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Original Research

Inhomogeneity of stiffness and density of the extracellular matrix within the leukoplakia of human oral mucosa as potential physicochemical factors leading to carcinogenesis

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ABSTRACT

Oral leukoplakia is a clinical term relating to various morphological lesions, including squamous cell hyperplasia, dysplasia and carcinoma. Leukoplakia morphologically manifested as hyperplasia with epithelial dysplasia is clinically treated as precancerous condition. Nevertheless, there is a lack of good markers indicating the transformation of premalignancies towards cancer. A better understanding of the mechanical environment within the tissues where tumors grow might be beneficial for the development of prevention, diagnostic, and treatment methods in cancer management. Atomic force microscopy (AFM) and immunohistology techniques were used to assess changes in the stiffness and morphology of oral mucosa and leukoplakia samples at different stages of their progression towards cancer. The Young's moduli of the tested leukoplakia samples were significantly higher than those of the surrounding mucus. Robust inhomogeneity of stiffness within leukoplakia samples, reflecting an increase in regeneration and collagen accumulation (increasing density) in the extracellular matrix (ECM), was observed. Within the histologically confirmed cancer samples, Young's moduli were significantly lower than those within the precancerous ones. Inhomogeneous stiffness within leukoplakia might act as "a mechanoagonist" that promotes oncogenesis. In contrast, cancer growth might require the reorganization of tissue structure to create a microenvironment with lower and homogenous stiffness. The immunohistology data collected here indicates that changes in tissue stiffness are achieved by increasing cell/ECM density. The recognition of new markers of premalignancy will aid in the development of new therapies and will expand the diagnostic methods.

Introduction

Oral leukoplakia is clinically described as a white colored oral lesion that is not linked to another disease process. Usually, these lesions are largely asymptomatic; however, they are clinically relevant due to an increased risk of cancer development, either in or close to the area of leukoplakia or elsewhere in the oral cavity or the head and neck region [5]. Oral leukoplakia is associated strictly with chronic trauma, smoking, hot temperatures, human papillomavirus (HPV) infection or dysbiosis. Changes in the oral microbiome have been proposed to contribute to oncogenesis in 7–15% of oral cancer cases that cannot be explained by known risk factors [36]. Histologically, the term leukoplakia refers to a large spectrum of lesions beginning from simple squamous cell hyperplasia, hyperkeratosis and parakeratosis, squamous cell dysplasia and oral squamous cell carcinoma (OSCC) (both in situ and invasive) [28]. Therefore, the final histopathology is necessary to assess the nature of leukoplakia. The malignant transformation rate displays excessive disparity, and until now, there has been no good marker available to assess the risk of such transformation [58,60]. Common alterations in chromosomal regions and genes, including FBXL5, UGT2B15, UGT2B28, KANSL1, GSTT1 and DUSP22, as well as some putative genes associated with hallmarks of malignancy are believed to play significant roles in predicting the evolution of lesions in term of leukoplakia to squamous cell carcinoma [58]. Malignant transformation has also been found to be

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associated with the status of SMAD4 expression [61], a balance in DNA repair proteins [3], the presence of keratin 10 in saliva [10], ALDH1 and podoplanin contents [32] as well as GLUT-1-specific staining with an oral brush biopsy [7]. The timely detection and treatment of high-risk premalignancy is very important in the prevention of any type of cancer [9,15]. Any additional test that could enrich standard histopathological methods and improve cancer detection and prevention would be very valuable. In recent years, tissue stiffness has become a powerful candidate as a highly specific mechanomarker that indicates pathophysiological changes within the tissue [52]. Because stiffness at the cellular level is associated with cell cytoskeletal remodeling, it was previously suggested that mechanical markers such as cell stiffness might be observed (recorded) before histopathological detection [42]. The mechanical properties of single cells and whole tissues change during diseases such as atherosclerosis, diabetes and many kinds of cancer, including bladder, breast and prostate cancers [19,21,33,51]. The early detection of premalignant conditions and lesions is also very important in the case of oral cancers, where despite significant advances in cancer treatment, the overall 5-year survival rate has not significantly increased over the past decades [4,11]. In this study, an assessment of stiffness in tissues collected from the oral cavity, including healthy mucosa, oral squamous hyperplasia with low grade dysplasia (leukoplakia) and oral cancer, has been performed using atomic force microscopy (AFM), in addition to histology that revealed a pattern of high inhomogeneity within leukoplakia samples. Thus, high inhomogeneity might be considered as mechanomarker of cancerous transformation, and as a tissue environmental factor (mechanoagonist) associated with cancer development.

Materials and methods

Tissue samples

Tissue samples were collected from three groups of patients. Group I consisted of 6 patients (3 women and 3 men) diagnosed with squamous cell hyperplasia with low grade dysplasia (leukoplakia), group II included 4 patients (2 women and 2 men) diagnosed with OSCC, and group III (control group) consisted of 3 patients (1 woman and 2 men) with healthy mouth mucosa. All the patients underwent a routine examination at the Clinic of Periodontal and Oral Mucosa Diseases, Dental Practice in Bialystok, Poland. Patients reported no alcohol abuse or tobacco use. Collected tissues were immediately placed into tubes filled with medium (DMEM + 10% FBS) and transported on ice (~4 °C) for an average of 1 h before the AFM measurements were conducted. For the patients from group I (leukoplakia), two types of samples were always collected: mucosa obtained from the healthy margin of the leukoplakia (0.5 cm from the lesion, without any major pathological changes) and tissue obtained from the healthy buccal mucosa. Altogether, this yielded four types of tissues for further examination: normal tissues, free margin of the leukoplakia, samples from the squamous cell hyperplasia with low grade dysplasia (leukoplakia) and oral carcinoma tissues (Table 1).

