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Original article

Different methylation levels in the KLF4, ATF3 and DLEC1 genes in the myometrium and in corpus uteri mesenchymal tumours as assessed by MS-HRM

Dušan Braný[a], Dana Dvorská[a,*], Marián Grendár[b], Marcela Ňachajová[c], Peter Szépe[d], Zora Lasabová[e], Pavol Žúbor[c], Jozef Višňovský[c], Erika Halášová[a]

[a] Division of Molecular Medicine, Biomedical Center Martin, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia
[b] Bioinformatic Unit, Biomedical Center Martin, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava
[c] Department of Gynaecology and Obstetrics, Martin University Hospital, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava
[d] Department of Pathological Anatomy, Martin University Hospital, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava
[e] Division of Oncology, Biomedical Center Martin, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava

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ABSTRACT

Mesenchymal tumours of the corpus uteri comprise common benign lesions – leiomyomas and very rare malignant variants – sarcomas. It can be difficult to distinguish between the particular types of mesenchymal tumours pre-surgically. Primarily, leiomyomas and the very aggressive leiomyosarcomas can be easily misdiagnosed when using only imaging devices. Therefore, a reliable non-invasive marker for these tumour types would provide greater certitude for patients that the lesion remains benign. Our collection comprises 76 native leiomyomas, an equal number of healthy myometrium samples and 49 FFPE samples of various types of sarcomas. The methylation level was assessed by MS-HRM method and we observed differences in the methylation level between healthy, benign and (semi)malignant tissues in the *KLF4* and *DLEC1* genes. The mean methylation levels of leiomyomas compared to myometrium and leiomyosarcomas were 70.7% vs. 6.5% vs. 39.6 % (*KLF4*) and 66.1% vs. 14.08% vs. 37.5% (*DLEC1*). The *ATF3* gene was differentially methylated in leiomyomatous and myometrial tissues with 98.1% compared to 76.6%. The AUC values of the predictive logistic regression model for discrimination between leiomyomas and leiomyosarcomas based on methylation levels were 0.7829 (*KLF4*) and 0.7719 (*DLEC1*). Finally, our results suggest that there should be distinct models for the methylation events in benign leiomyomas and sarcomas, and that the *KLF4* and *DLEC1* genes can be considered potential methylation biomarkers for uterine leiomyomas.

1. Introduction

Mesenchymal tumours of the corpus uteri are a very heterogeneous group of cancerous diseases. The benign variants of these tumours, called leiomyomas (also known as myomas or fibroids) can affect 40–60 % of reproductive-age women, and this incidence increases up to 80% in women of African origin [1]. At least 50% of these tumours remain asymptomatic and do not generally endanger the patient's life [2], but when they became symptomatic, they can considerably decrease the quality of life. Complications caused by leiomyomas depend on tumour location, size, multiplicity, woman wish to remain fertile and many other attributes [3]. The most common adverse effect is the abnormal uterine bleeding, which occurs in up to 75% of cases [4]. Other symptoms include pelvic pain, menorrhagia, dysmenorrhea, anaemia, infertility, dystopia during gravidity, or even ectopic gravidity and miscarriage [5]. The malignant variants of mesenchymal tumours, called sarcomas are quite rare, but very aggressive tumour types [6]. They comprise only 3–8% of all malign uterine tumours [7]. The incidence of sarcomas in United States is less than 7 in 100 000 women [8] and this is slightly less in Europe with approximately 4.9 in 100 000 women [9]. The most common types are leiomyosarcomas, accounting for almost 60% [10,11]. In addition, sarcomas are more common in women of the African origin, and these have lower five-year survival-rate than other ethnic groups [12]. Although there are substantial differences between leiomyomas and sarcomas in their risk-prone behaviour and incidence, the following aspects connect these two tumour

* Corresponding author at: Division of Molecular Medicine, Biomedical Center Martin, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Malá Hora 4C, 036 01 Martin, Slovakia.
E-mail addresses: brany@jfmed.uniba.sk (D. Braný), dvorska@jfmed.uniba.sk (D. Dvorská).

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types: both have relatively unknown genetic, as well as epigenetic background of their origin and the symptomology of benign leiomyomas and sarcomas is substantially similar, especially in the case of very aggressive leiomyosarcomas [11]. This similarity makes pre-surgical differentiation, based on standard imaging devices very difficult. It is mostly necessary to perform invasive surgical procedure with subsequent histological examination to assess adequately the malignant potential of the corpus uteri mesenchymal tumours [11]. Unfortunately, this surgical procedure is not popular with patients, and it is often rejected; almost 80% of American women do not like invasive surgery, and over 50% prefer to preserve the uterus [13]. Moreover, the surgical intervention has also some limitations. For example, myomectomy does not exclude future tumorigenesis, and its laparoscopic accomplishment can comprise morcellation of the tumour, and this remains risk-bearing through potential spread of the malignant mass [14]. From the patient's viewpoint, the most suitable solution is a non-surgical, medicament treatment that preserves fertility.

Currently, medicament treatment mostly utilises selective progesterone receptors modulators (SPRM), and still, even in smaller amounts, also gonadotropine-releasing hormones anologues (GnRH) [14,15]. However, this treatment often only foregoes surgical intervention [14–16], and while it is capable to reduce the size of the tumour, it is mainly performed to minimise the secondary effects of the leiomyomas; especially the abnormal uterine bleeding [14,15]. In general, current leiomyoma medical treatment is not optimal, and scientists are therefore looking for new approaches to improve it. There is a variety of ways of achieving this goal. One promising method is analysing the DNA methylation status of appropriate genes' promotor regions [17–20]. DNA methylation is one of the most important epigenetic modifications to ensure proper regulation of gene expression under physiological conditions, but it also has an important role in genetic imprinting and interplay with histone modifications [21]. Methylation abnormalities, therefore, have great impact on tumorigenesis of various cancer types [22], and these include (mostly) oncogene hypo-methylation and (mostly) tumour suppressor gene hyper-methylation [21–23]. However, there are still differentially methylated regions in the human genome with unknown function [23], and although abnormal DNA methylation effects on tumorigenesis are accepted, the impact of methylation changes in a wide range of genes and cancer types is still insufficiently known. Finally, while DNA methylation is one of the most analysed epigenetic modifications and is primarily connected with methyltransferases disfunction [21], the entire mechanism, and especially the "triggers" of abnormal methylation, remains in same aspects unknown.

One aim of this study based on the analysis of DNA methylation of promotor regions is the identification of methylation biomarker, which can be used as a discriminating factor for different tissue types. [24]. Here, it is most important that the potential of the methylation biomarkers can be utilised with the liquid biopsy approach. This possibility is based on the following facts – the ctDNA elements released into the blood stream should usually have the same methylation pattern as the primary tumour, from which they were released [25–28], and it is also documented, that these elements are released by the corpus uteri mesenchymal tumours [29–32]. A further application of the methylation biomarker is to improve medical treatment using demethylation agents [33]. However, this option is mainly theoretical because current use of these agents affects the entire cell by repressing methyl-transferases activity [33,34]. This approach is not optimal, because non-physiological repression of an important cell regulation element can be very inappropriate. Therefore, an important future goal is to make the demethylation agents more specific for clinical application. In other words, the potential application of demethylation agents for treating patients should be based mainly on targeting particular genes – methylation biomarkers.

Herein, we selected the following ten potential cancer-related genes with important roles in cell growth and division, apoptosis, formation of the extracellular matrix and vascularisation: *KLF4*, *MGMT*, *BCL2*, *EGR1*, *DLEC1*, *TIMP3*, *BIRC5*, *ANXA11*, *ATF3*, and *CTGF*. Many of these genes have previously been observed to be abnormally methylated in different types of tumours. These especially include, *KLF4* in gastrointestinal [35] and cervical cancer [36], *MGMT* in brain tumours [37,38], *DLEC1* in hepatocellular carcinoma [39], colon and gastric cancer [40], Hodgkin as well as Non-Hodgkin lymphomas [41] and prostate cancer [42], *TIMP3* in gastric [43], ovarian [44] and oral cavity cancer [45]. *BCL2* was hyper-methylated in colorectal carcinoma [46] and hypo-methylated in breast carcinoma [47]. In addition, the *BIRC5* methylation levels were increased from low to high grade endometrial cancers and they correlated with elevated expression of *BIRC5* protein product [48] and similar results were observed also in bladder carcinoma [49]. Finally, the abnormal methylation of *CTGF* gene has been connected to hepatic fibrosis [50] and diabetes mellitus [51].

