Cytology based cancer screening has significantly reduced the incidence and mortality of cervical cancer. High-risk human papilloma viruses (HPV) are definite aetiological agents of almost all cervical carcinomas and HPV-testing further improves efficacy of primary screening compared to cytological screening (Ács et al. 2012) . The HPV test however is less specific than the cytology test. Great efforts have been made to identify and introduce novel biomarkers with the aim to improve the specificity of screening using different technologies (Benczik et al. 2013, Varga et al. 2016). Several genetic and epigenetic alterations have been described in cervical cancer, such as changes in microRNA pattern (Galamb et al. 2015). $P16^{INK4A}$ has been proposed as a biomarker in combination with Ki67 (CINtec® PLUS) for transforming HPV infection (Sobel et al. 2015). Previously, our group described increased expression of the tight junction (TJ) protein claudin1 (CLDN1) in premalignant and malignant cervical lesions. These findings are consistent with the fact that TJs are disassembled during tumorigenesis and that overexpressed claudins may have roles in motility, invasion and survival (Zinner et al. 2013). Based on previous facts, our project focused on the detection of CLDN1 in histological and cytological material so as to develop a diagnostic test, to investigate the expression of TJ proteins in stem/progenitor cervical reserve cells, and to analyse the changes in the expression of microRNAs in diffferent stages of cervical carcinogenesis.

Studies on the development of claudins during cervical carcinogenesis

In our earlier studies, we were the first to report the characteristic changes of ***claudins*** (CLDN) observable in the premalignant and malignant lesions of the cervix. Analysing histological samples, we confirmed the significantly increased expressions of **CLDN1** and 7 in the early stages of cervical dysplasia, which further increased with progression. The aim of our recent study was to demonstrate whether *increased CLDN1 expression could be observed* on the surface epithelium on *cytological* specimens, whether the enhanced reaction may be used as a *diagnostic tool* to screen for cancer and whether, apart from the portio epithelium, other cervical cells, particularly *stem cell/progenitor cell types*, display changes in claudin pattern as well.

(a) Claudin1 (CLDN1) expression in cytological and histological samples, compared with CINtec® PLUS reaction

For the preparation of specimens, the so-called liquid based cytology (LBC) method was used in parallel with traditional smears, and evaluations were done according to the Bethesda classification. Samples of considerably higher quality were achieved using the LBC method, for which the cytological and histological assistants enlisted for this project underwent an appropriate course (including both technique and evaluation). This allowed better evaluation of immunohistochemical reactions. The obtained results were presented at several congresses (Sobel et al. and Szekerczés et al. abstracts, 2015) and have also been published as scientific papers (Benczik et al. 2013, Benczik et al. 2015, with two further papers currently under preparation).

First, CLDN1 immunohistochemical reaction was performed on the LBC preparations in parallel with the cytological evaluation and HPV typing. Analyses revealed sensitivity to be significantly high, whereas specificity proved to be low, therefore we seeked other options.

As an approach to increase specificity, combined immunohistochemical reactions of CLDN1, *EZH2 (histone methyl transferase) and Ki67 (proliferation marker)* were used and compared with the commercially available CINtec® PLUS reaction. Our studies revealed that Ki67 was a more reliable reaction, while EZH2 gave less consistent results in the cytology samples. During the combined use of the two reactions, peroxidase labelling and diaminobenzidine visualization (brown reaction) were accomplished for detection of CLDN1, and alkaline phosphatase as well as Fast Red chromogen (red) were used for nuclear Ki67 reaction. Cells were considered positive if CLDN1 on the cell membrane and Ki67 in the nucleus were expressed together. For comparison, the commercially available CINtec® PLUS reaction was used, which involved the double immunohistochemical reaction of $p16^{INK4a}$ and Ki67 (Appendix 1 Fig.1). CLDN1+Ki67 double immunohistochemical reaction was performed on LBC preparations in parallel with the CINtec® PLUS reaction, from which a few samples could not be evaluated due to technical reasons. At the beginning, the double immunohistochemical reaction was performed manually, later on we further developed the method for semi autoautomatic use..

Conventional cytological assessment was carried out in 687 cases and LBC evaluation was done in a total of 2844 samples. Table 1 shows the diagnosis of LBC samples.

Table 1. Diagnosis of LBC samples

Normal	CIN 1; ASCUS; LSIL	CIN 1-2	CIN 2	CIN 2-3	CIN 3	Carcinoma in situ	AGC	no evaluation	Summary
2370	377	32	25	10	7	2	1	20*	2844

Table 2 shows comparison of the reactions using the CINtec® PLUS (Sobel et al. 2015) and CLDN1+Ki67 tests in the LBC preparations of the same sample.

Table 2. Comparison of CINtec® PLUS and CLDN1+Ki67 reaction

CINtec® PLUS *(n =1 342)*	**CLDN1+Ki67** *(n=1342)*		
	positive *(n=186)*	**negative** *(n=922)*	**no evaluation** *(n=234)*
positive *(n=255)*	*149*	*56*	*50*
negative *(n=824)*	*21*	*765*	*38*
not done* *(n=263)*	16	101	146

* because of technical problem

In total **1342** smears could be evaluated from which **149 cases were found to be positive and 765 negative** by both methods. It is important to note that beside the 149 positive findings, CLDN1+Ki67 positive, but $p16^{INK4a}$+Ki67 negative reaction was observable in 21 cases, whereas conversely 56 such samples were detected. For statistical comparison, the CINtec® PLUS and CLDN1+Ki67 reaction based on the McNemar's test gave a p value of 0.058 (p value≤0,05). **Based on this result, there was no significant difference between the two molecular diagnostic tests.** Studying the concordance between the two methods, the Kappa coefficient value with a 95% confidence interval proved to be 0.747 (0.694-0.801), meaning good concordance. Standard error was 0.061, which shows the estimation of measurement accuracy. **Based on the assay values the two procedures show largely concurrent evaluations.** Hereinafter, we performed CINtec® PLUS and CLDN1+Ki67 double immunohistochemical reactions on 15 histological (conization) samples which showed

positive cytology and immunohistochemical results. The reactions gave identical results independent of the method used (Table 3).