Inclusion criteria for this study were age 18–72 years and a clinical diagnosis of keratosis with simultaneous overgrowth folds of the mucous membrane outside the lesion requiring a cut or with a clinically healthy mucous membrane of the mouth but an overgrown frenulum lip cheek. The exclusion criterion was previously received radiotherapy, chemotherapy or treatment with steroids. Each patient underwent a general medical and dental examination. Before excision of the material for the histopathological examination, patients were informed in an understandable way orally and in writing regarding the nature of the research. The respondents were asked to complete a “Patient Information” and "Informed Consent" form to participate in the study, with the option of resignation. The material for the tests was collected in accordance with the procedure of surgical removal of the slice/cut-outs of keratotic lesions or overgrown folds of the oral mucosa of clinically healthy tissues and subjected to further diagnosis. The procedure was performed by one physician. The test was carried out according to a specific protocol approved by the Bioethics Committee of Medical University of Bialystok (R-l-002/4/2019).

Table 1
Histological and nanomechanical characterization of collected tissue samples.

Group	Tissue sample source	No. of samples	No. of force curves analyzed
I	Squamous cell hyperplasia with low grade dysplasia (leukoplakia)	6	n = 2026
II	Oral squamous cell carcinoma	4	n = 3458
III	Healthy buccal mucosa (control)	3	n = 564
IV	Free margin of the squamous cell hyperplasia with low grade dysplasia (leukoplakia)	6	n = 1589

Tissue sample preparation

From each sample, a thick (~ 3–4 mm) around 5 × 5 mm slice was cut using a razor under liquid conditions and glued onto a 35-mm Petri dish using a small amount of cyanoacrylate adhesive. Small amount of medium was left on the top of the tissue to prevent drying. After gluing (~2 min), the sample was immersed in fresh DMEM, and AFM measurements were performed within 3 h at room temperature. After mechanical testing, tissue samples were gently detached and fixed in 10% buffered formalin. Fixed samples were then stained as described below.

AFM measurements

AFM measurements were performed using a Bruker/JPK Instruments system (model Nanowizard 4 Bioscience AFM) with the CellHesion module, allowing the Z axis to be extended up to 100 µm. Elasticity measurements of the tissue samples were taken with AFM working in force spectroscopy mode. The cantilevers used in the study were Arrow TL1Au cantilevers (Nanoworld) with a Ø 4.5 µm PS sphere and a nominal spring constant (k = 0.03 N/m). Constant force of 1 nN was applied for mechanical testing over the surface of the tissue. Up to 16 maps consisting of 8 × 8 points corresponding to a scan area of 10 × 10 µm spread around the sample were taken. Therefore, up to 1024 curves for each tissue sample were collected (Fig. 1).

AFM data collection and analysis

A colloidal tip mounted at the end of the cantilever was used to indent the sample, resulting in a change in the laser beam position at the photosensitive detector. The raw data included the cantilever deflection (d) versus the distance that the cantilever was moved towards the surface of the sample (*Z displacement*). The cantilever deflection was converted into force (F) using $F = d \times k$, where k is the spring constant of the cantilever. The apparent Young's (elastic) modulus of the tissue sample was calculated from the force vs distance curve using the Hertz model, where

$$F = \frac{4}{3}\frac{E}{1-\mu^2}\sqrt{R}\delta^{\frac{3}{2}}$$

Here, F is the loading force applied, μ is the Poisson's ratio of the sample equal 0.5, E is the Young's modulus of the sample, R is the radius of the probe and δ is the depth to which the sample was indented. The whole analysis was performed using JPK Data Processing software, and the final Young's modulus of a tissue was calculated taking into account all force curves recorded for a single tissue sample and is expressed in kilopascals (kPa).

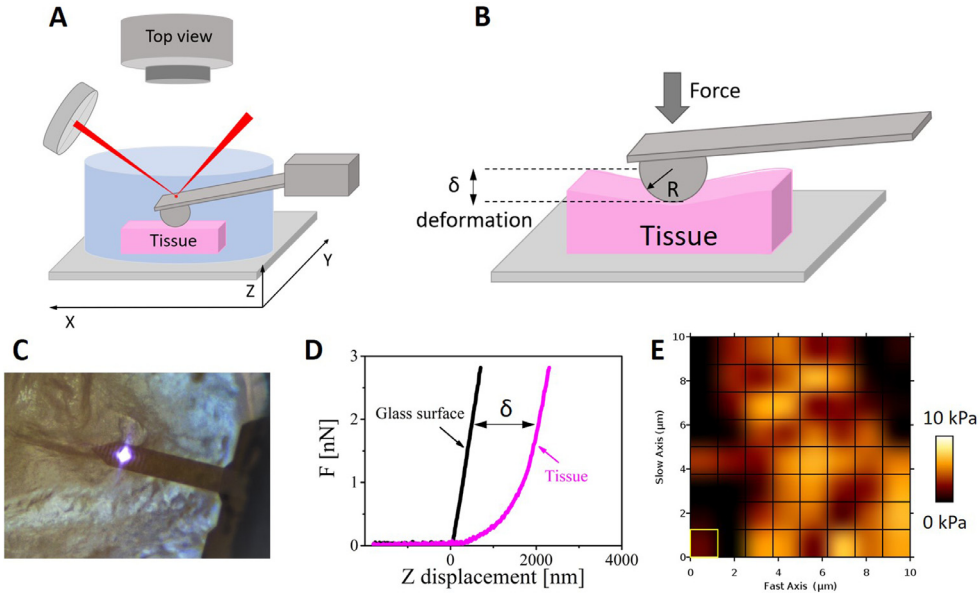

Fig. 1. Experimental setup. (a) Schematic of the AFM setup used to measure the stiffness of the tissue samples. (b) Idea of the colloidal probe indentation of the tissue sample where the force is applied perpendicular to the tissue surface. (c) Top view image of the AFM cantilever over the tissue sample before indentation. (d) Force vs distance curve recorded for the glass surface (reference, black) and the tissue sample (pink). During the data analysis, the two curves are subtracted to generate a force vs indentation curve that is subsequently fitted with the Hertzian model with the known radius of the indenter. (e) Representative 10 × 10 μm stiffness map collected from the tissue sample with brighter colors indicating higher stiffness values and darker colors indicating lover stiffness values.