1.1. The selected genes' functions

KLF4 is an important zinc-finger transcription factor, whose main function is to control progression through the cell cycle G1/S and G2/M boundary [52,53]. Nonetheless, the product of this gene is multi-factorial, since it is implicated in various molecular mechanisms regulating cell development and cell division, apoptosis and even programing of somatic cells [53]. Gene *EGR1* is another zinc-finger type transcription factor. It is an early response, nuclear regulator of transcription and mitogenesis [54]. Its biosynthesis is stimulated by various signal molecules including hormones, neurotransmitters and growth/differentiation factors [54] and it then acts as a very variable long-term downstream effector. This gene is, therefore considered as "binding point" in many signal cascades [55]. *ATF3* is leucine-zipper transcription factor. This gene contains the bZIP domain, required for interactions with other transcriptional factors such as AP-1, C/EBP and the Maf gene family [56]. It also forms homo-dimers with important regulators including c-Jun, junB and JunD [57]. Depending on the model of promoter, these dimers can activate as well as repress transcription [57]. *ATF3*'s role as a transcription effector or repressor is therefore difficult to generalise because it can be either oncogenic or tumour suppressive.

TIMP3 gene is a member of the family of inhibitor of matrix metalloproteinases; a group of peptidases which degrade the extracellular matrix. *TIMP3*'s functional product maintains the appropriate extracellular environment; but this is usually degraded in mesenchymal tumours, and especially in leiomyomas [1,5,7,9]. *TIMP3* also contributes to adipocyte differentiation [58] and can inhibit angiogenesis by blocking VEGF binding to VEGF2 receptor [59].

Our gene collection also includes two oncogenes - *BIRC5* and *BCL2*. *BIRC5* encodes the protein called survivin, which is the smallest member of IAP (inhibitor of apoptosis) family. Despite its name, this protein has other cell functions in addition to inhibiting apoptosis. These include cell division control, DNA reparation and angiogenesis [60]. Although survivin is expressed only minimally in normal healthy tissues, its activity is markedly increased in foetal and tumour tissues [60]. It is also likely, that survivin remains methylated in healthy tissues and that de-methylation causes its aberrant expression in tumour tissues. Finally, survivin can also interact with both the internal and external apoptotic pathways [60]. *BCL2*, is a well-known anti-apoptotic gene whose protein product regulates outer mitochondrial membrane permeability. Its main role is to "control" the activity of its own gene family members, including the pro- and anti- apoptotic proteins. *BCL2* typically inhibits caspase activity by preventing the cytochrome-c release or by direct binding to the APAF1 factor [61].

The *MGMT* gene encodes O-6-methylguanine-DNA methyltransferase which catalyses the removal of abnormal alkylation. This includes also methylation of the guanine's sixth oxide. This alkylation is effected by chemotherapeutic agents and mutagens [62] and causes abnormally methylated guanine binding to thymine instead of cytosine

[62]. Protein product of this gene can mediate the transfer of the methyl group from guanine to its own cytosine molecule; and this is subsequently proteolytically degraded [37]. *MGMT* is therefore often referred the "suicidal protein". Anyhow, it provides very effective one-step DNA repair system [62].

The *ANXA11* gene is a member of the annexin super-family of calcium-regulated phospholipid-binding proteins [63]. The main annexin function is to maintain the transfer of information between intracellular and membrane proteins [64]. *ANXA11* especially affects correct formation of the contractile ring during cytokinesis, $Ca2^{+}$ dependent signalling, vesicular transport and apoptosis. Finally, *ANXA11* dysfunction leads to destabilisation of the PDGFR and MAPK/p53 pathways [63–65].

The *DLEC1* gene is located in the 3p.22 hot-spot region [39] and is considered to act as a tumour suppressor by inducing cell-cycle arrest in the G1 phase [40]. While this gene's non-physiological activity has been observed in over 20 tissue types, especially in pancreatic and testicular tissues [41], its physiological expression can be partly renewed by application of 5-aza-2′-deoxycythidine, at least in HCC cell lines with hyper-methylated *DLEC1* [40]. Therefore, *DLEC1* can be considered a potentially epigenetically modifiable tumour suppressor gene

Finally, the *CTGF* gene encodes a metricellular protein with very complex function, and it interacts with various signalling pathways and contributes to cell adhesion and migration, angiogenesis, hypoxia-induced apoptosis, myofibroblast activation and most importantly, remodelling of the extracellular matrix [66]. This gene interacts specifically with integrins, lipoproteins, tyrosin-kinase and TGFβ receptors [67]. The *CTGF* gene is both oncogenic and tumor-supressive; and this dual character is apprehensible because of its wide range of interactions [67].

Our main goal herein was to assess the methylation indexes of healthy myometrial, benign leiomyomatous and malign leiomyosarcomatous tissues, so that we could then use these indexes as potential distinguishing factors for these groups. We then assessed the methylation indexes of other sarcoma types for comparative purposes. Anyhow, due to low sample numbers of other sarcoma types and their different origins, these results have less or more only informational character. Importantly, the study due to their long-term enlistment within mesenchymal tumours and the marked presence of mesenchymal elements [6] also includes carcinosarcomas, although are currently classified as endometrial tumours [68].

2. Materials and methods

2.1. Sample collection and clinical pathology characteristics

We obtained matched pairs of native samples of uterine leiomyomas, and healthy myometrium from 76 Slovak women who underwent hysterectomy for symptomatic fibroids at the Department of Obstetrics and Gynaecology at Martin University Hospital between 2014 and 2018. All these tumour tissues were histologically confirmed as benign lesions by an experienced histologist. Uterine leiomyosarcomas and other mesenchymal tumours types, except conventional leiomyoma, were obtained because of its rare occurrence from the formalin-fixed paraffin-embedded (FFPE) samples stored at the Department of Pathological Anatomy in Martin. Personal information and gynaecological anamnesis were obtained during the examination, or from the MEDEA database with patient consent. The study was approved by the ethical committee of the Jessenius Faculty of medicine in Martin (IORG0004721). Table 1 summarises the types and numbers of analysed tumours and Table 2 contains our patients file.

2.2. DNA isolation and bisulfite conversion

The native tumorous and healthy tissue samples were immediately after section transferred to the RNAlater stabilisation solution and frozen to -20 °C. The DNA from native samples was isolated by DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) according to protocol and the FFPE samples DNA was isolated by BLACKPrep FFPE isolation kit (Analytik Jena GmbH, Thuringia, Germany). Concentration was measured by the Nanodrop™ 2000 device (Thermo Fisher Inc, Wilmington, DE, USA). The qualitative parameters were assessed electrophoretically on 1.5% agarose gel. Samples with concentration of at least 100 ng/μl and good absorption ratios were selected for analysis. Subsequent bisulfite conversion of DNA (input amount 1 μg) was performed by the Epitect Bisulfite Kit (Qiagen Inc., Valencia, CA, USA) and the converted DNA was stored at -20°C.

Table 1
Numbers of analysed mesenchymal uterine tumour types (and myometrium).

Tissue/tumour type	n
Healthy myometrium	76
Conventional leiomyoma	76
Leiomyosarcoma	12
ESS[a]	4
Adenosarcoma	3
Carcinosarcoma	12
Adenomyoma	8
UPS[b]	5
STUMP[c]	5

[a] Endometrial stromal sarcoma, [b]Undifferentiated pleomorphic sarcoma, [c] Uterine smooth muscle tumors of uncertain malignant potential.

Table 2
Clinical and histopathological characteristics of the uterine leiomyoma group.

Clinical and histopathological characteristics of patients with conventional leiomyoma [a]	
Age	< 50 – 48 (63.2%); < 50 – 28 (36.8%)
Abortion	Yes – 16(21%); No – 60 (79%)
Pregnancy loss	Yes – 13(17.1%); No – 63 (82.9%)
Smoking	Yes – 15(19.7%); No – 61 (80.3%)
Age of Menarché	11 years (y) – 4 (5.2%); 12 y – 21 (27.7%); 13 y – 17 (22.4%); 14 y – 15 (19.7%); 15 y – 15 (19.7%); 16 y – 2 (2.7%); 17 y – 1 (1.3%); 18 y – 1 (1.3%)
AUT[b]	Yes – 59 (77.6%); No – 17 (22.4%)
Hypertension	Yes – 21 (27.6%); No – 55 (72.4%)
Diabetes mellitus	Yes – 6 (7.9%); No – 70 (92.1%)
Menstrual Cycle	Normal – 57 (75%); Abnormal – 19 (25%)
Non-surgical myoma treatment	SPRM – 22 (29%) GnRH – 5 (6.5%) No – 49 (64.5%)
Contraceptives	Yes – 28 (36.8%); No – 48 (63.2%)
Parity	0 – 17 (22.4%); 1 – 13 (17.1%); 2 – 29 (38.3%); 3 – 9 (11.8%); 4 – 4 (5.2%); 5 – 4 (5.2%)
Menopausal status	Pre – 54 (71.1%) Post – 22 (28.9%)

Note: [a] These data have no statistical significance in case of sarcomas and are not mentioned, [b] Abnormal uterine bleeding.

