Urinary measurement of epigenetic DNA modifications and 8-oxodG as a possible noninvasive markers of colon cancer evolution.

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Supplementary Materials

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Table S1. Correlation coefficients between the levels of DNA epigenetic modifications in urine - **control group**. Statistically significant correlations are indicated in red.

	5-hmdU	**5-mdC**	**5-hmCyt**	**5-fCyt**	**5-hmdC**	**8-oxodG**	**5-hmUra**
5-hmdU	R=1.0000 N=41 p= ---	R=-0.2364 N=31 p=0.200	R=0.5701 N=40 p<0.001	R=0.3642 N=41 p=0.019	R=0.0566 N=41 p=0.725	R=0.2600 N=41 p=0.101	R=0.2117 N=41 p=0.184
5-mdC	R=-0.2364 N=31 p=0.200	R=1.0000 N=31 p= ---	R=-0.2872 N=30 p=0.124	R=-0.2262 N=31 p=0.221	R=0.6698 N=31 p<0.001	R=0.1199 N=31 p=0.521	R=-0.0947 N=31 p=0.612
5-hmCyt	R=0.5701 N=40 p<0.001	R=-0.2872 N=30 p=0.124	R=1.0000 N=40 p= ---	R=0.3055 N=40 p=0.055	R=0.0326 N=40 p=0.842	R=0.2567 N=40 p=0.110	R=0.2309 N=40 p=0.152
5-fCyt	R=0.3642 N=41 p=0.019	R=-0.2262 N=31 p=0.221	R=0.3055 N=40 p=0.055	R=1.0000 N=41 p= ---	R=-0.0742 N=41 p=0.645	R=0.3079 N=41 p=0.050	R=0.3031 N=41 p=0.054
5-hmdC	R=0.0566 N=41 p=0.725	R=0.6698 N=31 p<0.001	R=0.0326 N=40 p=0.842	R=-0.0742 N=41 p=0.645	R=1.0000 N=41 p= ---	R=-0.0229 N=41 p=0.887	R=-0.0088 N=41 p=0.956
8-oxodG	R=0.2600 N=41 p=0.101	R=0.1199 N=31 p=0.521	R=0.2567 N=40 p=0.110	R=0.3079 N=41 p=0.050	R=-0.0229 N=41 p=0.887	R=1.0000 N=41 p= ---	R=0.3916 N=41 p=0.011
5-hmUra	R=0.2117 N=41 p=0.184	R=-0.0947 N=31 p=0.612	R=0.2309 N=40 p=0.152	R=0.3031 N=41 p=0.054	R=-0.0088 N=41 p=0.956	R=0.3916 N=41 p=0.011	R=1.0000 N=41 p= ---

Table S2.Correlation coefficients between the levels of DNA epigenetic modifications in urine - **inflammatory bowel disease group** (IBD).

	5-hmdU	**5-mdC**	**5-hmCyt**	**5-fCyt**	**5-hmdC**	**8-oxodG**	**5-hmUra**
5-hmdU	R=1.0000 N=38 p= ---	R=0.2532 N=21 p=0.268	R=0.6967 N=38 p<0.001	R=0.2508 N=35 p=0.146	R=0.5771 N=36 p<0.001	R=0.5006 N=38 p=0.001	R=0.4016 N=38 p=0.012
5-mdC	R=0.2532 N=21 p=0.268	R=1.0000 N=21 p= ---	R=0.1854 N=21 p=0.421	R=-0.0463 N=19 p=0.851	R=0.7745 N=21 p<0.001	R=0.1038 N=21 p=0.654	R=0.4011 N=21 p=0.072
5-hmCyt	R=0.6967 N=38 p<0.001	R=0.1854 N=21 p=0.421	R=1.0000 N=38 p= ---	R=0.3181 N=35 p=0.063	R=0.5124 N=36 p=0.001	R=0.4022 N=38 p=0.012	R=0.4596 N=38 p=0.004
5-fCyt	R=0.2508 N=35 p=0.146	R=-0.0463 N=19 p=0.851	R=0.3181 N=35 p=0.063	R=1.0000 N=35 p= ---	R=0.1442 N=34 p=0.416	R=0.0987 N=35 p=0.573	R=0.1337 N=35 p=0.444
5-hmdC	R=0.5771 N=36 p<0.001	R=0.7745 N=21 p<0.001	R=0.5124 N=36 p=0.001	R=0.1442 N=34 p=0.416	R=1.0000 N=36 p= ---	R=0.3146 N=36 p=0.062	R=0.5621 N=36 p<0.001
8-oxodG	R=0.5006 N=38 p=0.001	R=0.1038 N=21 p=0.654	R=0.4022 N=38 p=0.012	R=0.0987 N=35 p=0.573	R=0.3146 N=36 p=0.062	R=1.0000 N=38 p= ---	R=0.3713 N=38 p=0.022
5-hmUra	R=0.4016 N=38 p=0.012	R=0.4011 N=21 p=0.072	R=0.4596 N=38 p=0.004	R=0.1337 N=35 p=0.444	R=0.5621 N=36 p<0.001	R=0.3713 N=38 p=0.022	R=1.0000 N=38 p= ---

Table S3. Correlation coefficients between the levels of DNA epigenetic modifications in urine - **colorectal cancer patients**. Statistically significant correlations are indicated in red.

