly concerning observation is the AALD-induced shift toward earlier fracture occurrence in younger individuals [8, 11]. Further, the bone fracture mortality rate is substantially higher in patients with AALD [12], indicating the urgent need for adequate bone-assessing tools in the clinical management of these patients [8].

Since direct analysis of bone fracture risk is often challenging, many studies rely on measuring various surrogate markers of increased bone fragility [13]. The "gold standard" in the clinical assessment of bone fragility is the measurement of bone mineral density (BMD) using dual-energy X-ray absorptiometry [13]. Although this method provides reliable data on population-based fracture risk, it is known that BMD alterations can only partially explain increased bone fragility in each patient [14]. Therefore, modern research focuses on analyzing other characteristics at various hierarchical levels of bone tissue organization to understand further the increased bone fragility in the elderly and individuals with chronic diseases [15]. Previous studies reported that micro-scale alterations in the trabecular part of the lumbar vertebrae (thinner and less numbered trabeculae with reduced mechanical properties, deteriorated osteocytic network, and altered bone marrow adiposity) contribute to increased bone fragility in AALD [4, 9, 16]. Furthermore, it has been known that microdamage accumulation, including microcracks and micro-fractures, contributes to the aging-related bone strength decline [17]. Previous research indicates that bone microdamage is typically healed in the form of globular, woven bone formations known as microcalli [17, 18]. Still, bone micro-fracture healing events have not been previously investigated in AALD. Therefore, this study aimed to quantify the density of trabecular micro-fracture healing events (microcalli) in male individuals with pathohistological confirmation of AALD. Additionally, to analyze the effect of the liver disease stage on the density of trabecular microcalli, bone samples collected from individuals with initial and end-stage AALD were compared. Lastly, we aimed to estimate the potential association between micro-fracture healing events (microcalli) and disruption of trabecular microarchitecture in lumbar vertebrae of male individuals with pathohistological confirmation of AALD.

METHODS

Study design and study material

This cross-sectional study encompassed micro-computed tomography (micro-CT) images stored in the digital repository of the Center of Bone Biology, Faculty of Medicine, University of Belgrade (MFUB). Briefly, this study involved the analysis of micro-CT scans of 32 anterior mid-transverse portions of the first lumbar vertebrae that were collected during routine autopsies at the Institute of Forensic Medicine, MFUB, from deceased male adults (aged 33–75 years). The sample collection was approved by the MFUB Ethics Committee (approval no. 1322/V-1, approval date: 20.05.2021).

The collected bone samples were divided into the following groups:

1) AALD group (samples of male adults with different AALD stages, n = 13);

2) control group [samples of healthy male adults of the same age (± 5 years) without liver disease, n = 19].

The AALD group included bone samples from adult men with macroscopically visible signs of chronic alcoholism, with the visible presence of pathologically altered liver tissue at autopsy and positive hetero-anamnestic data on long-term alcohol consumption (more than three units of alcohol per day for more than five years) [9]. Pathohistological analysis of liver tissue confirmed the presence of different stages of AALD in these individuals (Figure 1), revealing that the initial AALD stage was