2.3. Methylation-sensitive high resolution melting analysis

The methylation levels of the selected genes' promoter regions were analysed by the MS-HRM method on the LightCycler 480® device (Roche Dignostic GmbH, Mannheim, Germany). The MS-HRM method is sensitive, and specific method, developed for SNP analysis, nonetheless, capable to detect also methylation changes.

2.4. Primer design for MS-HRM analyses

Our primers were designed to amplify the CpG-rich promoter sequence up to 1 500 base pairs upstream of the first exon. Sequences were obtained for each gene from the ENSEMBL® genome browser [69], and localisation of selected regions was verified by the USCS Blat tool

Table 3
Sequences of the primer pairs used in this study.

Gene	Primer sequence
KLF4	F: ATGGGTTAGAGAATTGGAGAGAATAAAG
	R: CTACCTAACCAATCCCCATATCT
ATF3	F: AGGTTAGATATGTATTGGAGTTTTAGAT
	R: CACAAAACAACCAACAACTATTACT
DLEC1	F: AGATAGGTTTGGTTGTGTTAATTAAAT
	R: TTTAAAACTCCTAATCTCCATAACAAC

[70]. The primer design was based on the recommendations published by the authors Smith et al. [71], and Wojdacz and Dobrowitz [72]. We employed the following freely available tools, suitable for primer design in the bisulfite converted sequences - the Primer Design and search tool [73], the MethPrimer [74], and the commercial Pyromark® Assay Design SW 2.0. We then used the ePCR primer search tool to determine if the designed primer pairs amplified only particular sequence in the genome [73] because this tool enables also to align the primer sequences against the bisulfite modified hg19 reference sequence. The primer pair sequences are listed in Table 3.

2.5. PCR amplification and detection of melting temperature by MS-HRM

The MS-HRM method requires dilution of DNA samples to identical concentrations. It is important to conduct this first step to minimise potential differences in the final fluorescence intensity. We therefore diluted our DNA samples to a final concentration of 20 ng/µl, and then prepared a solution for PCR pre-amplification and subsequent MS-HRM analysis. The total reaction volume was 10 µl and comprised 5 µl HRM Mastermix, (HotStarTaq® Plus DNA Polymerase, Epitect HRM PCR Buffer, EvaGreen® Dye and nucleotides – Qiagen GmbH); 2.5 µl RNase free water and 1 µl of the bisulfite converted DNA. The PCR reaction steps were: polymerase activation (95°C for 5 min); 45 cycles of: denaturation (95°C for 15 s.), annealing (*°C for 30 s) and extension (72°C for ** s). Here, the *annealing temperature was calculated according to the length and base composition of primer pairs and the ** extension time was 10 seconds for amplicons shorter than 150 bp, and one second was then added for each additional 10 base pairs in the longer amplicons. The HRM analysis parameters remained unchanged from the Epitect HRM PCR Handbook (Qiagen GmbH) protocol. We used the commercially available Methylated and Unmethylated DNA's as methylation standards (Qiagen GmbH), and these were diluted to achieve the 0%, 25%, 50%, 75%, and 100% methylation dilution series.

2.6. Statistical analyses

All biostatistical analyses were performed by R software [75], version 3.2.3. We conducted power analysis before the study to determine the sample size(s) required to control Type I and Type II error probabilities. Data was then visualised by boxplots and spaghetti plots, and normality of methylation was analysed by the quantile-quantile plot with bootstrap confidence interval. The differences in methylation levels between control groups and leiomyoma and sarcoma tissues were assessed by robust ANOVA with the post-hoc Tukey HSD test. The differences in leiomyoma tissue methylation levels in specific clinical states were assessed by Wilcoxon signed-rank test when there were two groups, and by robust ANOVA for more than two groups. The univariate logistic regression then estimated the risk by odds ratio, and the Youden index determined the optimal gene methylation cut-off value. The discriminative ability of the logistic regression models for classification between controls and leiomyoma, controls and sarcoma and leiomyoma and sarcoma was finally quantified by the ROC curve, and by the area under the ROC (AUC).

3. Results

While significant differences were noted in *KLF4*, *DLEC1* and *ATF3* gene methylation levels, there were no differences in the remaining seven genes - all their promoter regions were methylated to the same extent in all tissue types so there were not even statistically insignificant changes. The differences in *ATF3* methylation level were statistically significant in healthy myometrium and benign uterine leiomyoma tissues. *KLF4* and *DLEC1* methylation levels were statistically different also in leiomyosarcomas. In addition, the methylation level for the *KLF4*, and *DLEC1* genes was also quite variable in other sarcoma types. Three clinical parameters were statistically significantly associated with methylation level changes – hypertension in case of *ATF3* gene and post-menopausal status and diabetes-mellitus in case of *DLEC1* gene. (Table 2 lists all clinical parameters compared to methylation levels).

While the predictive logistic regression model was based on tumour methylation index and primarily discriminated leiomyomas from leiomyosarcomas, it also differentiated leiomyomas and leiomyosarcomas from myometrium. The model was afterwards used to find the optimal cut-off that would differentiate these tumour groups on the methylation index that optimizes the Youden index. Diagnostic accuracy of the logistic regression model, measures by AUC, was more than 0.7 for *KLF4* and *DLEC1* genes. Table 4 lists all the methylation mean and median values for the individual tumour types and the myometrium.

3.1. KLF4

The median methylation index values for the *KLF4* gene promotor region were significantly higher in uterine leiomyomas (ULM), than in healthy myometrium (MM) (75% vs. 0%; $P < 0.001$); and uterine

Table 4
Mean and median methylation level values for myometrium and specific tumour types.

	KLF4			*DLEC1*			*ATF3*		
Tissue type	Mean MI[a] (%) ± SD	Median MI (%)	P-value	Mean MI ± SD (%)	Median MI (%)	P-value	Mean MI (%) ± SD	Median MI (%)	P-value
Healthy myometrium	6.5 ± 13.8	0	< 0.01[b]	14.8 ± 15.4	25	< 0.01 [b]	76.6 ± 13.6	75	< 0.05 [b]
Conventional leiomyomas	70.7 ± 36.1	75	< 0.01[b]	66.1 ± 29.6	75	< 0.01 [b]	98.1 ± 13.7	100	< 0.05 [b]
Leiomyosarcomas	39.6 ± 22.5	37.5	< 0.001[c]	37.5 ± 25	37.5	< 0.001 [c]	77.1 ± 16.7	75	> 0.05 [c]
Adenosarcomas	41.6 ± 36.2	50	> 0.05[c]	25 ± 25	25	> 0.05 [c]	75 ± 25	75	> 0.05 [c]
ESS	37.5 ± 32.3	37.5	> 0.05[c]	50 ± 20.4	50	> 0.05 [c]	75 ± 20.4	75	> 0.05 [c]
Carcinosarcomas	47.9 ± 16.7	50	< 0.05[c]	33.3 ± 24.6	37.5	> 0.05 [c]	81.3 ± 12.9	75	> 0.05 [c]
UPS	45 ± 20.9	50	> 0.05[c]	20 ± 20.1	25	> 0.05 [c]	80 ± 11.2	75	> 0.05 [c]
Adenomyomas	25 ± 18.9	25	< 0.001[c]	31.3 ± 22.2	37.5	< 0.001 [c]	81.3 ± 17.7	75	> 0.05 [c]
STUMP	50 ± 25	50	> 0.05[c]	40 ± 28.6	50	> 0.05 [c]	85 ± 13.7	87.5	> 0.05 [c]

Note: [a] Methylation index; [b] P-value leiomyomas vs. myometrium, [c] P-value particular tumour vs. leiomyomas, ESS – Endometrial stromal sarcoma, UPS – Undifferentiated pleomorphic sarcomas, STUMP – Uterine smooth muscle tumours of uncertain malignant potential.

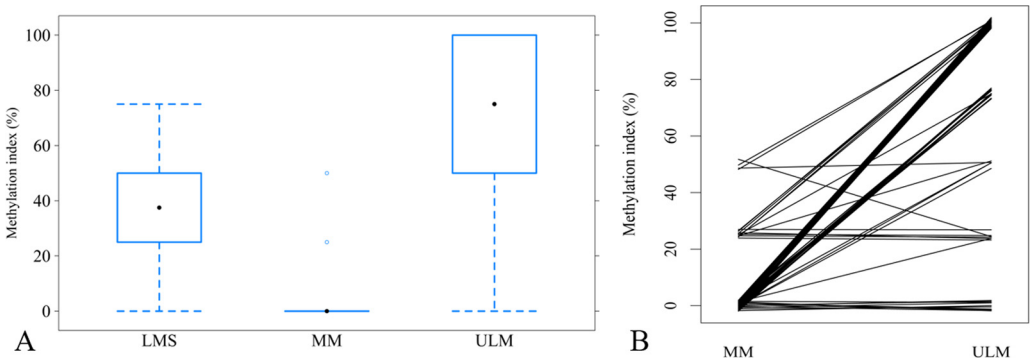

Fig. 1. Boxplot (A) illustrating the overall *KLF4* gene promoter sequence methylation levels in myometrial (MM), leiomyosarcomatous (LMS) and leiomyomatous (ULM) tissues (median is the black point) and the spaghetti plot (B) illustrating methylation levels of the *KLF4* gene promoter sequence in myomatous (MM) and leiomyomatous (LMS) tissues.