Histopathological and immunohistochemical analyses

All obtained samples were fixed in 10% buffered formalin and then processed in paraffin blocks. First, slides with an approximate thickness of 4 μm were cut and stained with hematoxylin and eosin. Additionally, in another set of experiments, the corresponding slides were stained using immunohistochemistry. For each type of reaction, the slides were incubated in PTLink (DAKO) to retrieve the antigen (high pH = 9.0). Subsequently, the following antibodies were used: Bcl-2 (DAKO M0887, mouse monoclonal antibody, 1:100 dilution), P16 CINtec (Histology Kit, Ventana Roche, clone E6H4, mouse monoclonal), p53 (DAKO mouse monoclonal antibody M 7001, clone DO-7) and collagen IV (mouse monoclonal antibody COL4A1 (5E10): sc-517572 Santa Cruz Biotechnology, 1:50 dilution). For the blood vessels assessment, we used CD31 antibody (CD31, Endothelial Cell (Dako Omnis Clone JC70A). Fibrosis was evidenced using Masson's trichrome staining in Ventana Benchmark and assessed semiquantitative according to the following scoring system: 0 – no fibrosis (lack of collagen deposits), 1 – mild fibrosis (focal fibrotic expansion network of collagen, mainly in the perivascular areas), 2 – moderate fibrosis (focal dense increase of collagen bands) and 3 – severe fibrosis (diffuse, strong, dense collagen band expansion in the stroma). In this type of staining, the collagen fibers are stained blue, the nuclei are stained black, and the background is stained red. The final reactions were evaluated under a light microscope (Olympus CX 45) by one pathologist.

Results

All the specimens, divided into 4 groups, were first mechanically tested using AFM. Mechanical testing involved multiple loading-unloading cycles over the surface of the tissue specimens with a constant force of 1 nN applied. Since strong local inhomogeneity in the mechanical properties of other types of tissues was previously reported, hundreds of points per tissue sample were investigated. Fig. 2a shows strong inhomogeneity between subsequent locations measured (stated as the map number), especially for squamous cell hyperplasia with low grade dysplasia and squamous cell carcinoma samples. Normal buccal mucosa tissue and healthy margin from the squamous cell hyperplasia with low grade dysplasia were both significantly softer than hyperplasia with dysplasia and squamous cell carcinoma samples. However, the small inset in Fig. 2b shows that differences in stiffness between normal tissue (black columns) and marginal tissue (gray columns) were distinguishable. Two-sample t-tests for differences in means showed $p < 0.001$ with Young's modulus equal to 1.1 ± 1.3 kPa (n = 564) for normal tissue and 0.9 ± 1.2 kPa (n = 1589) for marginal tissue (data not shown). Additionally, both types of tissue showed increased slight hyperplasia of the squamous epithelium, but lymphocytic infiltration was observed in the marginal tissue, with one sample showing dense inflammatory infiltration not present in normal tissues (Fig. 2c and black arrow in Fig. 2d). Samples from squamous cell hyperplasia with low grade dysplasia and squamous cell cancer samples showed dense lymphocytic infiltration (black arrow in Fig. 2e and 2f). Squamous cell hyperplasia with low grade dysplasia and squamous cell carcinoma samples were significantly stiffer than their normal counterparts, with a mean stiffness equal to 12.9 ± 20.9 kPa (n = 2026) for hyperplasia with dysplasia and 4.0 kPa ± 5.5 kPa (n = 3458) for squamous cell carcinoma tissue. Such a notable increase in the stiffness of samples with squamous cell hyperplasia with low grade dysplasia may suggest the existence of specific mechanical perturbations associated with tissue reorganization when in this premalignancy state. Since it is known that the development of premalignancy and malignancy is a multistep process involving the accumulation of genetic, epigenetic and metabolic alterations, we broadened our histopathological examination.

Evaluation of tissue vascularity and fibrosis

It is known that an increase in tissue stiffness may be associated with increased vascularization, ECM protein overexpression and increased matrix fibrosis and crosslinking [20,45]. Vascularity and fibrosis can be evaluated using collagen IV (basal membrane in blood vessels), Masson's trichrome staining and CD31 (blood vessels endothelial cells). Results of this analysis are presented on Figs. 3–4. As presented, significant fibrosis evaluated as moderate and severe was observed in squamous cell hyperplasia with low grade dysplasia, and trichrome staining showed increased collagen deposition (increased fibrosis) (Fig. 3). Nevertheless, this effect was variable among specimens, since in half of the analyzed samples, collagen deposit was greater than that in others. The accumulation of collagen fibers (stained blue) is clearly seen in Fig. 3f, and interestingly, we observed moderate and severe fibrosis in the squamous cell hyperplasia with low grade dysplasia specimens than in the squamous cell cancerous specimens, where the fibrosis was assessed as mild one. Although numerous blood vessels were visible in both normal and pathologically transformed tissues (black arrows in Fig. 3a, e and g), their number in all squamous cell hyperplasia with low grade dysplasia

