Methylation status of *KLF4* and *HS3ST2* genes as predictors of endometrial cancer and hyperplastic endometrial lesions

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Abstract. Endometrial carcinoma is one of the most common tumours in developed countries. In addition to the active role of genetic factors, epigenetic changes also have an important effect. The present study analysed the methylation status of kruppel like factor 4 (*KLF4*) and heparan sulfate-glucosamine 3-sulfotransferase 2 (*HS3ST2*) genes in three endometrial tissue types for carcinoma prediction. The sample comprised 91 women with histologically-confirmed endometrial carcinoma (64.16±9.64 years old), 36 women with hyperplasia (53.39±9.64 years old) and 45 with no signs or symptoms of malignancy (48.53±11.11 years old). The CpG dinucleotide methylation levels were examined by quantitative pyrosequencing, and the discrimination accuracy of the model was calculated using the Random Forest classification algorithm of the area under the ROC curve (AUC). The mean values of *KLF4* and *HS3ST2* methylation indices were 23.83±11.39 and 8.52±2.57 in the control samples; 30.40±8.52 and 33.76±20.66 in hyperplasia and 34.72±10.79 and 34.49±18.39 in the cancerous tissues. Multinomial logistic regression indicated that the *HS3ST2* CpG1 methylation status is a predictor of hyperplasia (P<0.05) and that the *KLF4* CpG2 dinucleotide can predict carcinoma formation (P<0.001). The AUC value of 0.95 indicates high discrimination accuracy of the CpG nucleotides methylation status model between the controls and the two other diagnoses. The results of the present study establish the likelihood that aberrations in *KLF4* and *HS3ST2* gene methylation levels are important in the development of endometrial hyperplasia and carcinoma, with hyperplasia an intermediate step between healthy and tumour tissues.

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Introduction

Although endometrial carcinoma (EC) is one of the most frequent malignant gynaecological diseases in developed countries, its occurrence in developing countries is lower (1). EC in North American and European female populations accounts for almost 6% of all cancer cases and 3% of all cases of cancer-associated mortality, and the incidence varies between 19 and 25/100,000 women (2). Although ~10% of diagnosed EC is hereditary (3), the remaining 90% is sporadic; with EC typical in older, postmenopausal women. Statistics also show that 15% of women are diagnosed before the age of 50 years, and 5% before the age of 40 years (4).

The formation of EC is associated with the presence of polycystic ovary syndrome (5), obesity (6), nulliparity (7), hyperinsulinaemia (8) and excessive exposure to oestrogen manifested in earlier menarche (9). The therapeutic use of tamoxifen in women with, or at high risk of, breast cancer is also potentiality implicated in increased EC incidence (10) and there are further inter-connections, including *in vitro*-fertilisation treatment and higher frequencies of miscarriages and abortions (11).

Endometrial hyperplasia is another important risk factor in EC as it can develop into adenocarcinoma. There are four types of endometrial hyperplasia: Simple hyperplasia (progressing to cancer in 1% of cases), complex hyperplasia (progressing to cancer in 3%), simple atypical hyperplasia (progressing to cancer in 8%), and complex atypical hyperplasia (progressing to cancer in 29%) (12).

Endometrial malignancies are traditionally classified under types I and II, dependent on hyperplasia formation (13). Type I distinguishes 70-80% of tumours and these are characterised as

oestrogen-dependent and preceded by hyperplasia formation. They are low grade, diploid and highly differentiated and more common in obese women. The remaining 20-30% is classified as Type II, which is considered oestrogen-independent and associated with atrophy and aneuploidy. These are high-grade, poorly differentiated and linked to higher metastatic risk and resultant poor prognosis (14).

Types of EC are differentiated by histopathological characteristics into endometrioid carcinoma (up to 75%, typically associated with type I tumours), serous carcinoma, carcino-sarcoma and clear-cell carcinoma (15). Although this classification remains the main diagnostic tool, studies have highlighted the importance of incorporating genetic profile and risk determination models (16-18). Molecular approaches may also describe EC biological characteristics and features more accurately, distinguish between subtypes, and substantially improve predictive and treatment approaches (16,18-22) as each EC subtype has a distinct mutation profile (17,18,23).

In addition to genetic factors, several epigenetic mechanisms are involved in endometrial carcinogenesis (24,25). Previous analyses have predominantly focused on methylation changes in the promoter regions of genes involved in oestrogen metabolism, the DNA mis-match repair system and signalling pathways, including human mutL homolog 1 (26,27), cyclin-dependent kinase inhibitor 2A (27), estrogen receptor 1 (28), progesterone receptor-B (29), phosphatase and tensin homolog (30), Ras association domain family member 1A (31), O-6-methylguanine-DNA methyltransferase (32) and adenomatous polyposis coli (33,34).

For the purpose of the present study, the kruppel like factor 4 (*KLF4*) and heparan sulfate-glucosamine 3-sulfotransferase 2 (*HS3ST2*) cancer-related genes were analysed. *KLF4* is part of the Kruppel-like gene family with 'zinc-finger' transcription factor. The main role of this gene is to maintain cell cycle integrity (35) and thus influence the growth, differentiation, proliferation and programmed apoptosis of somatic cells (36). *KLF4* inhibits cell proliferation as a control protein via the activation of p21, which normally inhibits cyclin-dependent kinases (37). It also acts as a mediator in arresting the cell cycle following recognition of damage in the G_1/S phase and eventually at the G_2/M checkpoint, with this process being mediated by p53 activity (35,38). The methylation levels of *KLF4* are generally lower in certain types of cancer, including oesophageal (39), pancreatic (40), lung (41), brain (42) and gastric cancer (43).

