Performance of DNA methylation analysis of *ASCL1, LHX8, ST6GALNAC5, GHSR, ZIC1* and *SST* for the triage of HPV-positive women: results from a Dutch primary HPV-based screening cohort.

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Supplementary Table 1. Original diagnosis versus revision diagnosis

		Revision diagnosis*					
		No dysplasia	CIN1	CIN2	CIN3	Cervical cancer	**Total**
Original diagnosis	No dysplasia	55	23	1	1	0	**80**
	CIN1	33	59	17	2	0	**111**
	CIN2	16	17	14	11	0	**58**
	CIN3	4	0	23	56	0	**83**
	Cervical cancer	0	0	0	0	3	**3**
	Total	**108**	**99**	**55**	**70**	**3**	**335**

*) revision diagnosis based on morphologic features combined with the interpretation of the p16INK4A and Ki-67 immunostainings, except for n = 57 based on morphologic features only.

ICC: 0.944 (95% CI 0.935-0.952)

Abbreviations: CIN1-3, cervical intraepithelial neoplasia grade 1-3;

Supplementary Table 2.

		Sensitivity			Specificity			PPV		NPV	
A	**Cytology**	**n/N**	**%**	**95% CI**	**n/N**	**%**	**95% CI**	**%**	**95% CI**	**%**	**95% CI**
	CIN3+	65/73	89.0%	(81.9-96.2%)	382/609	62.7%	(58.9-66.6%)	22.3%	(17.5-27.0%)	97.9%	(96.5-99.4%)
	CIN2+	111/128	86.7%	(80.0-92.6%)	373/554	67.3%	(63.4-71.2%)	38.0%	(32.4-43.6%)	95.6%	(93.6-97.7%)
B	**HPV16/18 genotyping**										
	CIN3+	46/64	71.9%	(60.9-82.9%)	369/555	66.5%	(62.6-70.4%)	19.8%	(14.7-25.0%)	95.3%	(93.3-97.4%)
	CIN2+	75/116	64.7%	(56.0-73.4%)	346/503	68.8%	(64.7-72.8%)	32.3%	(26.3-38.3%)	89.4%	(86.3-92.5%)
C	***ASCL1* / *LHX8* methylation***										
	CIN3+	57/73	78.1%	(68.6-87.6%)	455/609	74.7%	(71.3-78.2%)	27.0%	(21.0-33.0%)	96.6%	(95.0-98.2%)
	CIN2+	86/128	67.2%	(59.1-75.3%)	429/554	77.4%	(74.0-80.9%)	40.8%	(34.1-47.4%)	91.1%	(88.5-93.7%)
D	**Cytology + HPV16/18 genotyping**										
	CIN3+	61/64	95.3%	(90.1-100.5%)	240/555	43.2%	(39.1-47.4%)	16.2%	(12.5-19.9%)	98.8%	(97.4-100.2%)
	CIN2+	109/116	94.0%	(89.6-98.3%)	236/503	46.9%	(42.6-51.3%)	29.0%	(24.4-33.6%)	97.1%	(95.0-99.2%)
E	***ASCL1* / *LHX8* methylation* + HPV16/18 genotyping**										
	CIN3+	57/64	89.1%	(81.4-96.7%)	282/555	50.8%	(46.7-55.0%)	17.3%	(13.2-21.4%)	97.6%	(95.8-99.4%)
	CIN2+	96/116	82.8%	(75.9-89.6%)	269/503	53.5%	(49.1-57.8%)	29.1%	(24.2-34.0%)	93.1%	(90.2-96.0%)

* using a predefined threshold (70% specificity)

Abbreviations: CI, confidence interval; CIN, cervical intraepithelil neoplasia; n, number of; N, group size; NPV, negative predicted value; PPV, positive predicted value

Supplementary Table 3.