	5-hmdU	**5-mdC**	**5-hmCyt**	**5-fCyt**	**5-hmdC**	**8-oxodG**	**5-hmUra**
5-hmdU	R=1.0000 N=123 p= ---	R=0.3306 N=71 p=0.005	R=0.6651 N=119 p<0.001	R=0.1920 N=111 p=0.043	R=0.4375 N=114 p<0.001	R=0.1005 N=119 p=0.277	R=0.4055 N=118 p<0.001
5-mdC	R=0.3306 N=71 p=0.005	R=1.0000 N=72 p= ---	R=0.2763 N=68 p=0.023	R=0.1098 N=67 p=0.376	R=0.7105 N=68 p<0.001	R=-0.0005 N=68 p=0.996	R=0.3167 N=71 p=0.007
5-hmCyt	R=0.6651 N=119 p<0.001	R=0.2763 N=68 p=0.023	R=1.0000 N=119 p= ---	R=0.3846 N=107 p<0.001	R=0.3674 N=113 p<0.001	R=0.3657 N=118 p<0.001	R=0.4715 N=115 p<0.001
5-fCyt	R=0.1920 N=111 p=0.043	R=0.1098 N=67 p=0.376	R=0.3846 N=107 p=0.001	R=1.0000 N=112 p= ---	R=0.0195 N=102 p=0.845	R=0.2264 N=107 p=0.019	R=0.1676 N=108 p=0.083
5-hmdC	R=0.4375 N=114 p<0.001	R=0.7105 N=68 p<0.001	R=0.3674 N=113 p<0.001	R=0.0195 N=102 p=0.845	R=1.0000 N=114 p= ---	R=0.1042 N=113 p=0.272	R=0.3511 N=110 p<0.001
8-oxodG	R=0.1005 N=119 p=0.277	R=-0.0005 N=68 p=0.996	R=0.3657 N=118 p<0.001	R=0.2264 N=107 p=0.019	R=0.1042 N=113 p=0.272	R=1.0000 N=119 p= ---	R=0.2026 N=115 p=0.030
5-hmUra	R=0.4055 N=118 p<0.001	R=0.3167 N=71 p=0.007	R=0.4715 N=115 p<0.001	R=0.1676 N=108 p=0.083	R=0.3511 N=110 p<0.001	R=0.2026 N=115 p=0.030	R=1.0000 N=122 p= ---

Table S4.Correlation coefficients between the levels of DNA epigenetic modifications in urine - **precancerous patients (adenoma)**.

	5-hmdU	**5-mdC**	**5-hmCyt**	**5-fCyt**	**5-hmdC**	**8-oxodG**	**5-hmUra**
5-hmdU	R=1.0000 N=44 p= ---	R=0.3000 N=31 p=0.101	R=0.6702 N=44 p<0.001	R=0.2160 N=41 p=0.175	R=0.4703 N=41 p=0.002	R=0.2699 N=44 p=0.076	R=0.3790 N=44 p=0.011
5-mdC	R=0.3000 N=31 p=0.101	R=1.0000 N=31 p= ---	R=0.0368 N=31 p=0.844	R=0.1185 N=29 p=0.540	R=0.8273 N=31 p<0.001	R=0.1121 N=31 p=0.548	R=0.3614 N=31 p=0.046
5-hmCyt	R=0.6702 N=44 p<0.001	R=0.0368 N=31 p=0.844	R=1.0000 N=44 p= ---	R=0.3514 N=41 p=0.024	R=0.4026 N=41 p=0.009	R=0.4082 N=44 p=0.006	R=0.4632 N=44 p=0.002
5-fCyt	R=0.2160 N=41 p=0.175	R=0.1185 N=29 p=0.540	R=0.3514 N=41 p=0.024	R=1.0000 N=41 p= ---	R=0.1747 N=38 p=0.294	R=0.2329 N=41 p=0.143	R=0.3513 N=41 p=0.024
5-hmdC	R=0.4703 N=41 p=0.002	R=0.8273 N=31 p<0.001	R=0.4026 N=41 p=0.009	R=0.1747 N=38 p=0.294	R=1.0000 N=41 p= ---	R=0.2986 N=41 p=0.058	R=0.3595 N=41 p=0.021
8-oxodG	R=0.2699 N=44 p=0.076	R=0.1121 N=31 p=0.548	R=0.4082 N=44 p=0.006	R=0.2329 N=41 p=0.143	R=0.2986 N=41 p=0.058	R=1.0000 N=44 p= ---	R=0.3835 N=44 p=0.010
5-hmUra	R=0.3790 N=44 p=0.011	R=0.3614 N=31 p=0.046	R=0.4632 N=44 p=0.002	R=0.3513 N=41 p=0.024	R=0.3595 N=41 p=0.021	R=0.3835 N=44 p=0.010	R=1.0000 N=44 p= ---

Table S5.Correlation coefficients between the levels of DNA epigenetic modifications in urine for all analyzed cases.