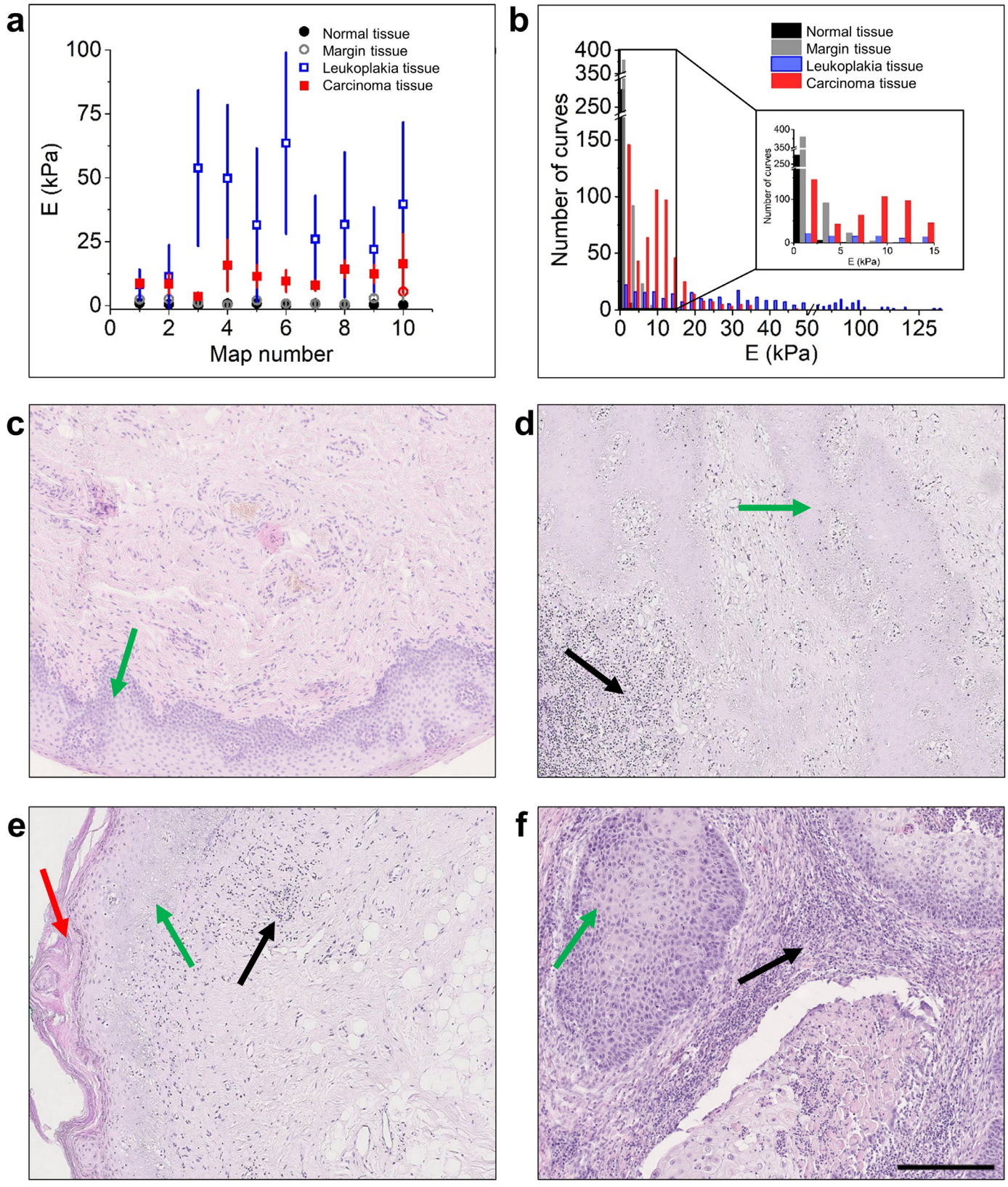

Fig. 2. Stiffness and histopathology of tested samples. Panels a and b show Young's modulus (E) values for visibly healthy and visibly changed samples. (a) Mean ± SD of local E (in kPa) and (b) E distributions for all force curves collected. The inset in Fig. 2b shows the distribution of E in the range of 0 to 15 kPa to highlight the difference between normal (healthy) tissue and tissue outside of the leukoplakia area. Panels c, d, e and f show representative images from hematoxylin staining. (c) Normal squamous epithelium in oral mucosa (d) tissue margin of the samples with squamous cell hyperplasia with low grade dysplasia (e) oral squamous cell hyperplasia with dysplasia and abundant lymphocytic infiltration within the stroma and (f) keratinizing squamous cell carcinoma G2 and dense lymphocytic infiltration. Arrows indicate regions with squamous epithelium hyperplasia (green), hyperkeratosis and parakeratosis (red) and lymphocytic infiltration (black). Scale bar: 250 μm.