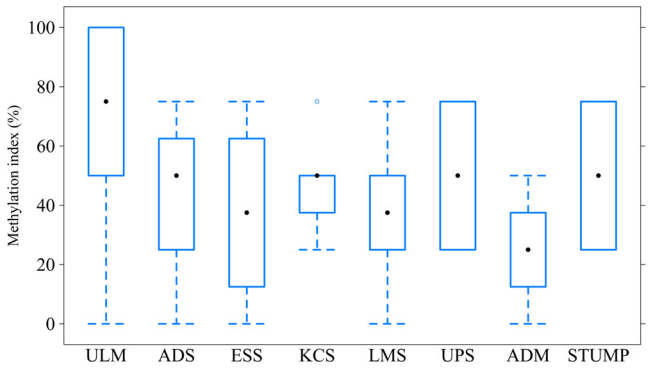

Fig. 2. Boxplot of the *KLF4* gene promotor sequence methylation indexes in the analysed tumour groups: ULM – Leiomyomas, ADS – Adenosarcomas, ESS – Endometrial stromal sarcomas, KCS – Carcinosarcomas, LMS – Leiomyosarcomas, UPS – Undifferentiated pleomorphic sarcomas, ADM – Adenomyomas, STUMP – Uterine smooth muscle tumours of uncertain malignant potential.

leiomyosarcomas (LMS) were more highly methylated than MM (37.5% vs. 0%; $P < 0.01$), but less than ULM (37.5% vs. 75%; $P < 0.001$) (Fig. 1A).

The methylation index differences in all analysed groups were tested by robust ANOVA (Fig. 2) followed by Tukey HSD post-hoc test. The following significant differences were established; between ULM and undifferentiated pleomorphic sarcomas (UPS) (75% vs. 50%; $P < 0.01$); ULM and adenomyomas (75% vs. 25%; $P < 0.01$); ULM and carcinosarcomas (75% vs. 50%; $P < 0.01$); carcinosarcomas and adenomyomas (50% vs. 25%; $P < 0.01$).

The ULM methylation levels were generally higher than in all other analysed tumour groups (75% vs.50%; $P < 0.001$) (Fig. 3A) and all malignant variants (75% vs. 50%; $P < 0.001$) (Fig. 3B).

Finally, no connection was observed between methylation status changes in the *KLF4* promoter region in ULM and any clinical or histological parameter (data not shown).

The AUC value of the predictive logistic regression model for discrimination between ULM and LMS based on the methylation levels was 0.7829 (Fig. 4A). The odds ratio (OR) was 0.9752 (95% CI, 0.945 – 0.989) and the cut-off value, based on the differences of methylation levels which should theoretically distinguish between the ULM and LMS was 62.5% with 71% specificity and 90.9% sensitivity (Fig. 4A). In addition, the AUC for the logistic regression model of ULM and MM was 0.9065 (Fig. 4B), with 37.5% cut-off value, 94.7% specificity and 77.6% sensitivity and 0.9382 OR (95% CI, 0.954 – 0.917). Finally, the AUC for discrimination between LMS and MM was 0.8756 with 12.5% cut-off value, 90.9% specificity, 78.9% sensitivity and 0.9752 OR (95% CI, 0.992 – 0.956) (Fig. 4C).

Power analysis then determined the sample size needed to control the probability of Type I and Type II error for the most important myometrium, leiomyomas and leimyosarcomas tumour groups. The minimum sample size for the gene *KLF4* and ULM vs. MM was 4; for ULM (n = 76) vs. LMS the minimal sample size of LMS group was 9; for MM (n = 76) vs. LMS the minimal sample size of LMS group was 3.

3.2. DLEC1

In the *DLEC1* gene, the promoter region methylation levels were significantly higher in ULM tissues, than in MM tissues (75% vs.25%; $P < 0.001$) and although uterine LMS were more highly methylated than the MM (37.5% vs.25%; $P < 0.01$), they were less methylated than ULM (37.5% vs.75%; $P < 0.001$) (Fig. 5A).

Fig. 6 highlights the statistically significant differences in methylation status between the ULM and adenomyoma groups (75% vs. 37.5%; $P < 0.01$), the ULM and carcinosarcoma groups (75% vs. 37.5%; $P < 0.001$), and the ULM and UPS groups (75% vs. 25% $P < 0.01$).

In addition, the *DLEC1* methylation levels gene in ULM were statistically significantly higher than in all other analysed tumour groups (75% vs. 25%; $P < 0.001$) (Fig. 7A) and malignant variants (75% vs. 25%; $P < 0.001$) (Fig. 7B)

The *DLEC1* gene AUC values of the predictive logistic regression model for discrimination between ULM and LMS was 0.7719 (Fig. 8A) with 0.9638 OR (95% CI, 0.945 – 0.989), 62.5% cut-off, 63.2% specificity and 58.3% sensitivity; between LMS and MM the AUC was 0.7632

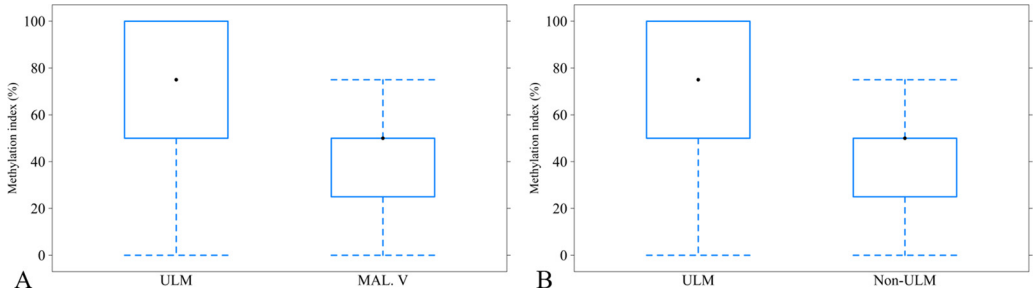

Fig. 3. Boxplots illustrating the *KLF4* gene promoter sequence methylation levels: (A) uterine leiomyomas (ULM) vs. all malign analysed tumour groups (MAL.V) and (B) leiomyomas vs. all other analysed tumour groups (Non-ULM).

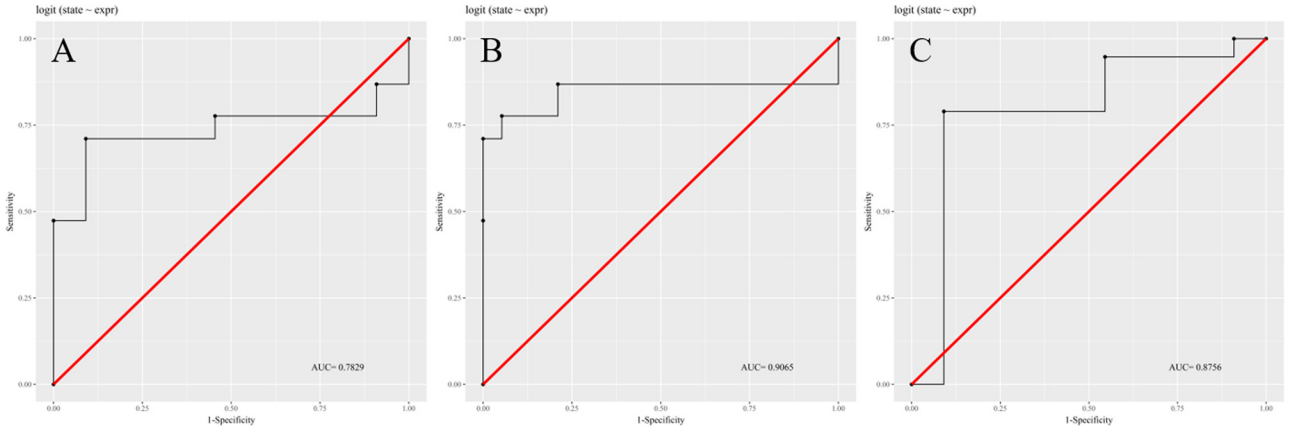

Fig. 4. ROC curve for the *KLF4* gene multinomial logistic regression, with AUC's describing the predictive performance of the model (A) leiomyomas vs. leiomyosarcomas; (B) leiomyomas vs. myometrium; (C) leiomyosarcomas vs. myometrium.