	5-hmdU	5-mdC	5-hmCyt	5-fCyt	5-hmdC	8-oxodG	5-hmUra
5-hmdU	R=1.0000 N=248 p= ---	R =0.2568 N=156 p=0.001	R=0.6616 N=243 p<0.001	R=0.2044 N=230 p=0.002	R=0.4254 N=234 p<0.001	R=0.2232 N=244 p<0.001	R=0.3612 N=243 p<0.001
5-mdC	R=0.2568 N=156 p=0.001	R =1.0000 N=157 p= ---	R=0.1502 N=152 p=0.065	R=0.0191 N=148 p=0.818	R=0.7331 N=153 p<0.001	R=0.0596 N=153 p=0.464	R=0.2881 N=156 p<0.001
5-hmCyt	R=0.6616 N=243 p<0.001	R=0.1502 N=152 p=0.065	R=1.0000 N=243 p= ---	R=0.3299 N=225 p<0.001	R=0.3713 N=232 p<0.001	R=0.3834 N=242 p<0.001	R=0.4180 N=239 p<0.001
5-fCyt	R=0.2044 N=230 p=0.002	R=0.0191 N=148 p=0.818	R=0.3299 N=225 P<0.001	R=1.0000 N=231 p= ---	R=0.0517 N=217 p=0.449	R=0.1871 N=226 p=0.005	R=0.1925 N=227 p=0.004
5-hmdC	R=0.4254 N=234 p<0.001	R=0.7331 N=153 p<0.001	R=0.3713 N=232 p<0.001	R=0.0517 N=217 p=0.449	R=1.0000 N=234 p= ---	R=0.1605 N=233 p=0.014	R=0.3280 N=230 p<0.001
8-oxodG	R=0.2232 N=244 p<0.001	R=0.0596 N=153 p=0.464	R=0.3834 N=242 p<0.001	R=0.1871 N=226 p=0.005	R=0.1605 N=233 p=0.014	R=1.0000 N=244 p= ---	R=0.2748 N=240 p<0.001
5-hmUra	R=0.3612 N=243 p<0.001	R=0.2881 N=156 P<0.001	R=0.4180 N=239 p<0.001	R=0.1925 N=227 p=0.004	R=0.3280 N=230 p<0.001	R=0.2748 N=240 p<0.001	R=1.0000 N=247 p= ---

Statistically significant correlations are indicated in red.

Figure S1. Selected correlations between the levels of DNA epigenetic modifications in urine – **control group**. Individual data points represent Box-Cox transformed values.