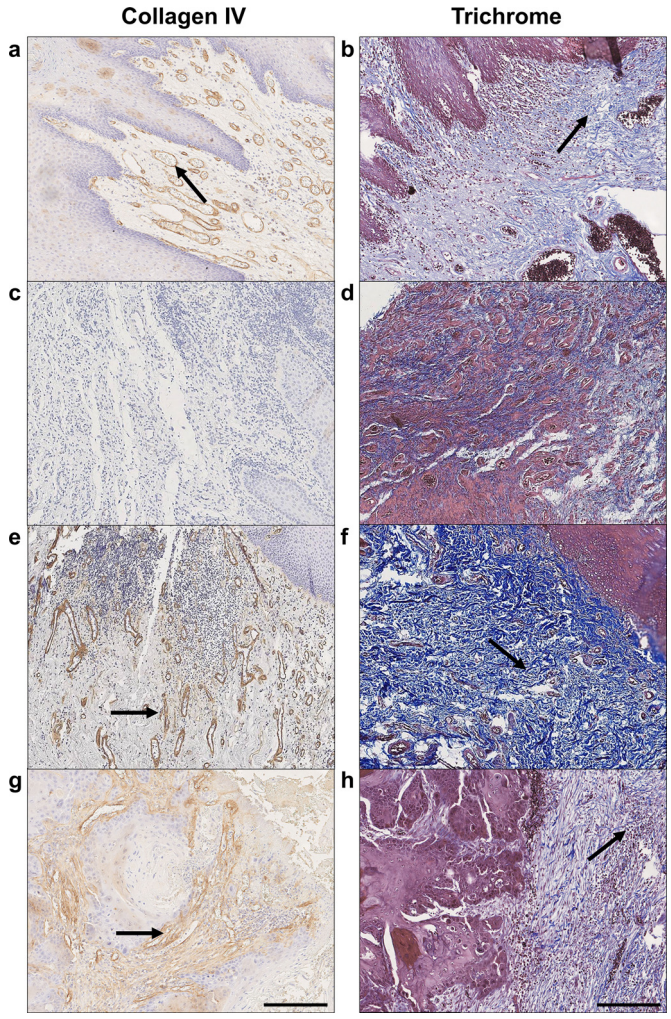

Fig. 3. Evaluation of vascularity using immunohistochemistry for collagen IV and Masson's trichrome staining for fibrosis. (a, b) Normal tissue, (c, d) margins, (e, f) squamous cell hyperplasia with dysplasia and (g, h) squamous cell carcinoma tissue. For collagen IV staining, arrows indicate numerous small and medium sized blood vessels within the stroma. For Masson's trichrome staining, arrows indicate fibrosis in the stroma. Scale bar: 250 μm.

samples were considerably increased when compared to margin area. In margin area, proliferation was slight and much less prominent than in the squamous cell hyperplasia with low grade dysplasia. Nevertheless, we observed two tissue specimens in which collagen IV and CD31 expression in both types of tissues was comparable. This finding may indicate that squamous cell hyperplasia with low grade dysplasia and its' margins phenotype may be similar.

By comparing the vascularity between leukoplakia and in cancer tissues, we observed a similar distribution of the small, proliferating blood vessels. Significant differences were observed between tissues obtained from the normal oral mucosa, squamous cell hyperplasia with dysplasia and margins, as well as in the type of blood vessels and in distribution within the stroma.

Expression of the p16 tumor suppressor protein

ECM dysregulation contributes to premalignancy and malignancy processes [18]. Many genetic modifications undoubtedly initiate and drive pathological transformation, influencing cell proliferation, death resistance, and chronic inflammation. $p16^{INK4a}$ is a tumor suppressor protein that is used as a prognostic biomarker for many types of cancer [1]. The overexpression of $p16^{INK4a}$ has been reported in OSCC tissues associated with human papillomaviruses (HPVs) [22]. Fig. 5 shows representative images of $p16^{INK4a}$ expression in normal, squamous cell hyperplasia with dysplasia and cancerous tissues. In all squamous cell hyperplasia with dysplasia samples, $p16^{INK4a}$ expression was observed mainly in the basal and parabasal layers of the squamous epithelium (Fig. 5c), with intense expression in 4 of 6 samples. This may indicate that in all cases, the cell cycle was ruptured as a result of HPV high-risk infection. $p16^{INK4a}$ was significantly overexpressed in squamous cell carcinoma tissues but stronger and more uniformly distributed over the surface of the tissue. This correlates with previous findings where $p16^{INK4a}$ overexpression was found to be positive in more than 70% of oral carcinoma lesions, whereas in squamous cell hyperplasia with dysplasia, $p16^{INK4a}$ overexpression was positive in only 57% (for 69 cancer and 21 squamous cell hyperplasia with dysplasia tissues tested) [56]. Tissues from normal-appearing mucosa or margins showed slight or no $p16^{INK4a}$ expression.

Evaluation of apoptosis

Bcl-2 and p53 proteins are important in the regulation of apoptosis [13]. Disturbances in the expression of apoptosis-associated proteins are widely observed in many human malignancies [13,37]. A strongly positive Bcl-2 immunoreaction was detected in all squamous cell hyperplasia with dysplasia samples, while only 50% of margin samples showed weak expression in basal cells of the squamous epithelium and in lymphocytes from mucosa biopsy (Fig. 6). Representative images in Fig. 6e and 6f shows an inverse correlation between Bcl-2 and p53 expression. Such observations have already been reported in several types of epithelial tumors, including esophageal squamous cancers, non-small-cell lung cancers and gastric carcinomas [29,70]. p53 expression was observed in the basal and parabasal layers of the squamous epithelium in 5 of 6 samples, with only a few p53 cells present in the remaining sample. Notably, p53 expression was also significant in margins and was especially prominent in 50% of the samples. The above findings may indicate inhibition of the apoptotic process and abnormal cell cycle regulation in such cells [66,68]. Nuclear expression of p53 protein was significantly stronger in squamous cell hyperplasia with dysplasia and squamous cell carcinoma samples comparing to margins and healthy oral mucosa.

In normal oral mucosa and in margins, we observed lack of p53 protein expression. Significant and strong nuclear expression was observed in the basal and parabasal layers of the squamous epithelium in squamous cell hyperplasia samples. Additionally, in squamous cell cancer samples, strong nuclear p53 protein expression was observed.

Bcl-2 protein expression was observed mainly in the lymphocytic infiltrate. The most intense infiltration was observed in squamous cell hyperplasia and squamous cell cancer samples. Dense lymphocytic infiltration is related to the activation of blood vessel proliferation via VEGF and TIA-1 and may explain the dense blood vessel channels within these lesions [39]. Similar p53 protein expression together with p16 expression determines abnormal cell proliferation within squamous cell hyperplasia with dysplasia and squamous cancer cells [46,48].