By contrast, the *HS3ST2* gene encodes the heparansulfate 3-*O*-sulfotransferase 2 enzyme, which is a key component in heparansulfate (HS) fine structure biosynthesis involved in multiple biologic activities (44). Each enzyme in this cascade has a tissue-specific role and serves as a substrate for the subsequent reaction. Therefore, change in even one enzyme, including heparansulfate 3-*O*-sulfotransferase 2, leads to the diverse HS structure (45) involved in several types of cancer (44,46).

The present case-control study is unique in that it involves comparison of *KLF4* and *HS3ST2* methylation status in EC, hyperplasia and normal endometrial tissue; by the investigation of other clinical and histopathological data roles in methylation status, and by the quantification of predictor diagnostic accuracy by the area under the ROC curve (AUC).

Materials and methods

Patients and clinical pathological characteristics. The sample group comprised 172 Caucasian women hospitalised at the Department of Obstetrics and Gynaecology at Martin University Hospital (Martin, Slovakia) between 2011 and 2017. Tissue was analysed from 91 patients with EC, 36 with hyperplasia and 45 with normal endometrial tissue. Each tissue underwent standard histopathological analyses at the Department of pathology at Martin University Hospital. This provided histological type, degree of differentiation (G), and parameters of tumour-node-metastasis classification (Table I). Information on personal and gynaecological anamnesis was obtained during medical examination. This comprised body mass index (BMI), hypertension, diabetes mellitus, age at menarche, parity, abnormal uterine bleeding and abortion, and smoking habit.

DNA isolation and bisulfide modification. Tissue samples were stabilised in RNAlater solution immediately following sectioning and frozen at -20˚C. DNA was then extracted by the column method (DNeasy Blood and Tissue Kit®, Qiagen GmbH, Hilden Germany). The qualitative parameters of the isolated DNA were assessed by 1.5% agarose gel electrophoresis and the DNA concentration was measured using a Nanodrop® device. Only samples with sufficient concentration of at least 100 ng/μl were considered for further processing. Genomic DNA (1 μg) was used for the bisulfite conversion performed using the Epitect bisulfite kit® (Qiagen GmbH): 1 μg of DNA dissolved in nuclease-free water was mixed with 85 μl of bisulfite mix and 35 μl of DNA protect buffer and amplified. The concentration of bisulfite modified samples was measured spectrophotometrically and samples were frozen to -20˚C.

Methylation analyses. The methylation levels of three CpG sites in the *HS3ST2* gene and six CpG sites in the *KLF4* gene were analysed by pyrosequencing (Pyromark Q96 ID device). This is a quantitative, precise real-time sequencing methodology. The visible light emitted in the final step of the enzymatic cascade was scanned using a CCD camera; with the rate of light emission retaining continuous proportion with the number of incorporated nucleotides.

Pyro-sequencing has PCR amplification and sequencing phases; DNA amplification required 25 μl total volume (Pyromark PCR Kit® Qiagen GmbH) containing 2X pyromark PCR master mix, 10X coral load concentrate, 1 μl 25 mM $MgCl_2$, 5X Q solution, 0.24 μM primer mix, RNase free water and bisulfide-modified DNA). The PCR reaction steps were as follows: Activation of polymerase (95˚C, 15 min); 45 cycles of: Denaturation (94˚C, 15 sec), annealing (56˚C, 30 sec), extension (72˚C, 30 sec) and final extension at 72˚C for 10 min. The amplicons were then assessed by 1.5% agarose gel electrophoresis.

The PCR product (20 μl) was mixed with streptavidin-coated sepharose beads (GE Healthcare Life Sciences, Chalfont, UK), binding buffer and nuclease free water in a total volume of 80 μl. The 5'-biotiniled strand for sequencing was immobilised, transferred to 0.4 M sequencing primer and binding buffer solution (Qiagen GmbH) and incubated for 2 min at 80˚C. The samples were analysed by Pyromark Q96 ID and interpreted by Pyromark Q96 software v. 2.5.8 (Qiagen GmbH) via calculation of the C/T ratio and the peak-high of

Table I. Histopathological characteristics of the endometrial cancer and hyperplasia groups.

Characteristic	n	%
Endometrial cancer (n=91)		
Endometroid	78	85.7
Endometroid with squamous differentiation	9	9.9
Other[a]	4	4.4
Stage (pT)		
T0	1	1.1
T1a	35	38.4
T1b	35	38.4
T2	11	12.2
T3a	6	6.6
T3b	3	3.3
Lymph node metastasis (pN)		
N0	34	37.0
N1	6	6.5
Nx	51	56.5
Histological grade		
G1	19	20.9
G2	49	53.8
G3	23	25.3
Hyperplasia (n=36)		
Simplex hyperplasia	21	58.3
Simplex hyperplasia with atypia	5	13.9
Complex hyperplasia with atypia	10	27.8

[a]Endometroid with mucinous differentiation; clear cell carcinoma; undifferentiated endometrial sarcoma; serous adenocarcinoma.

each CpG site. The samples were analysed in duplicate, and controls comprised commercial methylated and unmethylated DNAs (diluted to series of 100, 75, 50, 25 and 0%). Commercially available Pyromark CpG assays® (Qiagen GmbH) provided methylation analyses of the following regulation sequences: *KLF4* 5'-CCCGACATACTGACGTGCTGG CGGGCCACGCGCGA-3'; *HS3ST2* 5'-TTGGCGAGATGT CGAGAGCGGGGGGA-3'.