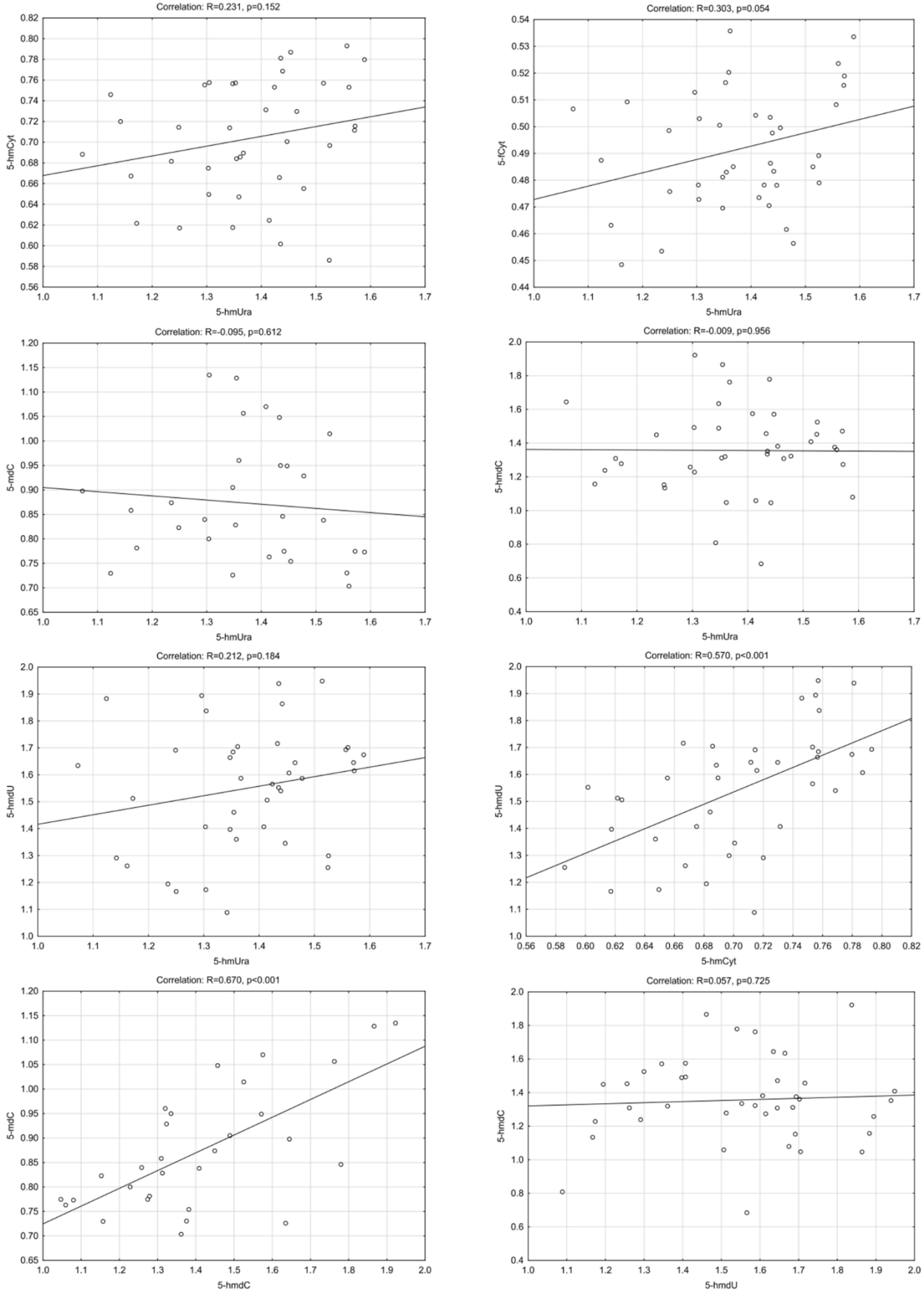
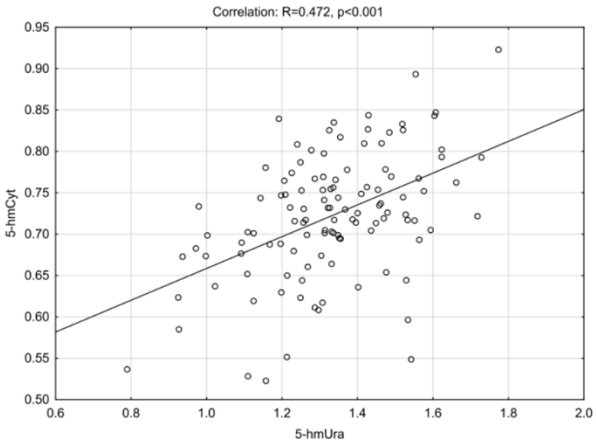
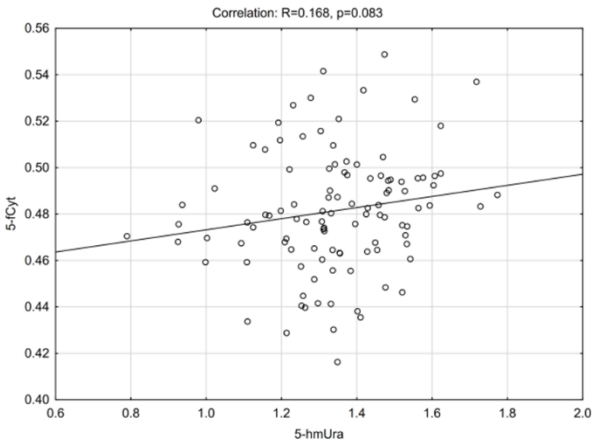
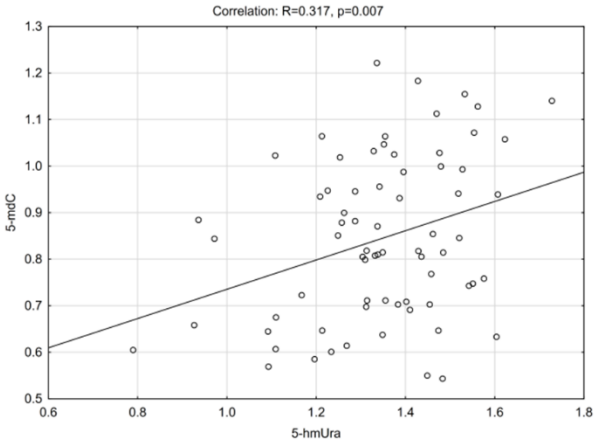
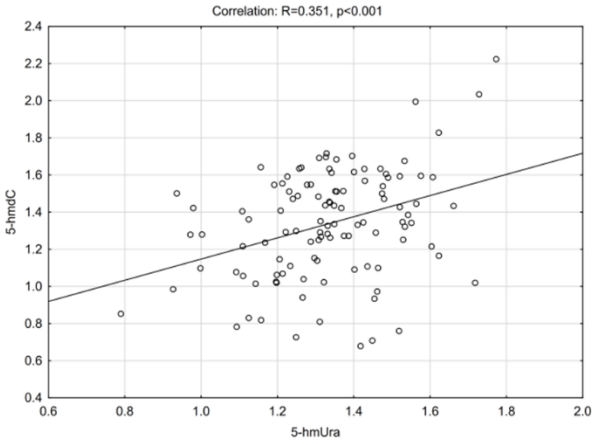