Discussion

Oral leukoplakia characterized by vascularization and inflammatory cell infiltration might lead to cancer development in 5 to 10% of cases, but little is known about the mechanical induction of cancer that occurs during leukoplakia transformation. A growing number of data collected over the past 10 years indicate that tissue remodeling associated with inflammation and fibrosis causes changes in rheological properties, and such changes can favor cancer development since a stiff microenvironment allows cancer cells to proliferate, migrate and modify nearby connective tissue cells [16,27,34,59,64]. During the course of pathological development, the composition and concentration of specific ECM components are dysregulated, leading to fibrotic disease and creating premalignant conditions, as has been shown for breast and liver fibrosis,

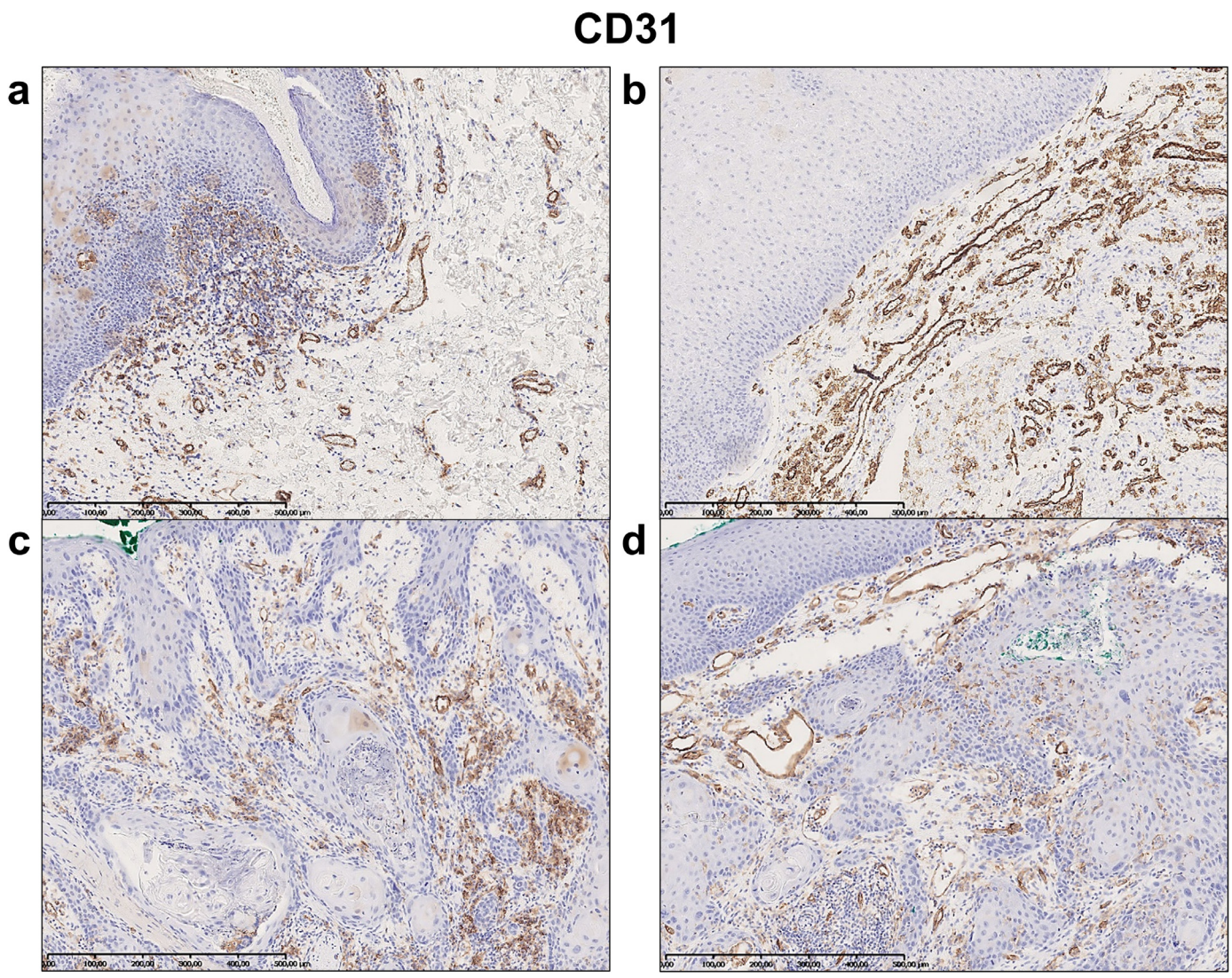

Fig. 4. Expression of CD31 on the endothelial cells (CD31 antibody; Dako Omnis Clone JC70A). (a) Margin of squamous cell hyperplasia with dysplasia with CD31 expression in endothelial cells within the blood vessels. (b) Squamous cell hyperplasia with low grade dysplasia. Numerous blood vessels with CD31 expression in endothelial cells. Images display the normotypic blood vessels and numerous proliferating small blood vessels composed only from the endothelial cells (immature blood vessels). (c, d) Squamous cell carcinomas with normotypic and proliferating small blood vessels. Scale bar: 500 μm.

where increased tissue stiffness contributed to malignant transformation [43,62]. In agreement with this general trend, our data indicate that an increase in tissue stiffness during leukoplakia formation might serve not only as a mechanomarker of subsequent cancer transformation but also as a mechanoagonist governing leukoplakia (in this study assessed as squamous cell hyperplasia with dysplasia transformation towards the cancer stage). We observed increased stiffness in all tested squamous cell hyperplasia with dysplasia samples, with dense fibrosis within the stroma, and an increased number of small blood vessels and abnormal regulation of the cell cycle, resulting in apoptosis inhibition. However, it should be emphasized that a nanoscale assessment of tissue rheology performed using AFM is difficult to directly correlate with molecular and biochemical changes that occur in individual cells or within the defined area of the ECM. In some samples, we also noted very similar processes of tissue remodeling between squamous cell hyperplasia with dysplasia and its margins. This observation indicates that the same cellular and molecular changes may be present in mucosa characterized as leukoplakia and margins, potentially influencing environmental factors and/or indicating different stages of squamous cell hyperplasia with dysplasia development. Numerous bacterial species in the oral cavity are involved in chronic inflammation that leads to the development of oral carcinogenesis. Bacterial products and their metabolic byproducts may induce permanent genetic alterations in epithelial cells of the host that drive the proliferation and/or survival of epithelial cells. *Porphyromonas gingivalis, Fusobacterium nucleatum* and *Treponema denticola* induce the production of inflammatory cytokines and cell proliferation and the inhibition of apoptosis, cellular invasion, and migration through host cell genomic alterations [14]. From a practical point of view, our findings indicate that the removal of leukoplakia tissue located within the margin of healthy tissue might not be sufficient; moreover, only a visual assessment is performed during the procedure. Autofluorescence devices are widely used to examine oral lesions. The technique described enables clinicians to measure the extent of these lesions beyond their visible margins. Autofluorescence detects nondysplastic areas that consist of mucosal inflammation and parakeratosis. It does not predict premalignancy [26,54]. In a previous study, a noncontact, three-dimensional curved shape measurement showed high surface roughness of squamous cell carcinoma and leukoplakia that affected the tongue [47]. This inhomogeneity in surface roughness, indicative of abnormal epithelial hyperplasia, is in strong agreement with our data indicating rheological