Statistical analysis. The methylation levels were visualised by swarmplots (47) (Figs. 1 and 2). The data was not Gaussian, so robust one-way analysis of variance (ANOVA) (48) was used instead of simple ANOVA to determine the equality of the CpG methylation population median levels across the diagnostic groups. Rejection of the ANOVA hypothesis was followed by Tukey's HSD post hoc test. Spearman's correlation coefficient was used to quantify the strength of the linear association between quantitative variables.

The methylation index (MI) was calculated as the mean-percent-methylation across all gene CpG sites (three CpG sites of the *HS3ST2* gene and six CpG sites of the *KLF4* gene). The promoter methylation status was theoretically classified as unmethylated (0-9%), methylated (10-29%) and highly methylated (30-100%) (49,50). Multinomial logistic regression then modelled dependence of the diagnosis on age, BMI, menarche, parity, CpGs and smoking. The model identified CpGs which are statistically significant predictors of diagnosis, while controlling the effect of other predictors.

The Random Forest algorithm assessed the diagnostic accuracy of the predictors. The subset of important predictors was identified by the minimum tree depth criterion in the nested cross-validation feature selection (51). The diagnostic accuracy was quantified by the ROC curve and summarised by the AUC. Finally, the Younden criterion identified the optimal sensitivity and specificity. Categorical variable independence was established using a χ^2 test. The analyses were performed in R ver. 3.2.1. (52) and IBM SPSS ver. 21.

Results

Gynaecological anamnesis and risk factors. The statistically different characteristics in the EC, hyperplasia and control groups are listed in Table II. The statistics were also age-adjusted to eliminate age-effect on the variables examined.

The following statistically significant differences were observed in gynaecological anamneses and reproductive characteristics: 90% of women with cancer were postmenopausal and 65.4% of the control group were premenopausal; 84.6% of women without children had cancer and the remaining 15.4% nulliparous women were in the control group, thus indicating the importance of nulliparity in EC. The statistics regarding abnormal bleeding were dominated by women from the cancer group (65.0%), followed by 18.3% of women with hyperplasia and 16.7% of controls. Menarche age was the only variable to have no significant impact on diagnostic typology in the study (P>0.05).

Analyses of other risk factors, including BMI, DM and hypertension, indicated higher cardiovascular risk, in addition to EC, as these metabolic parameters are also important cardiovascular risk variables. Women with cancer dominated the hypertension category (70.4% of cancer patients, 13.0% with hyperplasia and 16.7% of controls, P=0.002) and the diabetes mellitus category (76.0% prevailing in cancer sufferers, 12.0% in the hyperplasia group and 12.0% in the control group, P=0.043). The mean BMI values of classified women with cancer in class II on the obesity scale indicated severe obesity, and the women with hyperplasia were in class I obesity. The BMI values were not influenced by age (P=0.735), however, GLM analysis revealed they were influenced by diagnosis (P<0.001). Smoking results showed that 8.8% of women with cancer smoked; plus 25% with hyperplasia and 20% of controls (P=0.040).

Methylation levels. The MI of the two genes was statistically significantly different in the three study groups; with increasing tendency towards the EC group (Table III). The detailed comparison of groups using robust one-way ANOVA with Tukey's HSD test confirmed a statistically significant difference in the *HS3ST2* and *KLF4* genes between median methylations levels of normal tissues, vs. hyperplasia, normal tissues, vs. cancer, and hyperplasia, vs. cancer. The only exception was *HS3ST2* gene hyperplasia and cancer coincidence, where no significant difference was observed (P=0.847).