			Sensitivity			Specificity			PPV		NPV		Relative risk	
A		***ASCL1* / *LHX8* methylation***	**n/N**	**%**	**95% CI**	**n/N**	**%**	**95% CI**	**%**	**95% CI**	**%**	**95% CI**		**95% CI**
	CIN3+	NILM	5/8	62.5%	(29.0-96.0%)	294/382	77.0%	(72.7-81.2%)	5.4%	(0.8-10.0%)	99.0%	(97.9-100.1%)	5.32	(1.30-21.85)
		low-grade cytology	9/15	60.0%	(35.2-84.8%)	140/183	76.5%	(70.4-82.6%)	17.3%	(7.0-27.6%)	95.9%	(92.7-99.1%)	4.21	(1.58-11.26)
		high-grade cytology	43/50	86.0%	(76.4-95.6%)	21/44	47.7%	(33.0-62.5%)	65.2%	(53.7-76.6%)	75.0%	(59.0-91.0%)	2.61	(1.34-5.07)
	CIN2+	NILM	9/17	52.9%	(29.2-76.7%)	289/373	77.5%	(73.2-81.7%)	9.7%	(3.7-15.7%)	97.3%	(95.5-99.1%)	3.59	(1.43-9.05)
		low-grade cytology	17/38	44.7%	(28.9-60.5%)	125/160	78.1%	(71.7-84.5%)	32.7%	(19.9-45.4%)	85.6%	(79.9-91.3%)	2.27	(1.30-3.96)
		high-grade cytology	60/73	82.2%	(73.4-91.0%)	15/21	71.4%	(52.1-90.8%)	90.9%	(84.0-97.8%)	53.6%	(35.1-72.0%)	1.96	(1.31-2.94)
B		**HPV16/18 genotyping**												
	CIN3+	NILM	5/8	62.5%	(29.0-96.0%)	240/347	69.2%	(64.3-74.0%)	4.5%	(0.6-8.3%)	98.8%	(97.4-100.2%)	3.62	(0.88-14.87)
		low-grade cytology	8/15	53.3%	(28.1-78.6%)	111/168	66.1%	(58.9-73.2%)	12.3%	(4.3-20.3%)	94.1%	(89.8-98.3%)	2.07	(0.79-5.46)
		high-grade cytology	33/41	80.5%	(68.4-92.6%)	18/40	45.0%	(29.6-60.4%)	60.0%	(47.1-72.9%)	69.2%	(51.5-87.0%)	1.95	(1.05-3.61)
	CIN2+	NILM	10/17	58.8%	(35.4-82.2%)	236/338	69.8%	(64.9-74.7%)	8.9%	(3.6-14.2%)	97.1%	(95.0-99.2%)	0.63	(0.24-1.62)
		low-grade cytology	19/37	51.4%	(35.2-67.5%)	100/146	68.5%	(61.0-76.0%)	29.2%	(18.2-40.3%)	84.7%	(78.3-91.2%)	1.92	(1.08-3.39)
		high-grade cytology	46/62	74.2%	(63.3-85.1%)	10/19	52.6%	(30.2-75.1%)	83.6%	(73.9-93.4%)	38.5%	(19.8-57.2%)	1.36	(0.98-1.88)
C		**ASCL1 / LHX8 methylation* + HPV16/18 genotyping**												
	CIN3+	NILM	7/8	87.5%	(64.6-110.4%)	185/347	53.3%	(48.1-58.6%)	4.1%	(1.1-7.1%)	99.5%	(98.4-100.5%)	7.70	(0.96-61.97)
		low-grade cytology	12/15	80.0%	(59.8-100.2%)	87/168	51.8%	(44.2-59.3%)	12.9%	(6.1-19.7%)	96.7%	(93.0-100.4%)	3.87	(1.13-13.26)
		high-grade cytology	38/41	92.7%	(84.7-100.7%)	10/40	25.0%	(11.6-38.4%	55.9%	(44.1-67.7%)	76.9%	(54.0-99.8%)	0.72	(0.21-2.48)
	CIN2+	NILM	13/17	76.5%	(56.3-96.6%)	182/338	53.8%	(48.5-59.2%)	7.7%	(3.7-11.7%)	97.8%	(95.8-99.9%)	3.58	(1.19-10.76)
		low-grade cytology	26/37	70.3%	(55.5-85.0%	79/146	54.1%	(46.0-62.2%)	28.0%	(18.8-37.1%)	87.8%	(81.0-94.5%)	2.29	(1.20-4.35)
		high-grade cytology	57/62	91.9%	(85.2-98.7%)	8/19	42.1%	(19.9-64.3%)	83.8%	(75.1-92.6%)	61.5%	(35.1-88.0%)	2.18	(1.09-4.37)