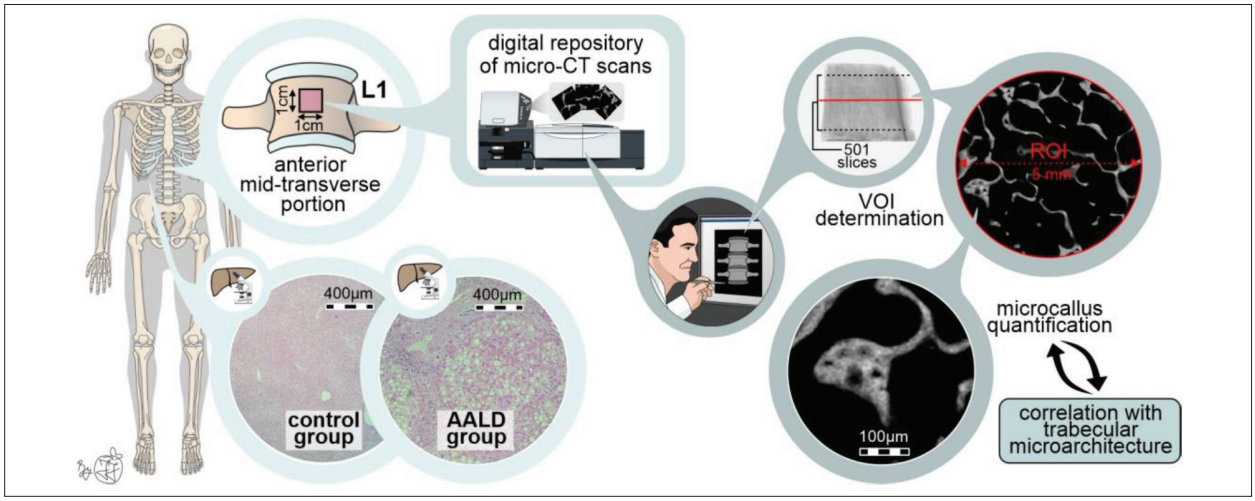

Figure 1. Schematic representation of methodology used in the present study; the figure is hand-generated using vector graphic editor software (CorelDRAW Graphics Suite v.X5, Ottawa, Canada) and represents the author's original work; AALD – alcohol-associated liver disease; L1 – first lumbar vertebra; micro-CT – micro-computed tomography; VOI – volume of interest; ROI – region of interest

present in seven individuals (FLD group), while the end-stage AALD was present in six individuals (ALC group). The control group included healthy age-matched men (± 5 years) without macroscopically visible liver disorders and with negative hetero-anamnestic data about long-term alcohol consumption. Pathohistological analysis of liver tissue confirmed the absence of pathological changes in liver tissue in these individuals (Figure 1).

Study exclusion criteria were a positive history of endocrine and metabolic diseases affecting the skeletal system (e.g., hyperparathyroidism, hypogonadism, thyroid function disorder, diabetes, chronic kidney disease), hereditary musculoskeletal changes, and the presence of solitary and/or metastatic cancerous, infectious and/or inflammatory lesions, as well as the use of drugs that significantly affect bone metabolism (e.g., antiepileptics, cytostatics, corticosteroids, hormonal therapy, vitamin D, and bisphosphonates). Also, clinical or hetero-anamnestic data on intravenous drug abuse and conditions characterized by immobility were criteria for exclusion from the study in potential donors of all groups.

Our micro-CT scanning was performed according to previously published recommendations to ensure adequate imaging quality standards [19]. Using 3D-histomorphometry software (Bruker CTAn Micro-CT Software 2020 1.20.30.0, Bruker, Billerica, MA, USA), all assessments were standardized so that the central 501 sections were analyzed (central section ± 250 sections, Figure 1). Two researchers, independently of each other, manually analyzed the presence of trabecular microcalli in the selected trabecular volume of interest (VOI). These VOIs were generated using centrally-positioned circular regions of interest (ROI), with a diameter of 5 mm (Figure 1). The microcallus quantification was done based on previous reports [18]. In short, trabecular microcalli were defined as globular formations made of woven bone (Figure 1), and its density was quantified per unit of vertebral bone volume (#/mm^3), while the mean results of the two investigators were used for statistical analysis. Further, to estimate the association between microcalli and trabecular microarchitecture, standard vertebral microarchitectural parameters [bone volume ratio (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular pattern factor (Tb.Pf)] were generated using 3D histomorphometry software (Bruker CTAn Micro-CT Software 2020 1.20.30.0, Bruker), as previously described [20].

Statistical analysis

Since the Kolmogorov–Smirnov test confirmed a normal distribution, the Student's t-test for two independent samples was used to assess the significance of the intergroup differences, with a significance level of 5% and a 95% confidence interval ($p < 0.05$). A one-way analysis of variance (ANOVA) with a Bonferroni *post hoc* test was used to assess the significance of the difference between different AALD stages and the control group. Further, the Pearson correlation assessment estimated the correlation between the density of trabecular microcalli and trabecular microarchitectural parameters in the obtained vertebral samples. Non-commercially available statistical EZR software (Easy R on R Commander) was used for data analysis.

RESULTS

The demographic data of included individuals and results of pathohistological assessment of liver tissue samples were presented in Table 1.