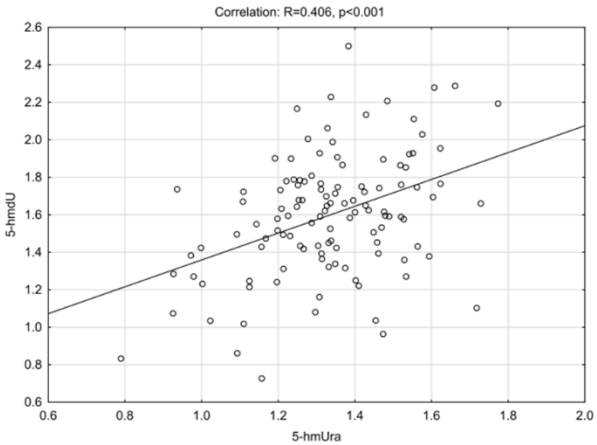
Figure S2. Selected correlations between the levels of DNA epigenetic modifications in urine – **colorectal cancer group**. Individual data points represent Box-Cox transformed values.

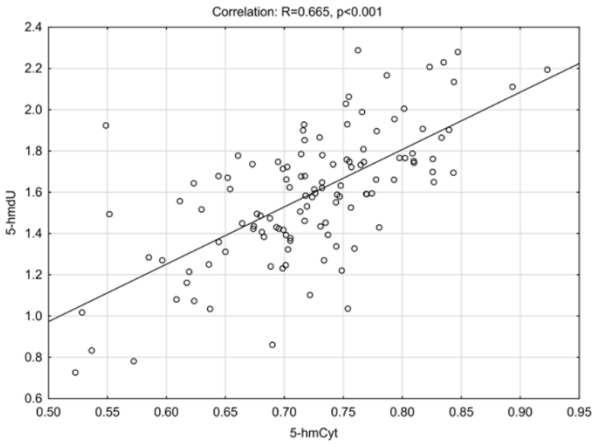


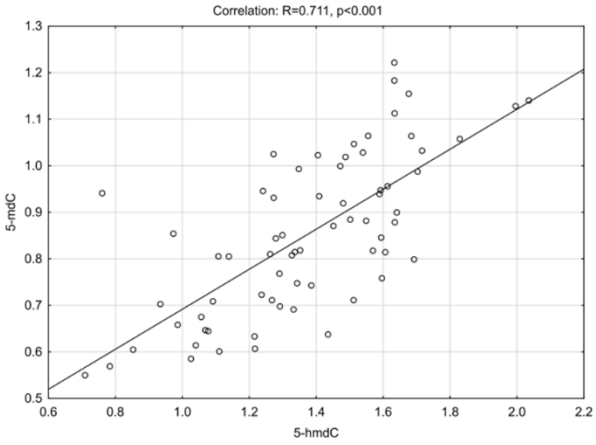












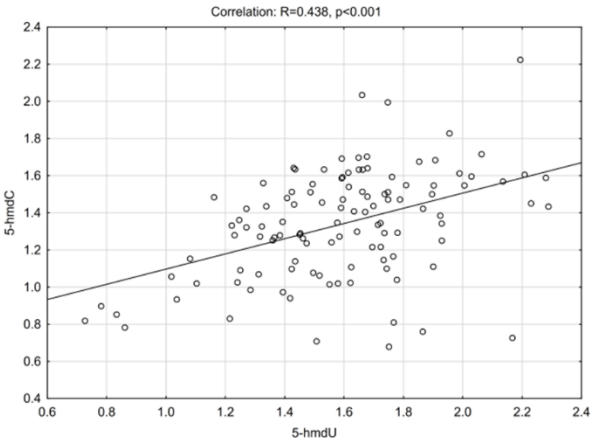


Figure S3. Selected correlations between the levels of DNA epigenetic modifications in urine – **precancerous patients (adenoma)**. Individual data points represent Box-Cox transformed values.