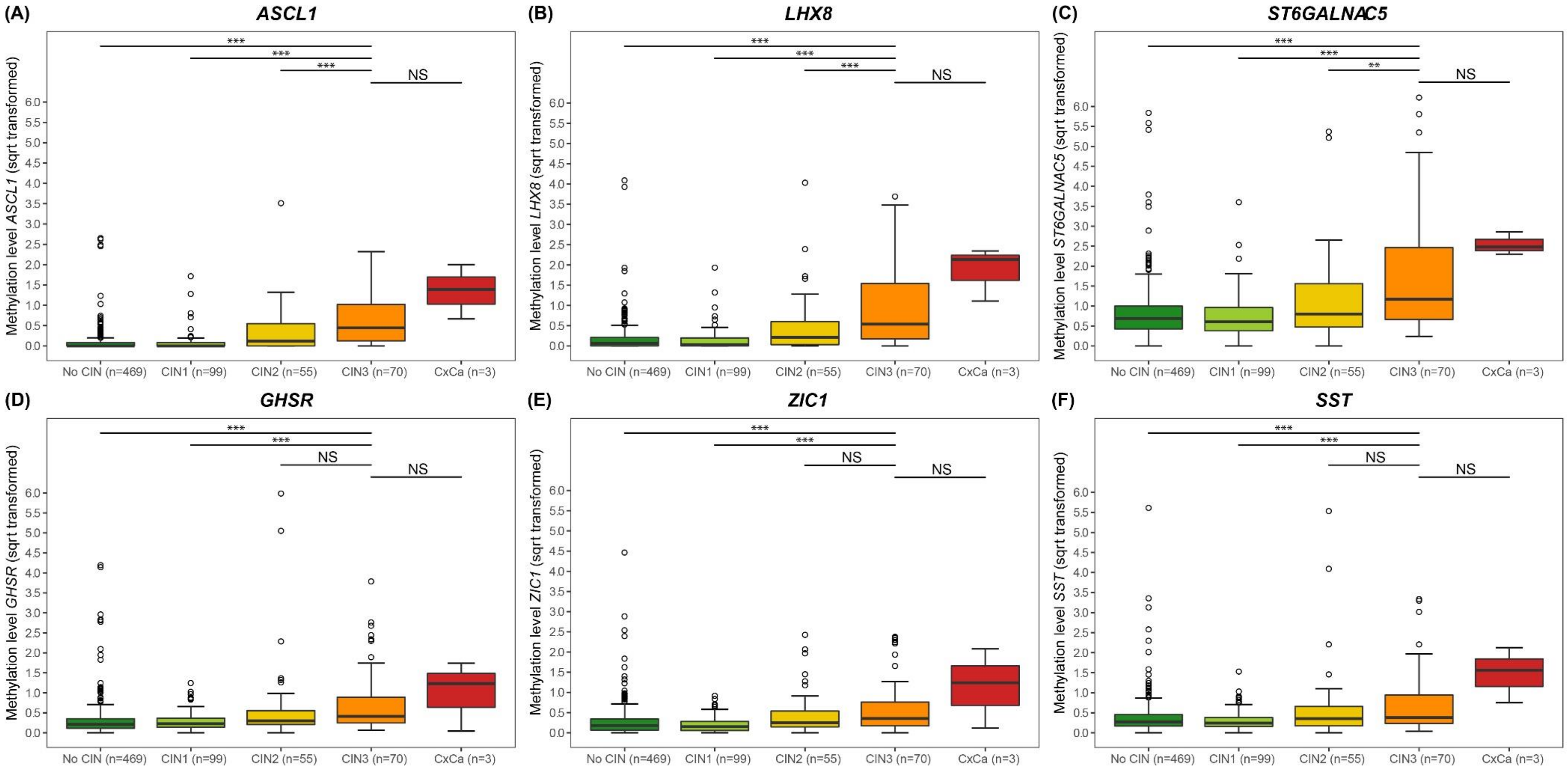
* using a predefined threshold (70% specificity)