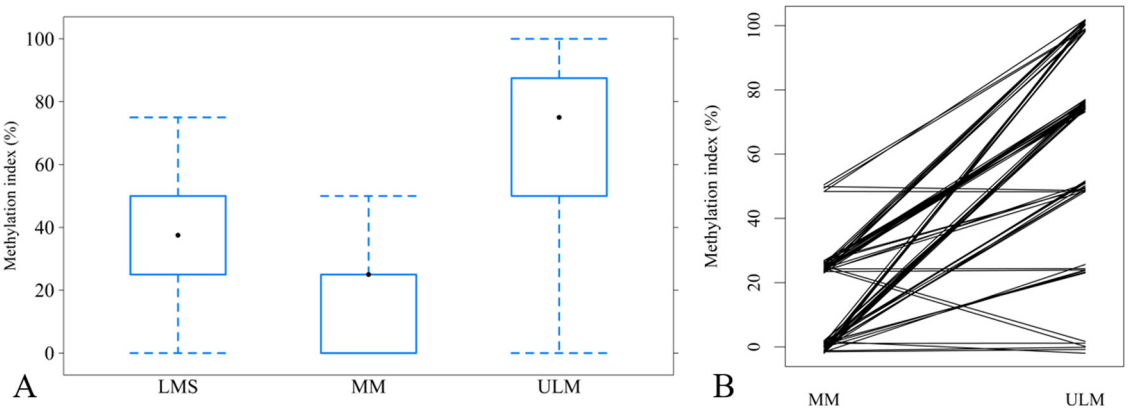

Fig. 5. Boxplot (A) illustrating the overall *DLEC1* gene promoter sequence methylation levels in myometrial (MM), leiomyosarcomatous (LMS) and leiomyomatous (ULM) tissues and the spaghetti plot (B) illustrating *DLEC1* gene promoter sequence methylation levels in myomatous (MM) and leiomyomatous (LMS) tissues.

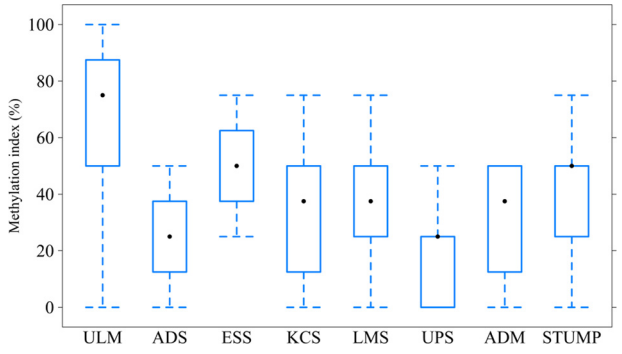

Fig. 6. Boxplot of the *DLEC1* gene promoter sequence methylation indexes in the analysed tumour groups: ULM – Leiomyomas, ADS – Adenosarcomas, ESS – Endometrial stromal sarcomas, KCS – Carcinosarcomas, LMS – Leiomyosarcomas, UPS – Undifferentiated pleomorphic sarcomas, ADM – Adenomyomas, STUMP - Uterine smooth muscle tumours of uncertain malignant potential.

(Fig. 8B) with. 0.938 OR (95% CI, 0.898 – 0.970), 37.5%, cut-off, 50% specificity and 93.4% sensitivity. Lastly, between ULM and MM the AUC was 0.9064 (Fig. 8C) with. 0.9267 OR (95% CI, 0.945 – 0.903), 37.5% cut-off, 81.5% specificity and 63.1% sensitivity

In addition, *DLEC1* gene promoter region median methylation was statistically significantly lower in patients with diabetes mellitus ($P < 0.05$) (Fig. 9A), and in post-menopausal women ($P < 0.05$) (Fig. 9B).

For the gene *DLEC1,* the minimal sample size ULM vs. MM was 3; for ULM (n = 76) vs. LMS the minimal sample size of LMS group was 9; for MM (n = 76) vs. LMS the minimal sample size of LMS group was 8.

3.3. *ATF3*

The methylation levels in the *ATF3* gene differed from those for the previous two genes. The main reason for this difference was the high methylation level in the control tissue. Higher methylation levels were detected in ULM tissues than in MM samples (100% vs. 75%; $P < 0.001$). These were also higher than in LMS, but without statistical significance (100% vs. 75%; $P > 0.05$). Interestingly, the LMS samples were only slightly more highly methylated than healthy tissues (75% vs. 75%; $P > 0.05$) (Fig. 10). Lastly, the differences in methylation index between other mesenchymal tumour groups were not statistically significant (data not shown).

The ULM methylation status was also higher than in all other analysed tumour groups (100% vs. 75%; $P < 0.05$), including the malign variant (100% vs. 75%; $P < 0.05$). (Fig. 11A, B)

We also discovered statistically significant methylation increase in hypertensive patients despite the *ATF3* gene's relatively homogenous methylation levels (Fig. 12)

The predictive logistic regression model values for discrimination were: 0.6508 AUC between ULM and LMS (Fig. 13A), 0.9688 OR (95% CI, 0.929–1.009), 87.5% cut-off value, 63.2% specificity and 58.3% sensitivity; 0.7348 AUC between ULM and MM (Fig. 13B), 0.9329 OR (95% CI, 0.957 – 0.907), 87.5% cut-off value, 81.5% specificity and 63.1% sensitivity. 0.5998 AUC between LMS and MM (Fig. 13C), 0.9759 OR (95% CI, 0.934–1.019), 62.5% cut-off, 71.0% specificity and 90.9% sensitivity.

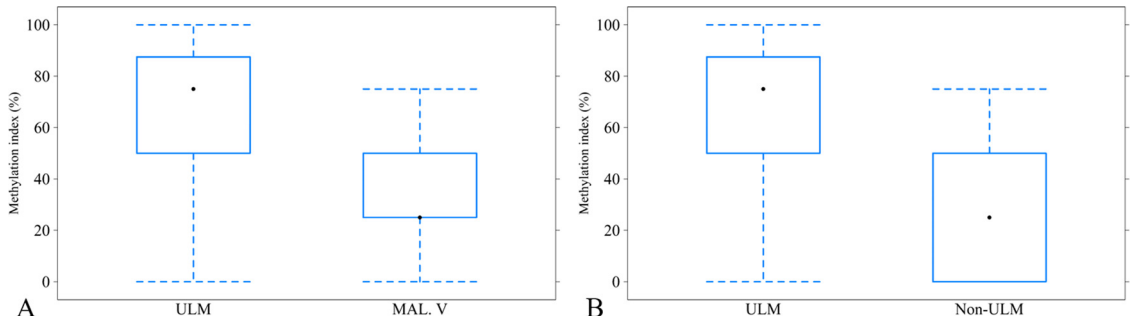

Fig. 7. Boxplots illustrating *DLEC1* gene promoter sequence methylation levels: (A) uterine leiomyomas (ULM) vs. all malignant analysed tumour groups (MAL.V) and (B) leiomyomas vs. all other analysed tumour groups (Non-ULM).

For the gene *ATF3* and ULM vs. MM the minimal sample size was 11; for ULM (n = 76) vs. LMS the minimal sample size of LMS group was 341; for MM (n = 76) vs. LMS the minimal sample size of LMS group was 25.

The *KLF4*, *DLEC1* and *ATF3* genes' AUC values, Odds ratios and Cut-off values are all summarised in Table 5.

4. Discussion

The molecular genetic- and especially the epigenetic background of the corpus uteri mesenchymal tumours in comparision to other cancer types have been analysed less frequently. Knowledge of the tumorigenesis mechanisms therefore needs improvement, especially for leiomyomas, because these have extremely high incidence rate and affect many women globally [1–3]. Although these tumours do not endanger patients' lives and are usually easily removed surgically, they can significantly decrease quality of life [1–3]. Unfortunately, many female patients prefer to avoid surgical excision [14,15] and there is also a lack of medical consensus on how to treat leiomyomas; where some physicians prefer surgical procedure alone and others wish to improve medicament treatment as much as possible [14–16]. While this latter approach could be beneficial in the future, it is currently insufficient in many parameters and still requires a lot of financial support and research. Nonetheless, the high leiomyoma incidence also makes surgical treatment very expensive [14–16,76].

There are many ways how to improve the medical treatment, and one important method is intentional alteration of methylation status by demethylation agents. For example, cytidine, decitabine and hydralasine are the most used demethylation agents in clinical practice for the occasional treatment of chronic myeloid leukaemia or myelodysplastic syndromes, but they are not suitable for solid tumours treatment [34,77]. In addition, the greatest disadvantage of these agents is planar effect in cell, which can cause various side effects [34,77]. Thus, the demethylation agents require improvement before widespread clinical use, especially for targeting methylation biomarkers.