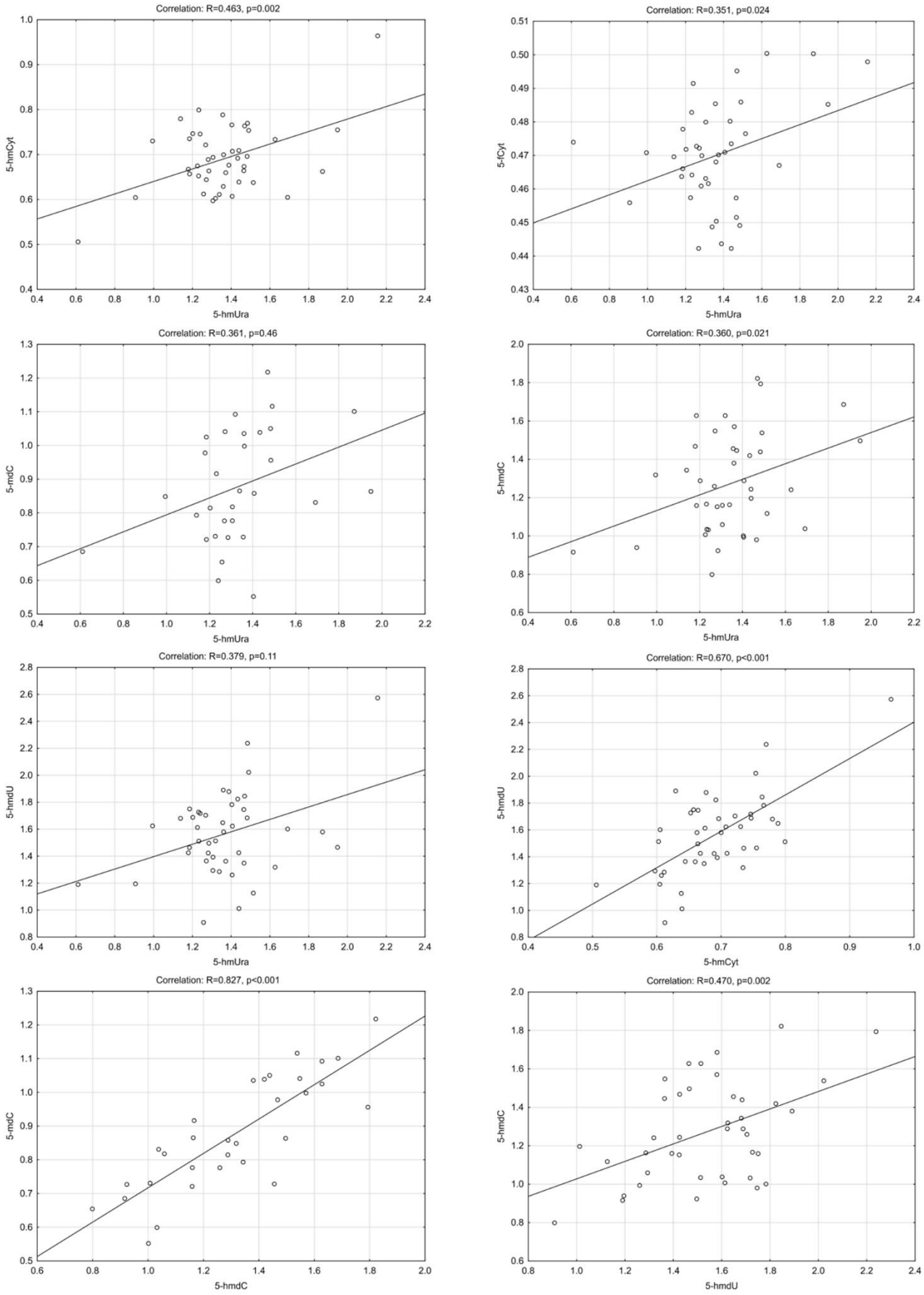
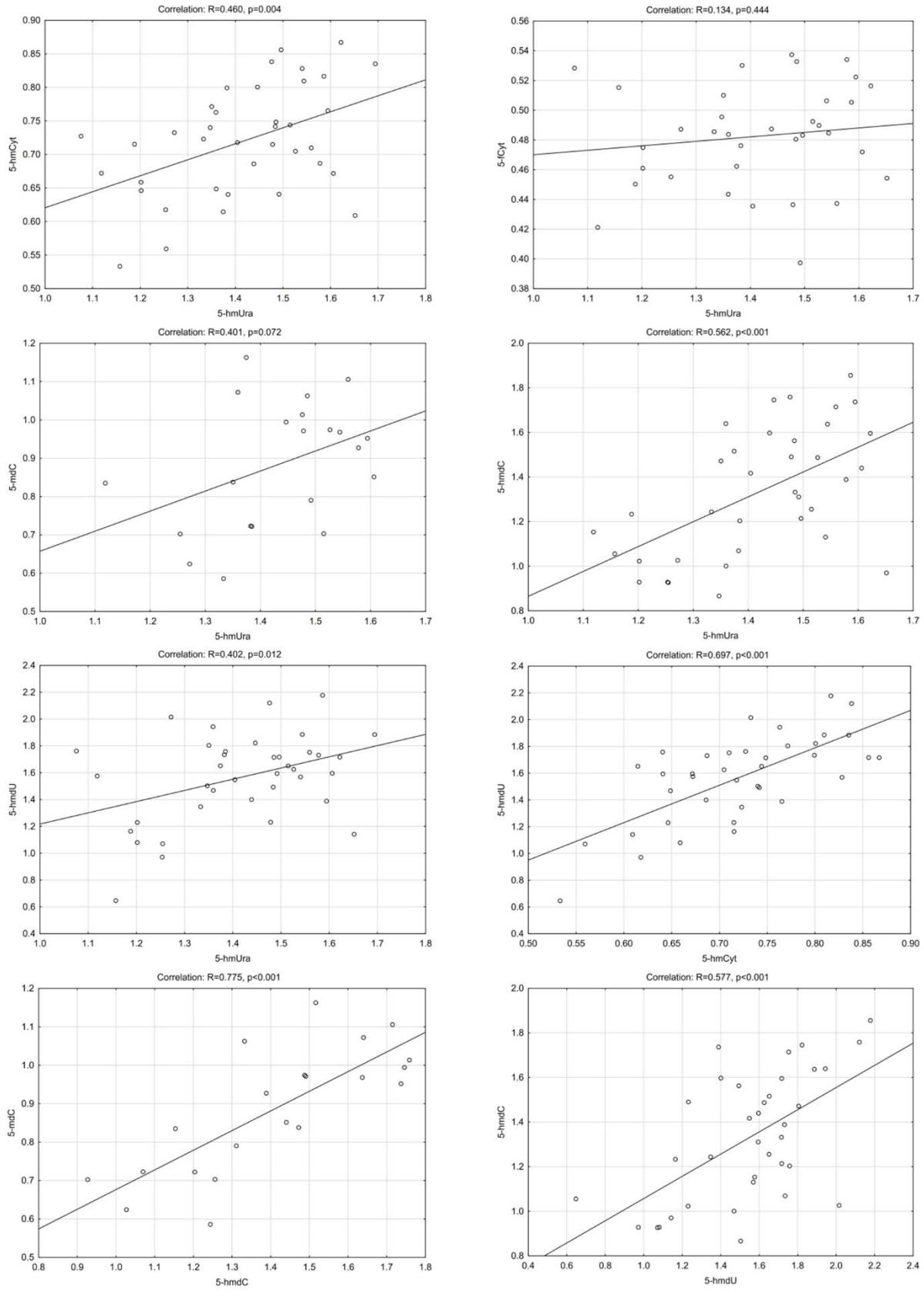