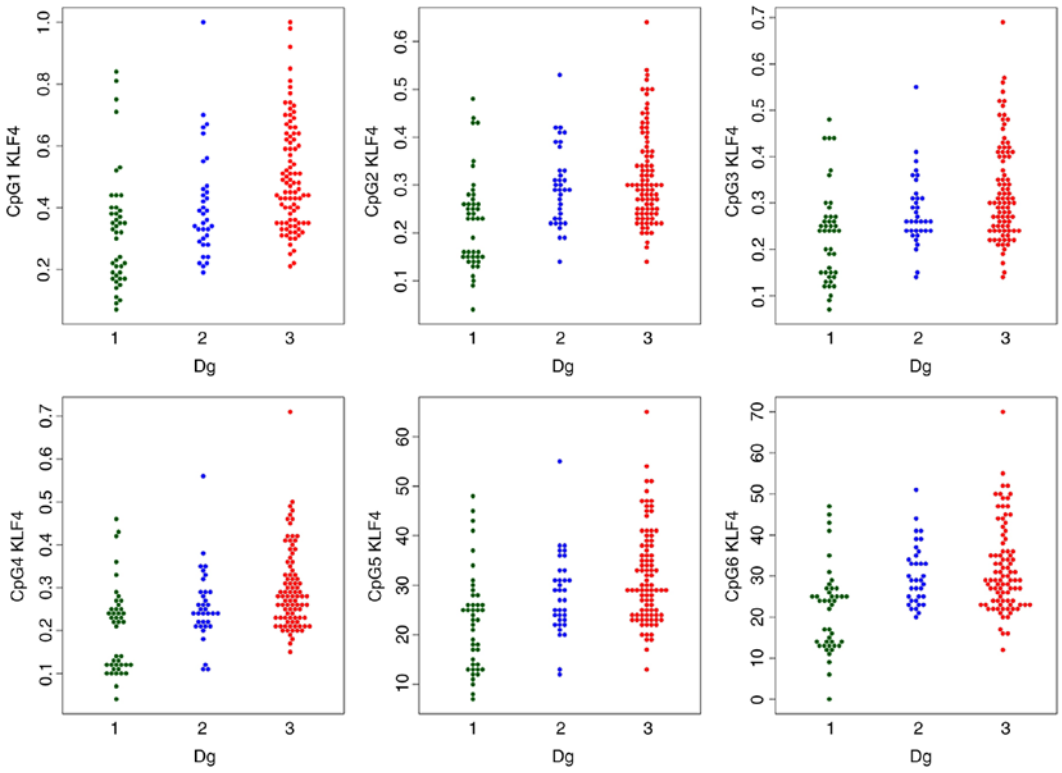

Figure 1. Swarmplots of the methylation status of *KLF4* CpG sites by diagnosis. 1, control; 2, hyperplasia; 3, endometrial cancer. Dg, diagnostic group; *KLF4*, kruppel like factor 4.

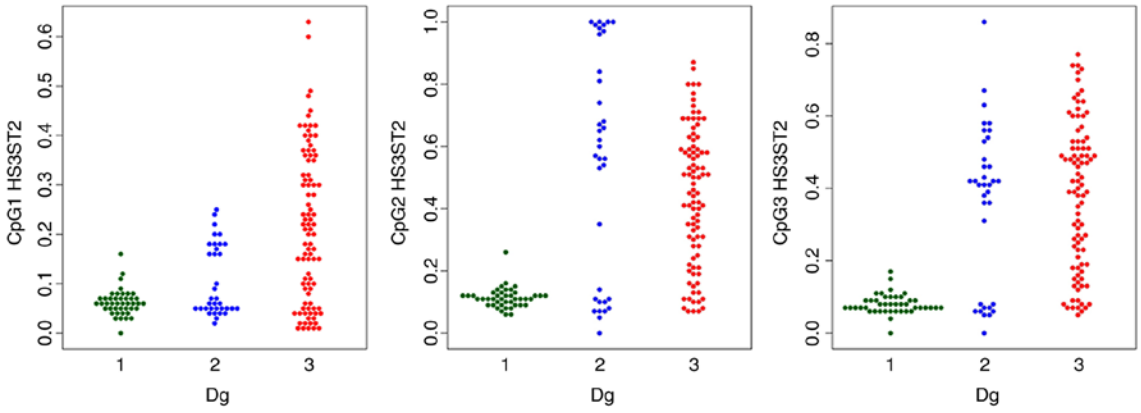

Figure 2. Swarmplots of the methylation status of *HS3ST2* CpG sites by diagnosis. 1, control; 2, hyperplasia; 3, endometrial cancer. Dg, diagnostic group; *HS3ST2*, heparan sulfate-glucosamine 3-sulfotransferase 2.

The comparison of each CpG site methylation highlighted statistically significant differences between the cancer, hyperplasia and control samples in the *KLF4* gene CpG1, CpG3, CpG4 and CpG5 sites, and the *HS3ST2* CpG1 site. Differences in *KLF4* gene CpG2 and CpG6 and *HS3ST2* CpG2 and CpG3 were observed only in the control, vs. hyperplasia and control, vs. cancer groups, but not in the hyperplasia, vs. cancer group (Figs. 1 and 2).

The comparison of methylation in the EC histological subtypes and the hyperplasia subtypes in the two genes revealed no statistically different median methylation levels at P<0.05; nor were there differences in each CpG site or MIs. The *HS3ST2* CpG1 site methylation levels were different between tumour stage status (pT) (0.027), however, no increasing or decreasing tendency in stage severity was detected. Similarly lacking any tendency, significant differences were established in lymph node metastasis status (pN) in the *KLF* CpG1 site (P=0.045), in the grading of all *HS3ST2* CpG sites (CpG1 P=0.001, CpG2 P=0.008, CpG3 P=0.043) and in the *HS3ST2* MI (P=0.004) (data not shown).

Correlation analysis. Correlation analysis of the entire sample confirmed the association between mean *HS3ST2* and *KLF4* gene methylation values and age (r=0.316, P<0.001; r=0.317, P<0.001), BMI (r=0.386, P<0.001; r=0.191, P=0.013) and diagnosis (r=0.496, P<0.001; r=0.387, P<0.001). The CpG sites in each gene correlated significantly with each other;

Table II. Mean values of age, BMI and menarche in the three study groups.