Table 1. Basic autopsy data and pathohistological assessment of liver tissue samples

Demographic data	AALD group	Control group
Age (years)	58 ± 13	59 ± 6
BMI (kg/m^2)	25.6 ± 7.1	25.7 ±2.8
Hip BMD in osteoporotic range (n)	2/13	1/19
Hip BMD in osteopenic range (n)	6/13	4/19
Previous bone fracture (n)	0/13	0/19
Pathohistological assessment of liver tissue		
Piecemeal necrosis		
None (n)	2/13	5/19
Mild (n)	1/13	10/19
Moderate (n)	2/13	2/19
Marked (n)	2/13	2/19
Moderate + bridging necrosis (n)	3/13	0/19
Marked + bridging necrosis (n)	2/13	0/19
Multilobular necrosis (n)	1/13	0/19
Intralobular degeneration and focal necrosis		
None (n)	2/13	19/19
Mild (n)	7/13	0/19
Moderate (n)	3/13	0/19
Marked (n)	1/13	0/19
Portal inflammation		
None (n)	4/13	7/19
Mild (n)	6/13	8/19
Moderate (n)	2/13	4/19
Marked (n)	1/13	0/19
Steatosis		
None (i.e. < 5%, n)	1/13	11/19
Mild (i.e. 5–33%, n)	2/13	6/19
Moderate (i.e. 34–66%, n)	4/13	2/19
Marked (i.e. > 66%, n)	6/13	0/19
Fibrosis		
No fibrosis (n)	5/13	14/19
Fibrous portal expansion (n)	1/13	5/19
Bridging fibrosis (n)	0/13	0/19
Cirrhosis (n)	7/13	0/19

AALD – alcohol-associated liver disease; BMI – body mass index; BMD – bone mineral density

Micro-CT revealed the presence of a total of 86 trabecular microcalli in the analyzed samples. Representative micro-CT findings of trabecular microcalli in lumbar vertebrae of individuals with AALD are shown in Figure 2. Trabecular microcalli were absent in 38.4% of the AALD group and 26.3% of control samples.

As shown in Figure 3A, our data indicate a trend toward a decrease in the density of trabecular microcalli in the AALD group (1.8 ± 1.7/mm^3) compared to controls

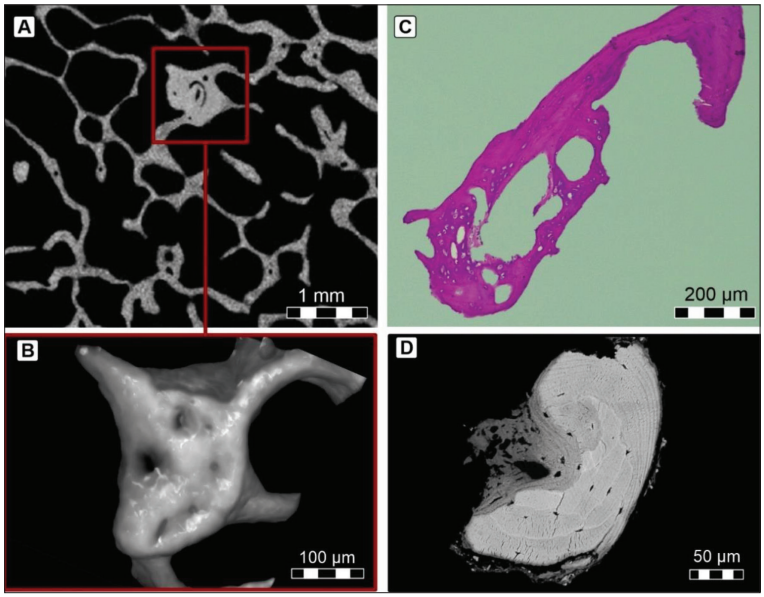

Figure 2. Representative findings of trabecular microcalli in individuals with alcohol-associated liver disease, obtained by micro-computed tomography (A – transverse cross-section, B – 3D reconstruction), C – optic microscopy, and D – quantitative back-scattered electron imaging; the figure represents the author's original work, arranged using vector graphic editor software (CorelDRAW Graphics Suite v.X5, Ottawa, Canada)