Figure S4. Selected correlations between the levels of DNA epigenetic modifications in urine - **inflammatory bowel disease group (IBD)**. Individual data points represent Box-Cox transformed values.



Methods

The determination of the epigenetic modifications and 8-oxodG levels in urine.[1]

Two-dimensional ultra-performance liquid chromatography with tandem mass spectrometry (2D UPLC–MS/MS) was used for the epigenetic modification analysis of urine samples (with the exception of 5-hmUra). Urine samples were spiked with a mixture of internal standards at a 4:1 volumetric ratio. The 2D-UPLC−MS/MS system consists of a gradient pump and autosampler for one-dimensional chromatography, and a gradient pump and tandem quadrupole mass spectrometer with a UNISPRAY ion source was used for two-dimensional chromatography. Both systems were coupled with a column manager equipped with two programmable column heaters and two 2-position 6-port switching valves. The at-column dilution technique was used between the first and the second dimensions to improve the retention in the trap/transfer column. The sample molecules were then adsorbed to the packing material as very narrow bands that could be eluted with well-resolved, small-volume peaks. A diluting stream of water (0.5 mL/min) was pumped with a Waters 515 isocratic pump and mixed with the first-dimension column effluent using a UPLC low-dead-volume tee valve. The following columns were used: CORTECS UPLC T3 Column (1.6 μm, 3 mm × 150 mm) with a CORTECS T3 VanGuard precolumn (1.6 μm, 2.1 mm × 5 mm) for the first dimension, a Waters ACQUITY UPLC CSH C18 (1.7 μm, 2.1 mm × 100 mm) for the second dimension, and a Waters XSelect CSH C18 column (3.5 μm, 3 mm × 20 mm) as the trap/transfer column. The chromatographic system was operated in heart-cutting mode, which means that selected portions of effluent from the first dimension were loaded onto the trap/transfer column by 6-port valve switching, which served as an “injector” for the second dimension of the chromatography system. Mass spectrometric detection was conducted with a Waters Xevo TQ-S tandem quadrupole mass spectrometer equipped with a UniSpray ionization source. The following common detection parameters were used: source temperature, 150°C; nitrogen desolvation gas flow, 1000 L/h; nitrogen cone gas flow, 150 L/h; desolvation temperature, 500°C; and nebulizer gas pressure, 7 bar. Collision-induced dissociation was obtained with argon (6.0 at 3 × 10-6 bar pressure) as a collision gas. The instrument response to all compounds was optimized by the infusion of 10 μM genuine compounds dissolved in water (10 μL/minute) in the mobile phase A stream via the mass spectrometer fluidics system operating in the “mixed” mode using MassLynx 4.1 software IntelliStart feature. The quantitative and qualitative transition patterns and the specific settings of the detector are summarized in Table S5. The chromatographic system was operated with MassLynx 4.1 software from Waters. Quantitative analyses were performed using the TargetLynx application. All samples were analyzed with three to six technical replicates. Due to the low sensitivity of the method used, the level of 5-hmUra was determined by high-performance liquid chromatography for pre-purification followed by gas chromatography with isotope dilution mass spectrometric detection (LC/GC–MS), as previously described [2].