Factor	Control (n=45)	Hyperplasia (n=36)	Cancer (n=91)	χ^2/P-value
Age (years)				
Mean ± SD	48.53±11.11	53.39±9.64	64.16±9.64	P<0.001
Median	47.00	52.00	65.00	
Menarche				
Mean ± SD	13.22±2.01	13.17±1.87	13.21±1.42	P=0.375
Median	13.00	14.00	13.00	
BMI				
Mean ± SD	28.09±4.82	32.05±5.07	35.57±3.81	P<0.001
Median	28.41	32.16	35.60	
Menopausal status, n (%)				
Pre	17 (65.4)	4 (28.6)	2 (4.0)	χ^2=42.22
Peri	4 (15.4)	3 (21.4)	3 (6.0)	P<0.001
Post	5 (19.2)	7 (50.0)	45 (90.0)	
Parity, n (%)				
Yes	43 (95.6)	36 (100.0)	80 (87.9)	χ^2=6.240
No	2 (4.4)	0 (0.0)	11 (12.1)	P=0.044
Bleeding, n (%)				
Yes	10 (38.5)	11 (78.6)	39 (78.0)	χ^2=13.09
No	16 (61.5)	3 (21.4)	11 (22.0)	P=0.001
Hypertension, n (%)				
Yes	9 (34.6)	7 (50.0)	38 (76.0)	χ^2=12.90
No	17 (65.4)	7 (50.0)	12 (24.0)	P=0.002
Diabetes, n (%)				
Yes	3 (11.5)	3 (24.1)	19 (38.0)	χ^2=6.303
No	23 (88.5)	11 (78.6)	31 (62.0)	P=0.043
Smoking, n (%)				
Yes	9 (20.0)	9 (25.0)	8 (8.8)	χ^2=6.415
No	36 (80.0)	27 (75.0)	83 (91.2)	P=0.040

BMI, body mass index; SD, standard deviation.

with correlation coefficients varying between 0.677 and 0.934 for the *HS3ST2* gene and between 0.850 and 0.944 for the *KLF4* gene. This indicated a correlation in single CpG site methylation status (Table IV).

Multinomial logistic regression. The P-values of the predictors in Table V highlight the importance of age, BMI and the *KLF4* CpG2 site as cancer predictors, and *HS3ST2* CpG1 methylation was a significant factor in hyperplasia prediction.

Selection of the diagnostic predictors and discrimination accuracy. The Random Forest classification algorithm plot schematically visualised and ordered variables by their importance in diagnostic prediction (Fig. 3). On considering CpG methylation status together with clinical variables, the BMI was identified as the most important predictor, followed by CpG1 *HS3ST2* dinucleotide methylation status, age and *HS3ST2* CpG3 and CpG2 dinucleotides. Other parameters, including smoking, menarche and parity, had no significant impact on diagnostic prediction and were therefore omitted from the final model. The Random Forest algorithm with nested cross validation determined the predictive performance of this model with the selected important variables. The highest AUC value of 0.961 was attained in the discrimination of controls from the other groups, followed by the discrimination between the EC group and other groups (AUC 0.945). The lowest AUC (0.845) was determined in the discrimination of women with hyperplasia from the other groups (Fig. 4).

The model produced exclusively from the CpG methylation status determined that the *HS3ST2* CpG sites were the most important predictors; followed by CpG1, CpG2 and CpG4 in the *KLF4* gene. The 0.95 AUC value indicated the perfect discrimination accuracy of the CpG model between normal tissue and other diagnoses.

Cut off value. The methylation status in the three diagnostic categories is shown in Fig. 5. Unmethylated *KLF4* gene status was detected only in normal tissue (4.9%). Although methylation and hypermethylation occurred at identical 50% frequency

Table III. Mean methylation values and medians of the MI and each CpG site in *KLF4* and *HS3ST2* genes according to diagnosis.

Site	Normal (n=43)	Hyperplasia (n=35)	Cancer (n=91)	P-value
MI KLF4[a]	23.83±11.39	30.40±8.52	34.72±10.79	
	25.33	29.50	31.83	0.001
CpG1	32.65±18.77	40.49±17.07	50.22±17.42	
	32.00	36.00	47.00	<0.001
CpG2	22.51±9.96	29.69±8.29	31.91±9.80	
	23.00	29.00	30.00	<0.001
CpG3	23.05±10.05	28.14±7.69	32.76±10.68	
	24.00	26.00	30.00	<0.001
CpG4	20.30±10.01	25.69±8.23	29.78±9.31	
	22.00	24.00	28.00	<0.001
CpG5	22.79±10.01	28.09±8.05	31.85±9.52	
	23.00	27.00	29.00	<0.001
CpG6	21.70±10.32	30.34±7.35	31.80±10.47	
	24.00	29.00	29.00	<0.001
MI HS3ST2[a]	8.52±2.57	33.76±20.66	34.49±18.39	
	8.33	36.67	35.00	<0.001
CpG1	6.21±2.63	10.43±7.11	21.59±15.35	
	6.00	6.00	21.00	<0.001
CpG2	11.33±3.26	54.77±36.14	43.88±21.81	
	11.00	60.00	45.00	<0.001
CpG3	8.02±2.76	36.09±21.96	38.00±20.26	
	7.00	41.00	40.00	<0.001

[a]MI was calculated as mean methylation value of analysed CpG sites; values are expressed as the mean ± standard deviation and median. MI, methylation index; *KLF4*, kruppel like factor 4; *HS3ST2*, heparan sulfate-glucosamine 3-sulfotransferase 2.