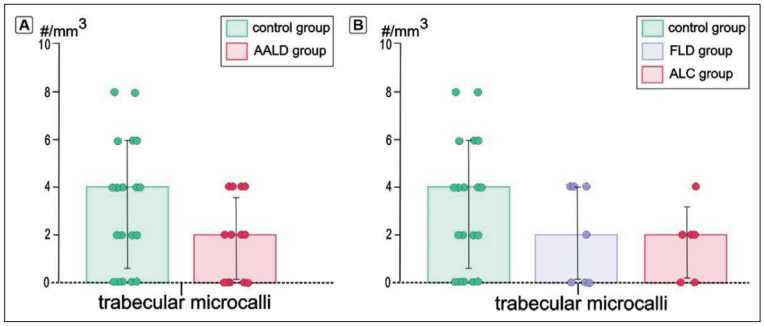

Figure 3. Comparative assessment of trabecular microcalli in individuals with alcohol-associated liver disease and the control group (A) and comparative assessment of trabecular microcalli in individuals with various stages of the disease (B); the statistical significance of the inter-group difference was estimated using the Student t-test (A) and ANOVA with Bonferroni *post hoc* test (B); bar graphs represent the data as mean ± standard deviation, including the distribution of individual data points; the figure represents the author's original work generated using Origin software [Origin(Pro), Version 2018. OriginLab Corporation, Northampton, MA, USA]; AALD – alcohol-associated liver disease; FLD – fatty liver disease (initial stage of alcohol-associated liver disease); ALC – alcoholic liver cirrhosis (end-stage alcohol-associated liver disease)

(3.3 ± 2.6/mm^3), but without reaching statistical significance ($p = 0.080$). As presented in Figure 3B, our data showed no significant difference in the density of trabecular microcalli in lumbar vertebrae collected from individuals with initial and end-stage AALD ($p > 0.05$).

The density of trabecular microcalli showed a strong positive correlation with age in all investigated individuals and controls, indicating an increasing trend in the density of trabecular microcalli with advanced age (Table 2). Furthermore, the density of the trabecular microcalli displayed a strong negative correlation with BV/TV and a moderate negative correlation with Tb.N in controls, suggesting that increased density of trabecular microcalli was noted in persons with lower BV/TV and Tb.N ($p < 0.05$). When analyzing samples from the AALD group only, our data indicated that density of trabecular microcalli displayed a strong positive correlation with BV/TV and mild positive correlation with Tb.N and Tb.Pf, implying that a trend toward a decrease in the density of trabecular microcalli in the AALD group was associated with deteriorated trabecular microarchitecture ($p < 0.05$, Table 2).

DISCUSSION

The concept of bone quality (features related to the bone material structure and composition) is increasingly considered important to heightened bone fragility without being noted in standard clinical tools [15]. Bone quality includes microdamage (micro-fractures, microcracks, and its healing events – microcalli), which accumulates due to physiological loading and mechanical stress occurring in daily life [21]. Numerous studies have focused on investigating the accumulation of ageing-related cortical bone microdamage, revealing that microcracks can dissipate energy and stimulate bone remodeling orchestrated by osteocyte mechanosensing mechanisms [21, 22]. Microdamage accumulation is more prevalent in interstitial bone, which is comparatively older than the surrounding tissues, and it is likely to have higher levels of non-enzymatic collagen cross-linking and lower water content [23]. Previous research devoted less attention to trabecular microdamage, but contemporary data suggest that if trabecular micro-fracture occurs extensively and if the micro-fracture healing rate is not adequate to repair the damage, the trabecular bone connectivity will be lost at the site of the micro-fracture, resulting in trabecular structural weakness, leaving a marked impact on bone strength and eventually leading to aging-related fragility fracture [17, 21]. However, it is essential to note that trabecular microdamage in patients with chronic diseases was rarely studied. To our knowledge, only one study revealed an increased density of vertebral trabecular microcalli in patients with chronic kidney disease [24]. The lack of microcalli research may have been due to the limited number of suitable techniques available for analyzing trabecular microcalli. These techniques include 2D histological assessment using optical microscopy or scanning electron microscopy, as well as 3D histomorphometric assessment using micro-CT (Figure 2). Since our previous studies revealed that the most prominent impairment in micro-scale trabecular vertebral features contributes to increased fracture susceptibility in AALD [4, 16, 25], we aimed to assess the density of trabecular microcalli in male individuals with pathohistological confirmation of AALD, using a high-resolution and non-destructive methodology.