Furthermore, there is also little information on the side effects of the GnRH analogues and SPRM's, which are most used drugs to treat leiomyomas. One exception is the GnRH analogue's effect on the *HOXA10* methylation status in endometrial tumour tissues in animal models whereby these changes affected final expression of this gene [78]. Therefore, increased knowledge of the effects of GnRH's and SPRM's on methylation changes during leiomyoma patient treatment would be needed.

While the proper application of demethylation agents for medicament treatment based mainly on their targeting of the methylation markers remains a challenge for the future, some methylation markers are already used in clinical praxis for early, non-invasive detection of various cancers. For example, the *NDRG4* and *BMP3* methylation assays implicated in the Cologuard® technology are beneficial for early detection of colorectal cancer [79,80], and the *SEPT9* methylation index can be used as a blood marker for the same disease [81]. Furthermore, methylation levels in the genes *GSTP1*, *RASSF1,* and the *APC* can help detect prostate cancer [82] and methylation levels in the *TWIST1* and *NID2* genes provided good results in predicting bladder cancer from urine samples [83].

Adequate methylation biomarkers have the potential to help non-invasively distinguish cancerous and healthy tissues when their methylation levels differ, and it is expected that they could also distinguish between different tumorous types. Although there are already assessed methylation biomarkers for corpus uteri mesenchymal tumours which can, at least theoretically differentiate healthy myometrium from leiomyomas and even leiomyomas from leiomyosarcomas dependent on their methylation levels [19,20], none of these markers have yet found clinical application.

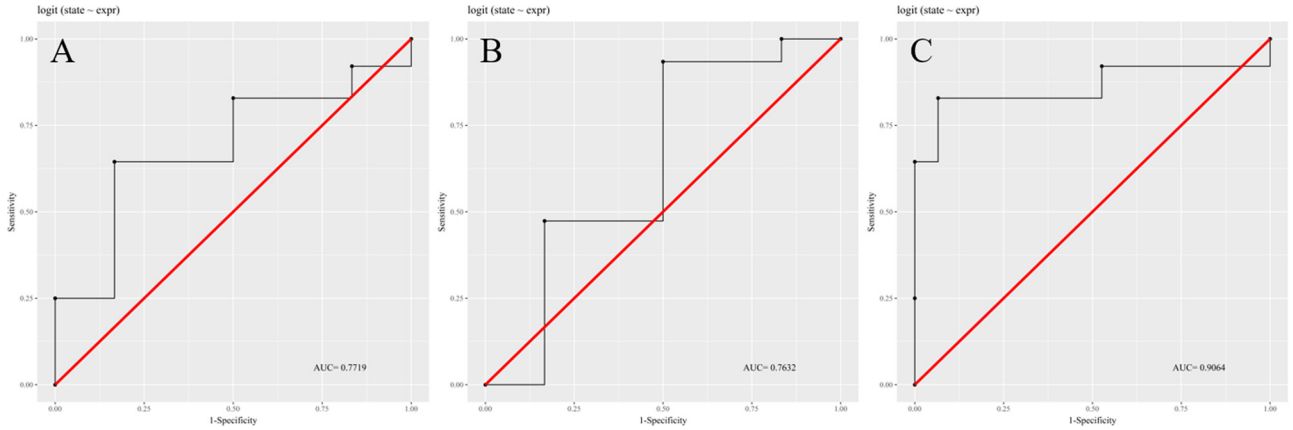

Fig. 8. ROC curve for the *DLEC1* gene multinomial logistic regression with AUC's describing the predictive performance of the model (A) leiomyomas vs. leiomyosarcomas: (B) leiomyosarcomas vs. myometrium; (C) leiomyomas vs. myometrium.

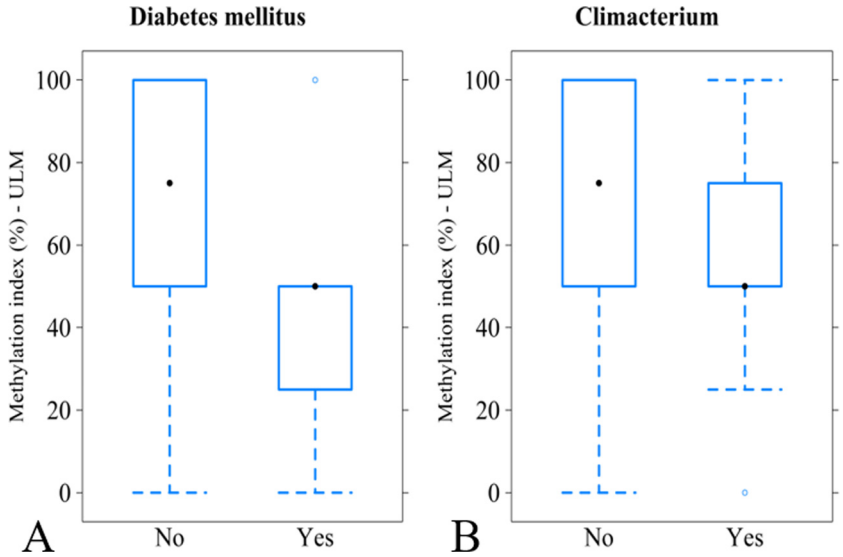

Fig. 9. Boxplots illustrating *DLEC1* promotor sequence methylation levels in leiomyoma tissues for specific clinical parameters: (A) patients with diabetes mellitus have statistically significant lower methylated promoter sequences; (B) post-menopausal women also have sequences lower methylated.

The methylation levels varied between *KLF4*, *DLEC1* and *ATF3*. Notably, *DLEC1* and *KLF4* methylation levels were markedly different between healthy and tumorous tissues. On the contrary, *ATF3* methylation levels were more similar. Furthermore, the AUC values of the *KLF4* and *DLEC1* gene higher than 0.7 can be considered as acceptable [84], and their cut-off values, theoretically, provide some possibilities for pre-surgical and liquid biopsy based differentiation between leiomyomas and leiomyosarcomas. These genes, however, should be employed in a wider methylation assay and not be used separately because of their lower sensitivity and specificity; and even this possibility remains mostly theoretical until further analysis. Nonetheless, diagnostic assays with lower predictive value may still be considered a plausible "starting point", because no mechanism to pre-surgically separate leiomyomas and leiomyosarcomas currently exists. Theoretically, while the "possibility of diagnosing, for example 8 patients out of 10 is better than none", it remains essential to improve assay diagnostic values as much a possible.

Methylation-based assays for non-invasive cancer diagnosis of uterine mesenchymal tumours using liquid biopsy depend on the methylation pattern that should not usually differ between the primary tumour and the released ctDNA [25–28]. However, identification of ctDNA released from the corpus uteri mesenchymal tumour is not optimal, because it is usually only incidental and collateral in different types of analysis and testing; such as non-invasive prenatal testing [29–32]. Therefore, it would be beneficial to perform more analyses to identify these elements and improve the possibility of enhancing the specificity and sensitivity of their identification in women with corpus uteri mesenchymal tumours. Modern sensitive laboratory and bioinformatical approaches could help in this regard.

Finally, it needs to be emphasized that in case of the *ATF3* gene, the predictive logistic regression model for discrimination of leiomyomas from leiomyosarcomas, and leiomyosarcomas from myometrium is only informative, and the gene's statistically significant differences in methylation levels have only been determined in leiomyomas and myometrium.

The highest differences in methylation levels were observed for the *KLF4* gene which acts as an important factor regulating the cell cycle at the G1/S and G/2 M transition [52,53]. However, methylation analysis of this gene in corpus uteri mesenchymal tumours has not yet been performed, and abnormal methylation index has been discovered in only one type of gynaecological tumour disease – cervix uteri carcinoma [36,85].

It is also quite difficult to determine *KLF4's* precise methylation effects because it has multiple functions. For example, *KLF4* acts as an antagonist of the Sp1 transcription factor which induces expression of *CYP1A1* gene [86]. This gene encodes the cytochrome p450 isoform implicated in oestrogen metabolism [86]. It is widely accepted that higher oestrogen levels are strongly associated with leiomyoma

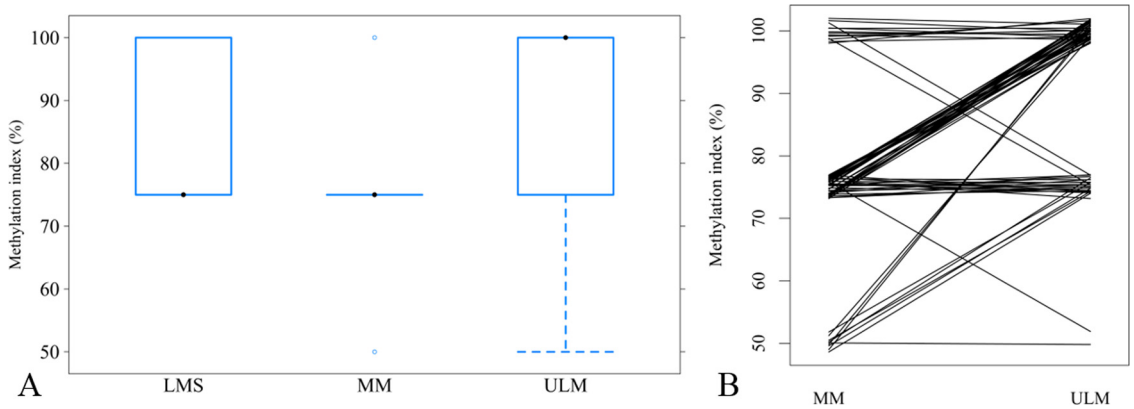

Fig. 10. Boxplot (A) illustrating overall *ATF3* gene promoter sequence methylation levels in myometrial (MM), leiomyosarcomatous (LMS) and leiomyomatous (ULM) tissues and the spaghetti plot (B) illustrating *ATF3* gene promoter sequence methylation levels in myomatous (MM) and leiomyomatous (LMS) tissues.