Table IV. Correlation analyses between CpG sites of *KLF4* and *HS3ST2* gene.

	KLF4					
	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6
HS3ST2						
CpG1		r=0.914	r=0.927	r=0.868	r=0.916	r=0.889
CpG2	r=0.677		r=0.933	r=0.850	r=0.923	r=0.914
CpG3	r=0.760	r=0.934		r=0.850	r=0.944	r=0.904
CpG4	-	-			r=0.900	r=0.873
CpG5	-	-	-	-		r=0.938

r, Spearman's correlation coefficient; P-value was <0.001 in all cases. *KLF4*, kruppel like factor 4; *HS3ST2*, heparan sulfate-glucosamine 3-sulfotransferase 2.

in hyperplasia, 35.2% of EC sufferers were identified as methylated and the remaining 64.8% were hyper-methylated. Additional results were as follows: i) *HS3ST2* gene analysis identified the high 71.8% unmethylated state in control tissue, but without hypermethylation; ii) there was high 64.9% hyper-methylation in the hyperplasia group and 62.1% in the EC group. These differences were statistically significant (P<0.001).

The Youden method calculated the cut-off value of class probability differentiating health status from the other two conditions. This was established at 0.5, with the diagnostic sensitivity and specificity of 88.8 and 60.5% for the *KLF4* gene and 53.3 and 83.5% for the *HS3ST2* gene. The corresponding cut-off for average methylation was 34.3 for *KLF4* and 41.0 for *HS3ST2*. The Random Forest algorithm also assessed each pair of diagnostic groups, and the average methylation cut-offs remained the same in each gene, whereas the specificity and sensitivity varied. The best AUC values were obtained in the diagnosis status models distinguishing health and cancer in

Table V. Multinomial logistic regression P-value coefficients of predictors in hyperplasia and cancer.

Predictor	Hyperplasia (P-value)	Endometrial cancer (P-value)
KLF4		
CpG1	0.464	0.106
CpG2	0.263	<0.001
CpG3	0.101	0.470
CpG4	0.846	0.892
CpG5	0.821	0.926
CpG6	0.058	0.164
HS3ST2		
CpG1	0.044	0.247
CpG2	0.434	0.806
CpG3	0.225	0.149
Age	0.932	0.015
Menarche	0.812	0.964
BMI	0.057	0.001
Smoking	0.365	0.897
Parity	0.302	0.787
Abort/UPT	0.600	0.402

KLF4, kruppel like factor 4; *HS3ST2*, heparan sulfate-glucosamine 3-sulfotransferase 2; BMI, body mass index.

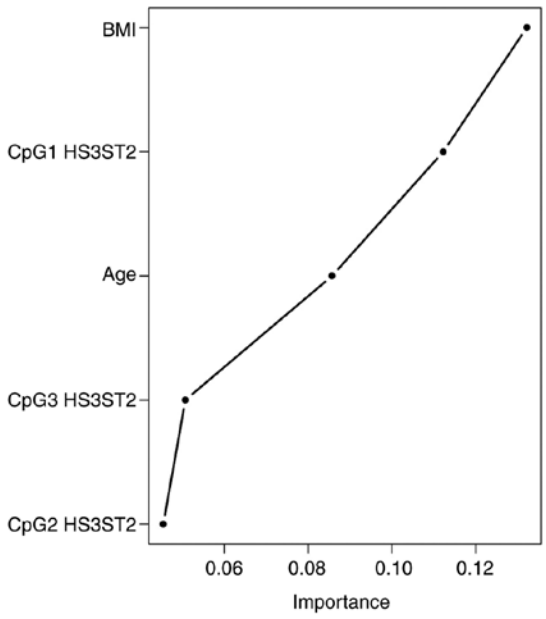

Figure 3. Importance plot with selected variables in diagnostic prediction. Importance increases to the right. *HS3ST2*, heparan sulfate-glucosamine 3-sulfotransferase 2; BMI, body mass index.

these genes; the *KLF4* gene recorded an AUC of 0.751, 76.74% specificity and 73.52% sensitivity, and *HS3ST2* returned an AUC of 0.789, 71.11% specificity and 86.81% sensitivity. It was not possible to differentiate between hyperplasia and cancer in the *KLF4* gene due to its AUC of 0.488 (data not shown).

Discussion

It is evident that aberrant DNA methylation is a common factor in endometrial carcinogenesis. Decreased DNA methylation occurs early in carcinogenesis, and promoter hypermethylation leads to gene silencing and loss of gene expression. Therefore, carcinogenesis can be induced when the tumour suppressor gene or a critical gene involved in the cell cycle or in DNA repair is affected (53,54).

Aberrant methylation. The methylation of three CpG sites in the *KLF4* gene and six CpG sites in the *HS3ST2* gene were analysed in the present study. These are novel genes, and there have been few reports on their association with hyperplasia and EC (55-58). The functions of the protein products of these genes indicate the likelihood of increased methylation levels in cancer tissues, however, aberrant methylation also requires detection in types of hyperplasia that can evolve into EC. Consequently, there was increasing tendency of average gene methylation from normal endometrial tissue through hyperplasia to cancer (Table III).