Table 2. Correlation between density of trabecular microcalli and trabecular microarchitectural parameters in lumbar vertebrae

Parameters	Microcalli and age	Microcalli and BV/TV	Microcalli and Tb.Th	Microcalli and Tb.N	Microcalli and Tb.Sp	Microcalli and Tb.Pf
Total sample (n = 32)						
Correlation coefficient (r)	**0.455**	-0.060	-0.211	0.173	-0.277	0.164
p-value	**0.009**	p > 0.05	p > 0.05	p > 0.05	p > 0.05	p > 0.05
AALD group (n = 13)						
Correlation coefficient (r)	0.410	**0.615**	-0.367	**0.578**	-0.147	**0.563**
p-value	p > 0.05	**0.049**	p > 0.05	**0.039**	p > 0.05	**0.045**
Control group (n = 19)						
Correlation coefficient (r)	**0.690**	**-0.634**	-0.327	**-0.400**	-0.128	0.205
p-value	**0.001**	**0.045**	p > 0.05	**0.035**	p > 0.05	p > 0.05

Pearson correlation was used to estimate the correlation between the density of trabecular microcalli and trabecular microarchitectural parameters in collected vertebral samples (significant correlations were presented in bold; AALD – alcohol-associated liver disease; BV/TV – bone volume ratio; Tb.Th – trabecular thickness; Tb.N – trabecular number; Tb.Sp – trabecular separation; Tb.Pf – trabecular pattern factor

Still, our data revealed only a nonsignificant declining trend in the density of trabecular microcalli in AALD (Figure 3), implying that AALD does not have a substantial impact on the healing process of trabecular micro-fractures and the formation of trabecular microcalli in the lumbar vertebrae. Considering that this result challenged our previous findings about the deteriorated vertebral bone quality in AALD, we relied on the Pearson correlation test to understand these puzzling findings [4]. In accordance with previous studies, our data revealed a strong positive correlation of microcalli density with age in all investigated individuals and control individuals, suggesting an increasing trend in the density of trabecular microcalli in advanced age (Table 2) [17, 21]. The density of the trabecular microcalli displayed a strong negative correlation with BV/TV and a moderate negative correlation with Tb.N in our control individuals (Table 2), implying that increased density of trabecular microcalli was associated with aging-related bone strength decline. Previous data suggested that neighboring microcalli are condensed near the endplates area, often appearing to form a line, corresponding to the mechanical loading pattern within the vertebral body [26, 27], while its mechanical characteristics were similar to that of a normal trabecular bone [28], indicating that microcalli might help maintain vertebral bone strength in the elderly [17]. Hahn et al. [26] reported that trabecular microcalli are most frequently found when vertebral BV/TV was less than 11% but without further correlation with declining BV/TV, indicating that this bone strength-maintaining mechanism is becoming ineffective in cases of severely declined microarchitecture. Since nine of our individuals with AALD (69.23%) had BV/TV lower than 11%, our data support this assumption (Table 2), implying that a trend toward a decrease in the density of trabecular microcalli in the AALD group was associated with the highly deteriorated trabecular microarchitecture [20, 29]. Subsequently, it is reasonable to assume that the decreased density of trabecular microcalli manifests reduced vertebral bone strength and subsequently increased vertebral fracture susceptibility in AALD [8].

Additionally, previous studies have not provided a clear answer regarding whether the stage of liver disease substantially impacts bone deterioration and, consequently, the healing process of micro-fractures. Some authors reported that skeletal damage was more pronounced in patients with advanced stages of liver disease and that the severity of skeletal changes is related to the disease stage [4, 20, 30]. Our data did not reveal a significant difference in the density of trabecular microcalli between individuals with initial and end-stage AALD (Figure 3), indicating that the AALD stage does not substantially affect the micro-fracture healing process in the lumbar vertebrae. These conflicting results may be due to differences in the study design (female subjects were predominantly examined in these previous studies), the type of chronic liver disease (cholestatic liver diseases were predominantly analyzed in previous studies), as well as the type of bone-assessing methodology (optic histomorphometry and peripheral quantitative CT were predominantly used in previous studies) or even interobserver differences when using the same methodological approach, which indicates that further research is necessary to elucidate this topic thoroughly [19].