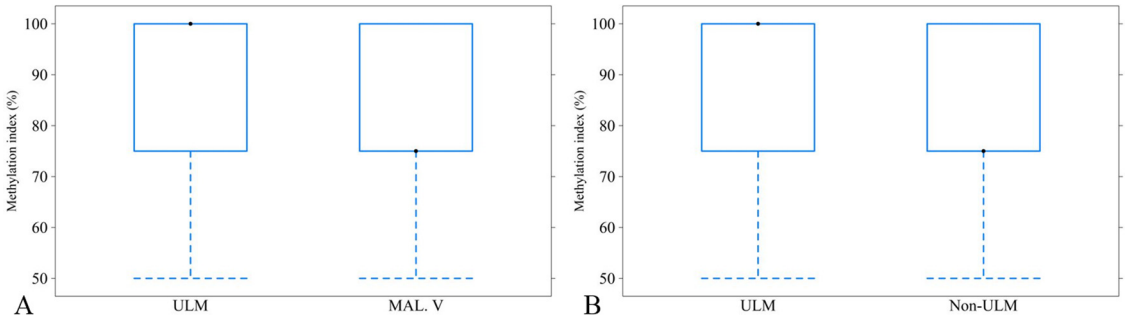

Fig. 11. Boxplots illustrating *ATF3* gene promoter sequence methylation levels: (A) uterine leiomyoma (ULM) vs. all malignant analysed tumour groups (MAL.V) and (B) leiomyoma vs. all other analysed tumour groups (Non-ULM).

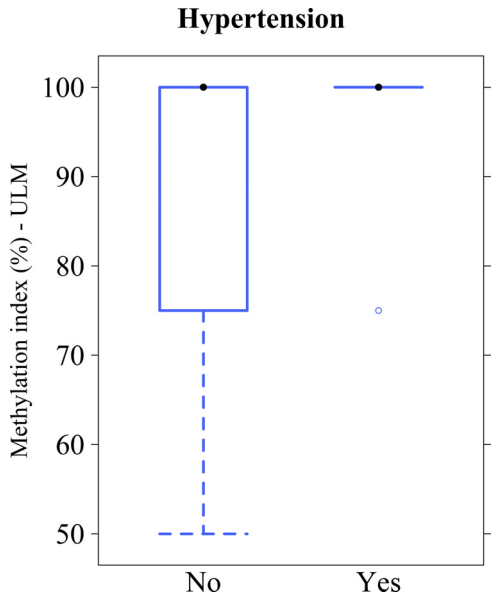

Fig. 12. Boxplots illustrating *ATF3* gene promoter sequence methylation levels in leiomyoma tissues for hypertension presence. Women with hypertension have very highly methylated sequences.

occurrence [5,87,88], and theoretically it can be expected that lower *KLF4* activity and subsequent higher *CYP1A* activity should be a protective mechanism, because *CYP1A* induces faster oestrogen metabolism. However, *CYP1A1* is also known under its Aryl-hydrocarbon-hydroxylase synonym, and its expression is transcriptionally up-regulated through the ligand-activated aryl hydrocarbon receptors (AhR), that are abundantly expressed in leiomyomatous tissue [89], and it is presumed that their activity should be markedly pro-oncogenic [90]. In addition to its oestrogen metabolism, the *CYP1A1* can stimulate bioactivation of procarcinogens and teratogenes, and it can have significant effect on tumorigenesis [91].

It is also exceedingly interesting that *KLF4* gene activity negatively correlates with the activity and positivity of the ERα receptors in breast cancer [92,93]. Here, the *KLF4* protein product is capable of direct binding to the ERα DNA binding region, and this represses binding between ERα and its responsive elements. While these interactions have been observed in breast cancer, it is important to realise that this tumour type is also greatly oestrogen dependent and ERα positive [94]. Therefore, it cannot be rejected that this same mechanism can occur also in mesenchymal uterine tumour tissue, because it is accepted that the level of ERα expression is higher in benign leiomyomas, and malign leiomyosarcomas [1,5,95]. Nonetheless, in malignant leiomyosarcomas it should not be associated with progression or recurrence [96]. Also, although ERα activity is higher in leiomyosarcomas than in myometrium, is lower than in leiomyomas [95]. Forasmuch as we observed higher methylation levels of *KLF4* gene in leiomyomas than leiomyosarcomas, this makes it possible that the activity of ERα receptors in both these tumours correlates with the *KLF4* methylation level. In other words, lower activity of *KLF4* caused by its methylation can increase activity of ERα receptors.

The *KLF4* can also repress the WnT/β-catenin signalling which is abnormally activated in leiomyomas [97,98]. The *MED12* is the most mutated gene in leiomyomas, and the WnT/β-catenin pathway regulates this gene's activity. Particularly, β-catenin physically and functionally targets the *MED12* subunit in Mediator to activate transcription [98,99]. In contrast, the physical interaction of *KLF4* protein with β-catenin represses its binding to the TCF complex required to begin this transcription [100]. In summary, if *KLF4* activity is decreased, it can be

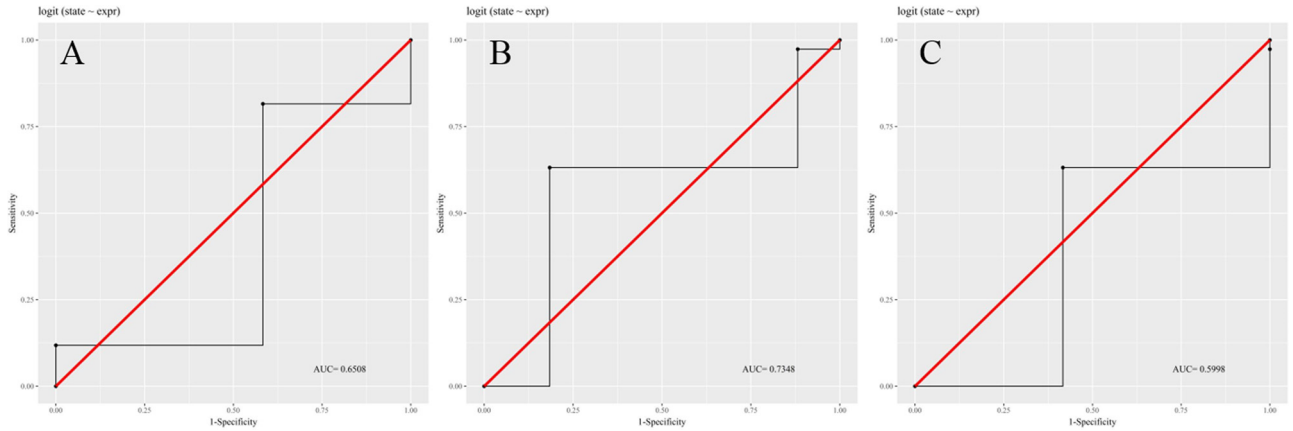

Fig. 13. ROC curve for the *ATF3* gene multinomial logistic regression with AUC's describing the predictive performance of the model (A) leiomyomas vs. leiomyosarcomas; (B) leiomyomas vs. myometrium; (C) leiomyosarcomas vs. myometrium.

Table 5
Summary of *KLF4*, *DLEC1* and *ATF3* gene AUC's, Odds ratios and Cut-off values.