Low-grade cytology: BMD equaling ASC-US/ASC-H/LSIL

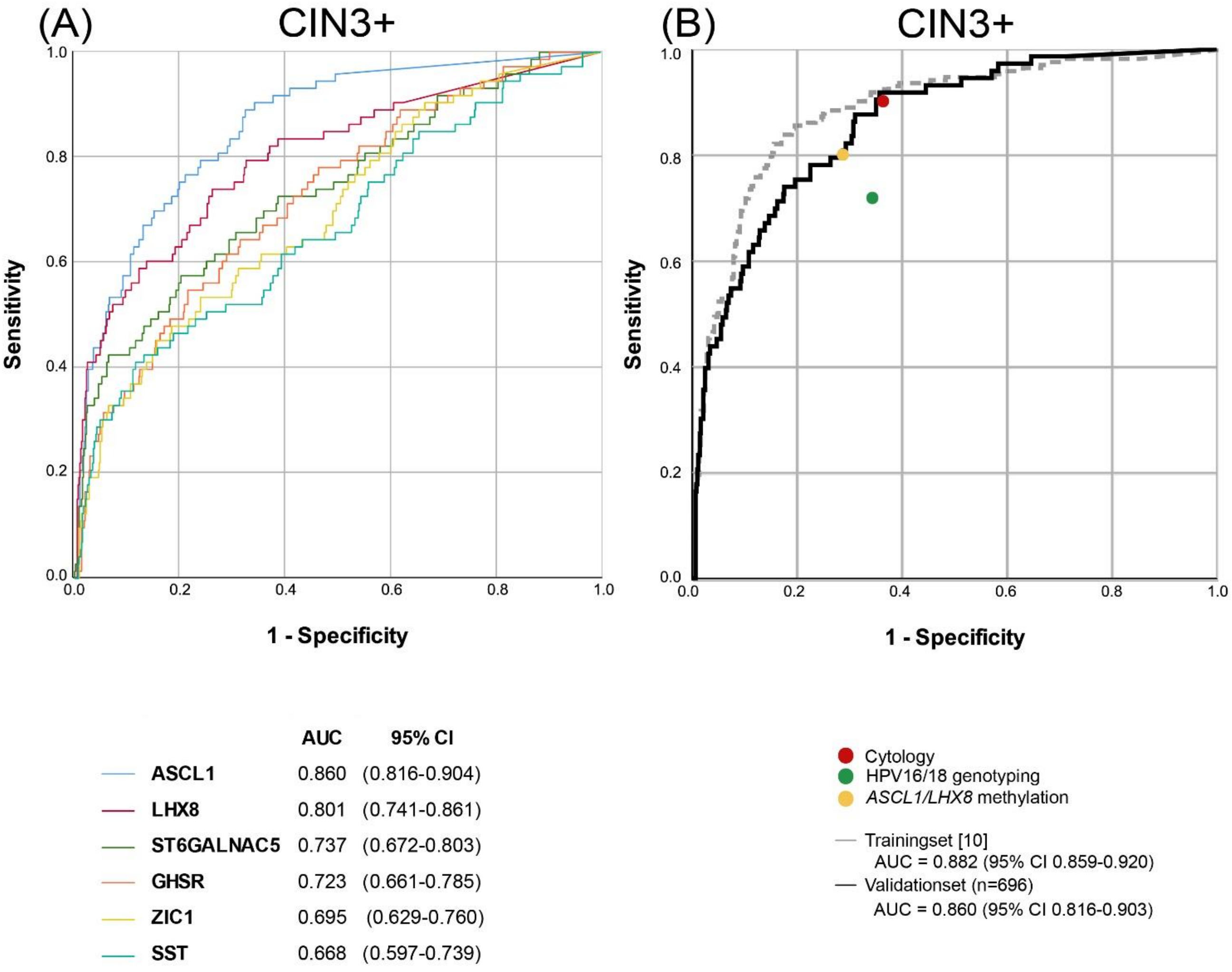
High-grade cytology: >BMD equaling HSIL

Abbreviations: ASCUS, atypical squamous cells of undetermined significance; ASC-H, atypical squamous cells that cannot exclude high-grade squamous intraepithelial lesions; CI, confidence interval; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; n, number of; N, group size; NILM, negative for intraepithelial lesion or malignancy; NPV, negative predicted value; PPV, positive predicted value

Supplementary Figure 1. Methylation levels increase with severity of underlying cervical disease – subgroup with revision diagnosis. DNA methylation levels of *ASCL1* (A), *LHX8* (B), *ST6GALNAC5* (C), *GHSR* (D), *ZIC1* (E) and *SST* (F) represented by the square-root transformed Cq-ratios (y-axis) in the different histology categories (x-axis). Differences between histological categories upon Kruskal-Wallis omnibus test, followed by post hoc testing using the Wilcoxon rank-sum test and Bonferroni multiple testing correction: *, p-value <0.05; **, p-value <0.01; ***, p-value <0.001; NS, not significant. ○ outlier sample. Abbreviations: CIN, cervical intraepithelial neoplasia; CxCa, cervical carcinoma; no CIN, women with no histology or normal histology.



Supplementary Figure 2. Diagnostic accuracy – subgroup with revision diagnosis. ROC curves and corresponding AUC for CIN3+ detection for (A) single marker classifiers *ASCL1, LHX8, ST6GALNAC5, GHSR, ZIC1, SST*; and (B) bi-marker panel *ASCL1/LHX8* (black line), including point estimates of cytology (red circle), HPV16/18 genotyping (green circle) and bi-marker panel *ASCL1/LHX8* with the predefined threshold calibrated at 70% clinical specificity (yellow circle). For comparison, the cross-validated ROC curve and corresponding AUC, as obtained in the hrHPV-positive training series is projected (grey line) [10]. Abbreviations: AUC, area under the curve; CIN, cervical intraepithelial neoplasia; ROC, receiver operating characteristic.



	AUC	95% CI
ASCL1	0.860	(0.816-0.904)
LHX8	0.801	(0.741-0.861)
ST6GALNAC5	0.737	(0.672-0.803)
GHSR	0.723	(0.661-0.785)
ZIC1	0.695	(0.629-0.760)
SST	0.668	(0.597-0.739)