The difference in methylation of each CpG and the MI in the two genes were statistically significant between normal tissue, hyperplasia and EC tissue. However, the difference between hyperplasia and EC tissues was less distinct as only one of the *HS3ST2* gene CpG sites and four of the six *KLF4* sites had statistically significant methylation levels at $P<0.05$. In addition, the comparison of hyperplasia and cancer MI revealed statistically significant difference only for the *KLF4* gene ($P=0.04$). These results accentuate the importance of these genes in the genesis of cancer and hyperplasia, and they further support hyperplasia as a pre-cancerous tissue form (59,60). Similar results were recorded by Nieminen *et al* (61), who observed increasing methylation tendency from normal endometrial tissue through simple hyperplasia to the complex type in 24 tumour-suppressor genes.

Although the methylation analyses of hyperplasia and carcinoma histological subtypes in the present study revealed no statistically significant differences in methylation levels, a difference was found between simple and complex hyperplasia. This inconsistency is likely due to unbalanced subtype incidence, as simple hyperplasia at 58.3% and endometroid adenocarcinoma at 85.7% formed the majority of the respective conditions, with the remaining subtypes registering at only minor frequencies. The small sample size in the present study limited statistical analyses to a certain extent; it was not possible to compare endometrial and hyperplasia sub-types, and the comparison of methylation mean values between hyperplasia and cancer was under-powered. Sample size determination analysis revealed the optimal sample size for *HS3ST2* and *KLF4* gene methylation is 11,268 and 80 individuals in each diagnostic group. In order to compare single CpG sites, the required number of individuals are as follows: *HS3ST2* CpG1 19, CpG2 118 and CpG3 1,921, and *KLF4* CpG1 50, CpG2 263, CpG3 64, CpG4 73, CpG5 87 and CpG6 603 in each diagnostic group. In the present study, only the analysis of *HS3ST2* CpG1 site methylation between hyperplasia and cancer had adequate statistical power, and it was not possible to determine whether hypermethylated hyperplasia tissue is a prerequisite step in the carcinoma cascade. A higher sample size is also essential in terms of the detailed analysis of aberrant methylation in

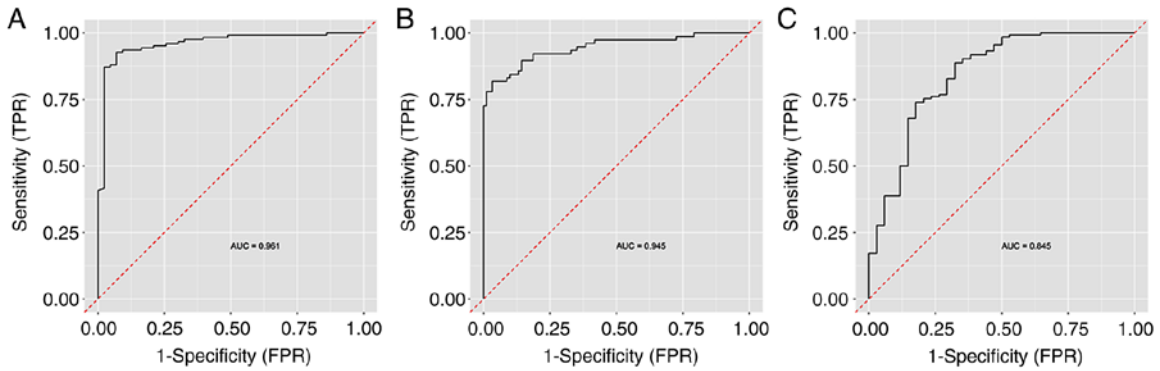

Figure 4. ROC with AUC describing the predictive performance of the model. (A) Control, vs. cancer and hyperplasia; (B) endometrial cancer, vs. hyperplasia and control; (C) hyperplasia, vs. cancer and control. AUC, area under the ROC curve.

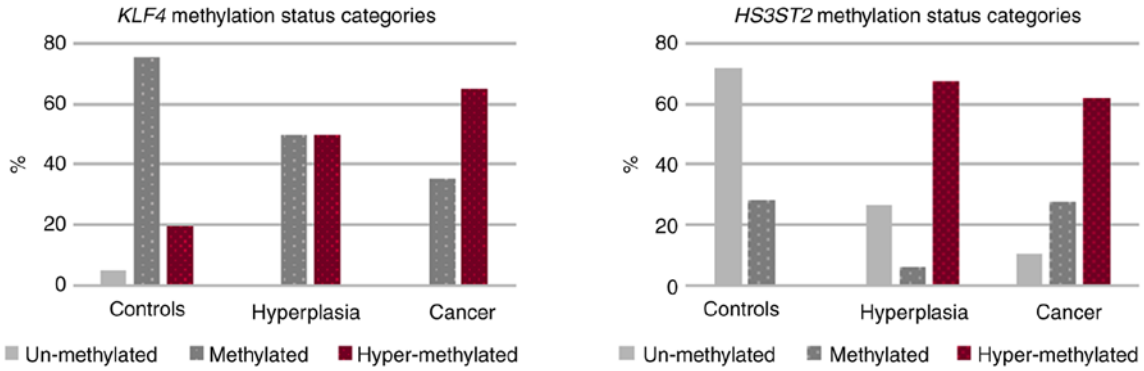

Figure 5. *KLF4* and *HS3ST2* methylation status categories according to diagnosis. *KLF4*, kruppel like factor 4; *HS3ST2*, heparan sulfate-glucosamine 3-sulfotransferase 2.

specific hyperplasia and cancerous subtypes. However, data from the preliminary analyses may be utilised by those who design investigations on the methylation status of these genes and include predominantly Caucasian subjects.