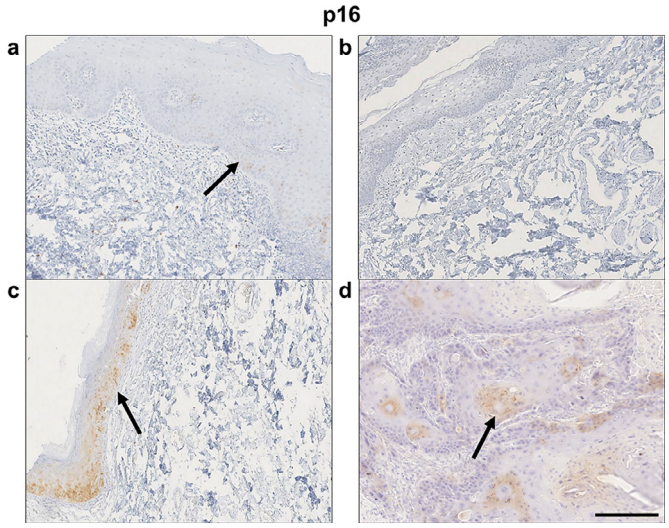

Fig. 5. Expression of the p16INK4a protein in tissue samples using CINtec E6H4. (a) Visibly healthy tissue, (b) margins, (c) squamous cell hyperplasia with dysplasia and (d) squamous cell carcinoma tissue. Arrows show expression in the squamous epithelium. Black bar: 250 μm.

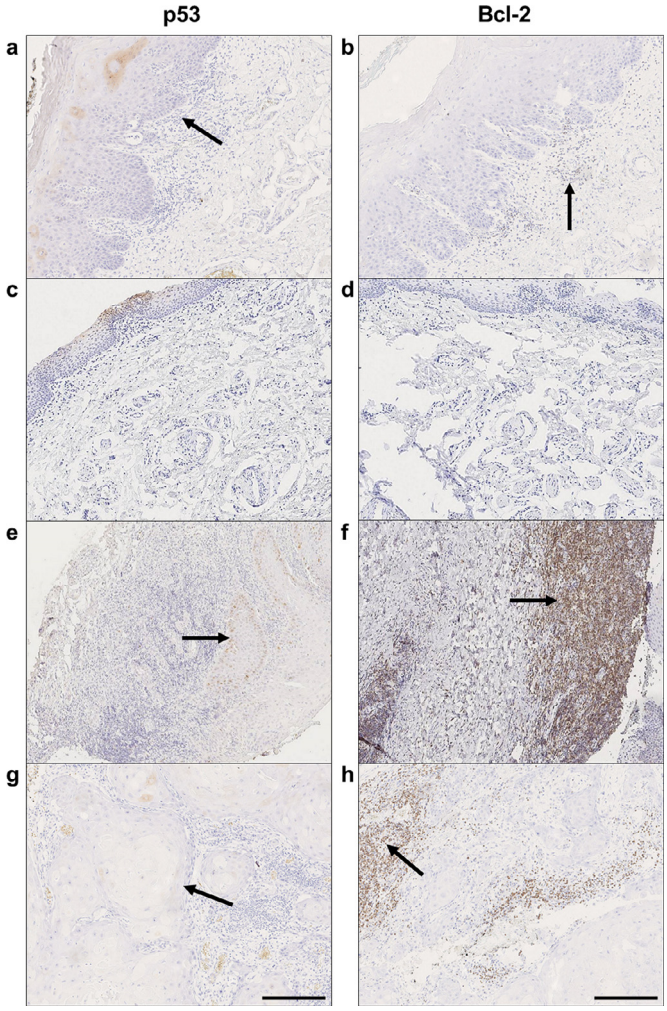

Fig. 6. Assessment of apoptosis using protein p53 expression and staining of the antiapoptotic protein Bcl-2. (a, b) Visibly healthy tissue, (c, d) margins, (e, f) squamous cell hyperplasia with dysplasia and (g, h) squamous cell carcinoma tissue. For p53 staining, arrows show expression in the basal layer of the squamous epithelium. For Bcl-2 staining, arrows show significant expression in numerous lymphocytes. Black bar: 250 μm.

inhomogeneity in leukoplakia and in tested samples of squamous cell carcinoma tissues.

Leukoplakia tissue, assessed as squamous cell hyperplasia with dysplasia represents a precancer condition and indicates that cancer development might be controlled by remodeling of the ECM that leads to an increase in tissue stiffness. Considering that we hypothesized that such an increase occurs mostly due to changes in collagen accumulation, an evaluation of cell signaling pathways governing this step of tissue remodeling might provide important data that will help to describe more precise mechanisms of cancer development, especially when alterations in the ECM take place. On the other hand, it can be assumed that the increased expression of MMPs (collagenases) sometimes observed in ECM should rather show less accumulation of collagen. Probably the observed accumulation of collagen indicates a higher rate of turnover of this protein in the analyzed samples, or that the increased local production is still higher then increases of MMPs activity.