Our study is limited by its cross-sectional cadaveric study design, which prevents us from following the progression of skeletal damage over time. Our findings may be biased due to the covariant effect of various confounding factors, which could not be avoided due to the scarcity of heteroanamnestic data. Although we utilized all available resources, the absence of significant inter-group differences in trabecular microcalli suggested that our assessment may have been underpowered due to the limited sample size. Our assessment may have limited informative value in evaluating bone metabolism, cellular indices, and mineral content of trabecular microcalli in AALD individuals, underscoring the need for further research. Lastly, this study exclusively focused on the density of trabecular microcalli in lumbar vertebrae of AALD individuals, thereby enabling only an indirect estimate of its effect on bone micro-fracture accumulation and healing.

CONCLUSION

Our data has revealed that the density of trabecular microcalli was not significantly altered in the lumbar vertebrae of men with different stages of AALD, suggesting that AALD does not have a substantial impact on the healing process of trabecular micro-fractures and the formation of trabecular microcalli in the lumbar vertebrae. Our data

implied that a trend toward a decrease in the density of trabecular microcalli in the AALD group was associated with the highly deteriorated trabecular vertebral microarchitecture. However, considering this study's limitations, further research is necessary to confirm our results.

ACKNOWLEDGMENT

The authors thank Prof. Dr. Nada Tomanović and the medical staff at the Institute of Pathology, Faculty of Medicine, University of Belgrade for their invaluable help in the pathohistological liver tissue assessments. Further, the authors thank Prof. Dr. Slobodan Nikolić, Prof. Dr. Vladimir Živković, Asst. Prof. Dr. Danica Đukić and medical staff from the Institute of Forensic Medicine, Faculty of Medicine, University of Belgrade for their help in collecting bone samples. Lastly, the authors thank Prof. Dr. Danijela Đonić for her administrative support.

Funding: J.J. and M.Đ. received financial support from the Ministry of Science of the Republic of Serbia [grant no. 451-03-1524/2023-04/18 and 451-03-66/2024-03/200110].

Conflict of interest: None declared.

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Трабекуларни микрокалуси слабинских кичмених пршљенова код одраслих мушкараца са алкохолном болешћу јетре – постмортална анализа методом микрокомпјутеризоване томографије

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САЖЕТАК

Увод/Циљ Претходне студије указале су на повећан ризик од прелома код особа са алкохолном болешћу јетре (АБД), али зарастање трабекуларних микропрелома (у виду микрокалуса) до сада није истраживано код ових болесника. Циљ наше студије био је да квантификује густину трабекуларних микрокалуса у слабинским кичменим пршљеновима мушких кадаверичних донора са патохистолошком потврдом различитих стадијума АБД-а.

Методе Користили смо снимке микрокомпјутеризоване томографије високе резолуције за процену густине трабекуларних микрокалуса у телима слабинских кичмених пршљенова прикупљених од 32 мушка одрасла кадаверична донора (старости између 33 и 75 година), који су били разврстани у две групе – АБД групу ($n = 19$) и контролну групу ($n = 13$). Патохистолошка анализа ткива јетре показала је да је седам особа имало почетни стадијум АБД-а (масна болест јетре), док је шест особа боловало од крајњег стадијума АБД-а (алкохолна цироза јетре).

Резултати У АБД групи уочен је тренд смањења густине трабекуларних микрокалуса ($1,8 \pm 1,7/mm^3$) у поређењу са контролном групом ($3,3 \pm 2,6/mm^3$), али без достизања статистичке значајности ($p = 0,080$; Студентов т-тест). Такође, није утврђена значајна разлика у густини трабекуларних микрокалуса код особа са различитим стадијумима АБД-а ($p > 0,05$; АНОВА, Бонферонијева *post hoc* анализа). Пирсонов тест корелације указује да је опадајући тренд густине трабекуларних микрокалуса повезан са пропадањем трабекуларне микроархитектуре уочене у АБД групи.

Закључак Наши резултати показују да густина трабекуларних микрокалуса није била значајно измењена код мушкараца са различитим стадијумима АБД-а, што указује да АБД нема значајан утицај на процес формирања трабекуларних микрокалуса слабинских кичмених пршљенова. Ипак, даља истраживања су неопходна како би потврдила ове резултате.

Кључне речи: алкохолна болест јетре; зарастање микропрелома; микрокалус; микрокомпјутеризована томографија; слабински кичмени пршљенови; мушкарци