Methylation cut-offs. The literature surveys identified a lack of consistency in determining methylation cut-off values. A number of authors depend on three categories; unmethylated 0-9%, methylated 10-29% and highly methylated 30-100% (49,50), whereas others rely on two; unmethylated <15% and methylated >15% (62-64). In addition, several studies use ROC curve analysis to establish the optimal methylation threshold in discriminating diagnostic categories, cancer types, treatment decisions and outcomes, and patient survival (62,65,66). This highlights the importance of methylation status, which may be useful as a biomarker in cancer management.

In the present study, ROC analysis was performed using the Random Forest Algorithm with nested cross validation, as this provides realistic results. It was established that the model distinguishing control samples from hyperplasia and cancer provided the highest discriminatory ability at AUC=0.961, and that hyperplasia comparison with the other two conditions had the least discriminatory ability at AUC=0.845.

Pyrosequencing method. Several methods are available to analyse promoter region methylation status, and selection of the optimal method depends on the following: Relevant gene identification, gene analysis range, robustness, DNA quantity, the inclusion of bisulfite conversion and the availability of detection devices. Kurdyukov and Bullock (67) compared several methods and described their exploitation in practice. In the present study, pyrosequencing was selected as it is the standard technique in cancer research, detects small differences in methylation, is suitable for heterogeneous samples and provides quantitative results (67,68). However, it was not possible to determine whether it provides information on allele specificity or hemi-methylation, which may differentiate *de novo* methylation events from maintenance factors (69,70).

Risk factors. Risk factor analysis investigated the significant effects of metabolic factors, including BMI, hypertension and diabetes mellitus, in EC development. The high mean BMI values indicated severe obesity in women with cancer (35.57±3.81), compared with those in the control group (28.09±4,82, P<0.001). This risk factor is also often connected with hypertension, hypercholesterolaemia and diabetes mellitus. The high occurrence of hypertension and diabetes mellitus was noted in cancer patients (76.0 and 38.0%, respectively) and in hyperplasia (50.0 and 24.1%, respectively); therefore, future lipid profile analysis is worthwhile in determining the cluster effect of these factors and their combination in metabolic syndrome formation (71). These three conditions, high BMI, hypertension and diabetes mellitus, are also the main risk factors in cardiovascular disease (CVD) (72). Although cancer is considered second only to CVD in recently

determined leading causes of mortality in Europe, America and Asia, current publications suggest that cancer, rather than CVD, is the most common cause of mortality (73-77). The results of the present study highlight the similarities and interactions between these diseases. The most common feature is inflammation as it contributes to both diseases and is specific in obesity, diabetes, hypertension and dyslipidaemia (74). Due to the high mortality rates of these diseases, it is paramount to unify preventive programmes to control and eliminate these risk factors and thus reduce risks of cancer and CVD.

Although smoking presents an unequivocal negative risk factor, certain independent studies have suggested that it may be a protective mechanism against the development of EC (9,78). The present study did not confirm association between smoking and EC; although the number of smokers in the different diagnostic groups was significantly different (P=0.040), the identical smoking rate of 34.6% was recorded in the control group and hyperplasia group; with 30.8% incidence in the patients diagnosed with cancer.

In conclusion, to the best of our knowledge, the present study is the first report discriminating EC from hyperplasia and normal tissue using the AUC and to analyse *KLF4* and *HS3ST2* methylation cut-off points. The CpG methylation model revealed perfect discrimination accuracy between the control samples and other diagnoses. The AUC value was marginally higher when clinical variables, including BMI and age, were included. The aberrant CpG1 dinucleotide methylation level in the *HS3ST2* gene regulation sequence was determined to be an important predictor in hyperplasia formation; similar to the *KLF4* regulation sequence CpG2 dinucleotide effect in EC prediction.

The present study also confirmed the prominent role of BMI and other metabolic risk factors in EC formation. As these factors are important also in CVDs, this study sample is considered at high risk in the terms of morbidity and mortality rates for the two most common causes of mortality, CVD and cancer. Therefore, the implantation of effective and mutual preventive programs is required.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PŽ, DB, DD and ZD conceived the study; DD, DB, ZD and VH performed laboratory analyses; MŇ, RF and TB were responsible for the collection of samples and clinicopathological data; ZD and MG performed statistical analyses, MG designed the figures; ZD, DB and DD designed and wrote the paper in consultation with PK, JH, EH and PŽ.

Ethics approval and consent to participate

The study was approved by The Regional Ethics Committee of the Jessenius Faculty of Medicine (code 169/2011, 1933/2016) and the research was performed in compliance with the Declaration of Helsinki. Informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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