In our samples such remodeling is occurring because of collagen accumulation and changes in its network arrangement might force increased cell density and compactness. This new tissue architecture might represent a potential “mechanoagonist”, acting as a ligand, inducing a cancerous pattern of tissue growth. Although the mechanism of the stiffness of this structural rearrangement by which the ECM is converted into biochemical signaling leading to uncontrolled cell growth is only hypothetical and it should be kept in mind that single cells are considered active objects that bind to extracellular matrix and possess all the machinery needed for mechanical recognition of their surrounding [23]. It is also highly recognized that stiffness modulates development, tissue homeostasis, and contributes to various pathological states [57]. A possible scenario might also implies a liquid crystalline ordering of precursor procollagen molecules prior to fibrillogenesis [31] .Therefore, based on our data and literature reports it is justified to say that remodeling of the ECM that leads to an increase in tissue stiffness might control cancer development. Although the phenomenon of “mechanical induction of cancer” is not proven by our results, we might assume that in 3D setting of leukoplakia on the microscopic cellular level some cells might be subjected to higher pressures, or find themselves in a situation of direct contact with the ECM of much greater stiffness than cells in healthy tissue. Recently, a new term “cancer mechanopathology” was introduced in the literature suggesting that mechanical confinement or solid mechanical stress can contribute to tumor growth, invasion and treatment [49]. Interestingly, an increase in liver stiffness was reported to precede liver fibrosis and potentially myofibroblast activation [30]. Moreover, it was already showed that brain tumors cause neuronal loss at the tumor margin, limited vessels perfusion and neuroinflammation just by using mechanical compression [40]. Also it is recognized that increased stiffness can favor cell proliferation and migration [55, 69]. Some newer studies exploring the cancerous transformation of cells are also in agreement with our assumptions. Panciera et al. investigated the possibility to reprogram healthy cells into tumors precursors engaging RTK/Ras cascade and explored how ECM stiffness affect this process. Indeed, authors were able to transform normal cell into tumorigenic ones, but only when cells were exposed to stiffer extracellular microenvironment than healthy tissue. Microenvironments with physiological softness prevented this oncogene-mediated conversion. Importantly, even subtle changes in rigidity of the surrounding ECM were able to induce cell reprogramming leading to tumor emergence, which was determined by disproportional responsiveness of cells to environmental stiffness upon oncogene exposure and regulation of YAP/TAZ activity [12,53]. Importantly, YAP signaling is activated mechanically by increased ECM stiffness in fibrotic tissues and promotes epithelial-mesenchymal transition (EMT) and proliferation of epithelial cells, being a possible molecular link between fibrosis and cancer [50] and most

recently, this signaling pathway was noted to promote oral squamous cell carcinoma proliferation by induction of PIEZO1 channels [35]. We believe that studies of oral submucosal fibrosis could complement our research, nevertheless we were not able to collect appropriate samples, since in our geographic region (Poland) such pathology was not yet reported. However, it could be an interesting concept for future studies in regions where oral submucosal fibrosis occurs. Increased stiffness of ECM were reported also to modulate the expression of EMT-related genes, leading to enhanced invasiveness of lung cancer cells [2]. Based on above, we believe that mechanical factors are able to support malignant transformation although we are aware that it is difficult to prove this directly in in vitro settings and tissues isolated from their mechanical environment.

Apart from this, involvement of changes in the expression of the $p16^{INK4a}$, p53 and Bcl-2 proteins should also be taken under the consideration [38,63]. The expression of p53 and loss of the expression of p16 are the earliest events in the malignant transformation process. In nondysplastic squamous cell hyperplasia, a combined alteration of p53/Ki67/p16INK4a was proven to increase the risk of progression [48,54]. The significant increase in the expression of the p53 and p63 proteins in OSCC, squamous cell hyperplasia with dysplasia, and oral submucous fibrosis suggests their role as surrogate markers of malignant transformation [67]. Indeed, changes in the expression of the p53 tumor suppressor known to provide a major barrier to cancerous transformation have been observed. However, the loss of BCL-2 gene expression in the basal cells of oral epithelial dysplasia and OSCC tissues that was previously reported was not confirmed in our study [44]. It has been reported that Bcl-2 promotes tumor cell metastasis with Twist1 when functioning as a complex [24]. Although it is known that the transduction of mechanical stimuli into biological signals, or mechanotransduction, is a ubiquitous phenomenon observed in many different aspects of cell physiology, its molecular mechanism during carcinogenesis is still unclear [17]. An increase in tumor stiffness directly activates biochemical pathways by mechanical induction, enhancing the cell cycle, cell motility and epithelial-mesenchymal transition [8]. Moreover, resulting increase in tumor solid stress can be associated with the activation of carcinogenic pathways as well as the ECM, which might be cancer primed or act as a barrier, and depending on biochemical and biophysical properties, the progression of metastasis can change [8,25,41,49,65]. It was recently discovered that different functions involving mechanotransduction as well as vascular development necessary for cancer growth occur with the aid of the mechanosensitive ion channels Piezo1 and Piezo2 [6]. Thus, understanding these mechanisms will be important for the future progress of new treatments. Despite advancements in cancer treatment, oral cancer has a poor prognosis and is often detected at a late stage. To overcome these challenges, investigators should search for early diagnostic and prognostic biomarkers.

Ethical approval

All procedures performed in the studies were in accordance with ethical standards. The study protocol was approved by the Bioethics Committee of Medical University of Bialystok (R-l-002/4/2019).

Informed consent

Written informed consent was obtained from all individual participants included in the study.

Data availability

The data generated during and analyzed during the current study are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Katarzyna Pogoda: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Mateusz Cieśluk:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Piotr Deptuła:** Data curation, Investigation, Writing – review & editing. **Grażyna Tokajuk:** Data curation, Resources, Writing – review & editing. **Ewelina Piktel:** Data curation, Formal analysis, Writing – review & editing. **Grzegorz Król:** Data curation, Formal analysis, Writing – review & editing. **Joanna Reszeć:** Data curation, Investigation, Writing – review & editing. **Robert Bucki:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

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