Statistical analysis

The results are presented as median values, interquartile ranges and non-outlier ranges. Variables with a normal distribution were analyzed as “raw” data, while variables with non-normal distributions were subjected to Box–Cox transformation prior to statistical analyses based on parametric tests. The association between pairs of variables was analyzed based on Pearson correlation coefficients for raw or normalized data as applicable. All statistical transformations and analyses were carried out with Statistica 13.1 PL software [Dell Inc. (2016). Dell Statistica (data analysis software system), version 13. software.dell.com.]. The results were considered statistically significant at p values less than 0.05.

Supplementary References

1. Rozalski R, Gackowski D, Skalska-Bugala A, et al. The urinary excretion of epigenetically modified DNA as a marker of pediatric ALL status and chemotherapy response. Sci Rep. 2021;11(1):21345.
2. Rozalski R, Gackowski D, Siomek-Gorecka A, et al. Urinary 5-hydroxymethyluracil and 8-oxo-7,8-dihydroguanine as potential biomarkers in patients with colorectal cancer. Biomarkers. 2015;20(5):287-291.

Table S6. Transition patterns and specific detector settings for all compounds analyzed by 2D UPLC–MS/MS in urine.

compound name		ionization mode	nominal molecular mass (Da)	pseudo-molecular ion formulation	nominal parent ion (Da)	nominal daughter ion (Da)	capillary (kV)	cone (V)	collision (eV)
5-hydroxymethylcytosine	quantifier	UniSpray+	141	$[M+H]^+$	142	81	1.3	40	18
	qualifier	UniSpray+	141	$[M+H]^+$	142	124	1.3	40	12
[D_3]-5-hydroxymethylcytosine	quantifier	UniSpray+	144	$[(M+3)+H]^+$	145	84	1.3	40	18
	qualifier	UniSpray+	144	$[(M+3)+H]^+$	145	127	1.3	40	12
5-hydroxymethyl-2'-deoxycytidine	quantifier	UniSpray+	257	$[M+H]^+$	258	124	1.3	35	22
	qualifier	UniSpray+	257	$[M+H]^+$	258	142	1.3	35	10
[D_3]-5-hydroxymethyl-2'-deoxycytidine	quantifier	UniSpray+	260	$[(M+3)+H]^+$	261	127	1.3	35	22
	qualifier	UniSpray+	260	$[(M+3)+H]^+$	261	145	1.3	35	10
5-carboxylcytosine	quantifier	UniSpray-	155	$[M-H]^-$	154	67	1.3	60	12
	qualifier	UniSpray-	155	$[M-H]^-$	154	110	1.3	60	12
[$^{13}C_{10}$, $^{15}N_2$]-5-carboxylcytosine	quantifier	UniSpray-	162	$[(M+7)-H]^-$	161	71	1.3	60	12
	qualifier	UniSpray-	162	$[(M+7)-H]^-$	161	116	1.3	60	12
8-oxo-2'-deoxyguanosine	quantifier	UniSpray+	283	$[M+H]^+$	284	168	1.3	30	18
	qualifier	UniSpray+	283	$[M+H]^+$	284	140	1.3	30	18
[$^{15}N_5$]-8-oxo-2'-deoxyguanosine	quantifier	UniSpray+	288	(M+5)+H+	289	173	1.3	20	15
	qualifier	UniSpray+	288	(M+5)+H+	289	145	1.3	20	15
5-formylcytosine	quantifier	UniSpray+	139	$[M-H]^-$	140	97	1.3	40	15
	qualifier	UniSpray+	139	$[M-H]^-$	140	70	1.3	40	15
[$^{13}C_{10}$, $^{15}N_2$]-5-formylcytosine	quantifier	UniSpray+	146	$[(M+7)-H]^-$	147	102	1.3	40	15
	qualifier	UniSpray+	146	$[(M+7)-H]^-$	147	73	1.3	40	15
5-methyl-2'-deoxycytidine	quantifier	UniSpray+	241	$[M-H]^-$	242	126	1.3	30	12
	qualifier	UniSpray+	241	$[M-H]^-$	242	224	1.3	30	12
[$^{13}C_{10}$, $^{15}N_2$]-5-methyl2'-deoxycytidine	quantifier	UniSpray+	253	$[(M+12)-H]^-$	254	133	1.3	30	12
	qualifier	UniSpray+	241	$[(M+12)-H]^-$	254	236	1.3	30	12