	KLF4			*DLEC1*			*ATF3*		
	ULM vs. LMS	ULM vs. MM	LMS vs. MM	ULM vs. LMS	ULM vs. MM	LMS vs. MM	ULM vs. LMS	ULM vs. MM	LMS vs. MM
AUC	0.7829	0.9065	0.8756	0.7719	0.9064	0.7632	0.6508	0.7348	0.5988
Odds ratio	0.9752 (95% CI, 0.945 – 0.989)	0.9382 (95% CI, 0.954 – 0.917)	0.9752 (95% CI, 0.992 – 0.956	0.9638 (95% CI, 0.945 – 0.989)	0.9267 (95% CI, 0.945 – 0.903)	0.938 (95% CI, 0.898 – 0.970)	0.9688 (95% CI, 0.929 – 1.009)	0.9329 (95% CI, 0.957 – 0.907)	0.9759 (95% CI, 0.934 – 1.019).
Cut-off value	62.5%; (0.710 spec.; 0.909 sens.)	37.5 % (0.947 spec.; 0.776 sens.)	12.5 % (0.909 spec.; 0.789 sens.)	62.5% (0.632 spec.; 0.583 sens.)	37.5% (0.815 spec.; 0.631 sens.).	37.5% (0.5 spec.; 0.934 sens.).	87.5% (0.632 spec.; 0.583 sens.)	87.5% (0.815 spec.; 0.631 sens.)	62.5% (0.710 spec.; 0.909 sens.)

ULM – Uterine leiomyomas, LMS – Uterine leiomyosarcomas, MM – Healthy myometrium, CI – Confidence interval, spec. – Specificity, sens. – sensitivity.

hypothesized that the WNT/β-catenin signalling, interacting with most mutated (mainly gain-off) leiomyoma gene, became abnormally active.

In addition, the *KLF4* gene maintains cell cycle integrity at the G1/S and G2/M checkpoints [53,53], and while cell-cycle damage is not excluded in leiomyoma development and progression, the key cell-cycle regulator dysfunction is more often connected with malignant tumour types [6,11,12,101]. An example here is the *TP53* gene, which is usually functional in benign leiomyomas but often dysfunctional in mesenchymal uterine sarcomas [102]. This gene's differential activity may then promote the differences in leiomyoma and leiomyosarcoma tumorigenesis.

There is still key information lacking on the *DLEC1* gene's precise function. This gene is localized in the 3p22.3 region which is often chromosomally aberrated [39,103] and is presumed to be tumour-suppressive [40]. Promoter methylation differences of this gene are known in hepatocellular cancer [39], in gastrointestinal [40] and prostate cancers [42] and in lymphomas [41]. Importantly, higher levels of this gene's methylation are previously reported in leiomyoma tissue, even from smaller and ethnically diverse group of women [104]. It is accepted, that *DLEC1* can repress the NF-κB signalling pathway, which becomes abnormally active due to this gene's methylation. This mechanism has been observed in prostate cancer [42]. Theoretically, this mechanism may also affect uterine mesenchymal tumours. This is possible because this signalling pathway has been demonstrated to be abnormal in uterine leiomyomas [97,105]. The NF-κB signalling pathway interacts with many intracellular elements and multiple cell receptors, and it is especially activated in response to inflammatory processes. Binding of estradiol to the ER receptors could also trigger activation of this signalling pathway [97], and this provides the further possibility that leiomyoma, and most likely also leiomyosarcomas, which have more expressed ER receptors, can have an abnormally stimulated NF-κB pathway; and this could at least potentially be due to the lower activity of its hyper-methylated *DLEC1* repressor.

In comparison to the *KLF4* and *DLEC1* genes, *ATF3* methylation levels were less variable, mainly because the control tissues were also highly methylated. It is considered that leiomyomas tumorigenesis can be initiated by many stress factors [106]. Furthermore, the heterogenity and tissue specificity of stress-related triggers can result in *ATF3* exhibiting either oncogenic or tumor-supressive character [56]. The *ATF3* gene has documented lower expression in leiomyomas [107], and our slightly higher methylation level in these tumours leads to the expectation of its tumour-supressive effect in these tissues. Moreover, it is known that the *ATF3* gene often acts as an oncogene only after dimerisation with c-Jun, which has low expression in leiomyoma tissues [107]. On the opposite, there are also assumptions that *ATF3* may not act as a tumour suppressor in leiomyoma tissues, because *ATF3* activation has also been demonstrated after interaction with the Smad pathways [108–110]. These pathways participate in transducing the signal from TGFβ and activin and myostatin receptors which affect cell growth and expression of extracellular components; and, most likely, also the repression of extracellular matrix (ECM) degradation components [111,112].

TGFβ expression is generally abnormally high in leiomyomas, and this results in excessive ECM components production and storage which should contribute to the abnormally fibrous character of these tumours [113,114]. Therefore, it is possible that *ATF3* and Smad3 interaction could cause the leiomyomas' fibrous character. This assumption is supported by decreased fibrosis in animal models following *ATF3* knockout [109]. However, this model in leiomyoma tissues is possible only if the resultant higher methylation either does not affect *ATF3* activity or affects it only in a range that does not drastically reduce the activity of the abundantly expressed TGFβ receptors.

Important TNFα signalling is also abnormal in uterine leiomyomas – both ligands and receptors are more highly expressed in these tissues [114], and this can potentially also result from *ATF3* activity [115]. TNFα was abundantly expressed in the animal models when *ATF3* was

knocked out [115]. Furthermore, TNFα stimulates *MMP2* gene expression, which is usually higher in both leiomyoma [116] and sarcoma tissues [117]. It is therefore presumed that the higher methylation level and decreased *ATF3* expression can affect its regulatory role in TNFα signalling and that the ECM becomes abnormal. In contrast to previous models, although this supports our assumption that the *ATF3* gene should be tumour-suppressive in leiomyoma tissues, the observed high methylation levels of the *ATF3* promoter sequence remain baffling. Therefore, determination of which model is more feasible requires more ample and ideal sequencing analysis of the whole *ATF3* promotor sequence.

The statistically significant lower *KLF4* and *DLEC1* gene methylation levels in leiomyosarcomas than in benign leiomyoma promotes the assumption that different methylation mechanisms occur in these tumour types. These differences are potentially due to the two tumours' variable oestrogen response, whereby oestrogen receptors should positively regulate *DNMT1* expression [95,118,119]. Therefore, the differences may be due to variable DNMT methyltranserases' activity and function. While higher DNMT activity is known in leiomyomas [120,121] information is still lacking on its activity in leiomyosarcomas. However, these remain still assumptions, and proof requires further analysis. Furthermore, our analyses suggest that leiomyosarcomas should arise *de novo*, as generally assumed [7], and not as consequence of leiomyoma malignant transformation. It is quite implausible that de-methylation of tumor-supressor genes and hyper-methylated transcriptional factors occurs during the malignant transformation process.

Significant differences in *ATF3* gene methylation levels in healthy patients and those with hypertension could theoretically connect hypertension with epigenetic changes as was previously suggested [122]. In addition, our results also indicate a role of *ATF3* gene for proper function of cardiovascular system. While some evidence for this is provided by the variable large-scale cardiovascular defects apparent in mice models when this gene is knocked out, and *ATF3* activity increased in mice with induced hypertension [123], substantiation of these phenomena is required. In addition, diabetes mellitus is demonstrably caused by epigenetic changes in various genes [124,125], and because there is insufficient knowledge of the character and precise functions of the *DLEC1* gene, it cannot be excluded that abnormalities in this gene affect also diabetes development.

Crujerias et al. [126] studied the relationship between the climacterium and epigenetic changes, and they monitored the methylation level in 1287 CpG sites in mammary carcinoma tissue. Interestingly, post-menopausal women with normal BMI registered no changes, but post-menopausal with higher BMI had significantly higher methylation levels. Anyhow, further studies have provided no information on BMI effects in different gynaecological tumours.

Finally, because uterine leiomyomas were generally more highly methylated than other tumour types, it is questionable, whether these differences are not due to the long-term fixation of sarcoma samples. It is generally accepted, that formalin fixation causes DNA fragmentation and leads to crosslinking [127]. Anyhow, MS-HRM method requires only very short fragments, that should remain preserved in adequate numbers even in FFPE samples. Furthermore, previously published studies have reported a good correlation of methylation calls between fresh tissues and FFPE samples [128,129]. Lastly, here we observed no decrease in PCR efficiency. Therefore, it is implausible that FFPE fixation alone causes the differences we achieved in methylation levels.

In conclusion, to the best of our knowledge this is the first report that determines the methylation levels of the more variable groups of uterine mesenchymal tumour types and one of the few that provides at least theoretical possibilities of employing methylation levels to distinguish between two types of mesenchymal uterine tumours – leiomyomas and leiomyosarcomas. This possibility alone should inspire clinicians to counteract the current lack of pre-surgical, non-invasive tumour diagnostic certainty. Finally, although sarcomas are extremely rare, finding as many markers as possible will help distinguish them from leiomyomas; and this would provide benefits and safety to the thousands of women who either cannot, or prefer not to, undergo surgical intervention, and reduce number of women whose malignant lesions are initially misdiagnosed as benign.

Conflict of interest

Authors declare that they have no conflict of interest.

Acknowledgements

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