Table 3. Results of cytology, CINtec® PLUS and CLDN1+Ki67 reactions on histological samples

		Histological diagnosis	LBC	CINtec® PLUS	CLDN1+Ki67
1.		CIN 2	CIN 2	+	+
2.		CIN 3	CIN 1-2	+	+
3.		CIN 3	ASC-H	+	+
4.		CERVICITIS CHRONICA, no dysplasia koilocytosis	CIN 1	-	-
5.		CIN 1	CIN 1-2	-	-
6.		CIN 3	ASCUS	+	+
7.		CIN 3	CIN 1-2	+	+
8.		CIN 3	CIN 2-3	+	+
9.		CIN 1 koilocytosis	CIN 1-2	+	+
10.		CIN 2	CIN 1-2	+	+
11.		CIN 3	CIN 2-3	+	+
12.		Carcinoma in situ	Squamous cell carcinoma	+	+
13.		CIN 3	CIN 2	+	+
14.		CIN 2	CIN 2	+	+
15.		CERVICITIS CHRONICA, atypia, no malignancy	Normal	-	-

Based on all the above, it can be concluded that in **both cytological and histological** samples, **CLDN1+Ki67 double immunohistochemical reaction** shows **sensitivity and specificity identical to** the commercially available tests. Summarizing our results, we were able to **develop the basis of a diagnostic test based on cellular biomarkers, using double labelling. Both the specificity and sensitivity of our CLDN1+Ki67 test are similar to the values obtained by the CINtec® PLUS test.** Compared with the cytological evaluation, the CLDN1+Ki67 test showed a specificity of 79.9 % and a sensitivity of 68.5 %, while specificity was 84.9% and sensitivity 64.9% with the CINtec® PLUS test. At present the reaction is being performed in a „semi-automated" manner, i.e. detection of CLDN1 is by means of an immunostainer, whereas Ki67 reaction is performed manually. It is expected that in the future, based on further studies we shall be able to develop the processability of samples farther and our test will be applicable as a supplement to cytological assessments and clarifications, or as a „triage" test for HPV molecular detection (Benczik et al. 2013,

Szekerczés et al. 2015, Benczik et al. 2016, Varga et al. 2016, Szekerczés et al. in preparation).

(b) Claudin1 expression characterizes human uterine cervical reserve cells *(Zinner et al. 2013)*

The function of endocervical reserve cells (RCs) is still not clearly defined. Epithelial cells are attached to each other by tight junctions, the dominant components of which are the claudins, expressions of which change in cancer, however, no data are available on the claudin pattern of RCs. Expressions of various claudins (1,2,3,4,7), occludin, cytokeratins 5/6, 7, p63 were analysed in 60 paraffin-embedded cervical samples, including cases of cervical intraepithelial neoplasia (CIN), and in normal samples. Immunohistochemical reactions were evaluated semiquantitatively and statistically. CLDN1 was expressed in reserve cells, suprabasal squamous epithelial cells and CIN, contrary to glandular and squamous basal cells, which were negative. CLDN1 expression was significantly higher in RCs and CIN than in parabasal cells. CLDN2 was positive in RCs, glandular cells as well as squamous basal cells and CIN, while parabasal cells were negative. CLDN4 and 7 were weakly positive, CLDN3 was negative in all cell types. Occludin was expressed in RCs, basal/parabasal cells and CIN, while glandular cells were negative. **This is a first report to describe the „intermediate" claudin pattern of RCs, demonstrating that it differs from both cervical glandular and squamous basal cells, but is similar to the strong CLDN1 expression detected during cervical carcinogenesis.**

Analysis of microRNA pattern in the different stages of cervical carcinogenesis

(Galamb et al. 2015, Szekerczés et al. 2017.)

The pattern of microRNAs is characteristic for the tissue and different cells and aberrant expression has been detected during carcinogenesis (Galamb et al. 2015). Several authors described altered expression of microRNAs in cervical cancer, however only few studies deal with the premalignant alterations in comparison with the surrounding normal epithelia. MicroRNAs (miRNA) are molecules that regulate gene expression at the posttranscriptional level and have been reported to be deregulated in cervical cancer and in precancerous cervical neoplasia. Thus, these molecules may fulfil a role in molecular characterization of cervical neoplasia. For this reason, our aim was to reveal miRNA expressional differences during cervical carcinogenesis.

First, we collected 34 samples from patients diagnosed with CIN1 (7), CIN2 (8) and CIN3 (19) from the Archives of the 2nd Department of Pathology of the Semmelweis University. For screening the expression of multiple miRNAs using TaqManArrays (TaqManArray Human MicroRNA Cards Set v2.0, Panel A and B, Life Technologies, USA) in precancerous cervical neoplasia samples and normal cervical tissues, only the CIN3 cases were selected, in which the molecular alterations are more characteristic. The results indicated the further examination of 9 miRNAs (Appendix 2).

Next, we selected 44 samples – pairs of precancerous cervical lesions (22) and corresponding normal tissues (22) – from patients diagnosed with CIN3 and determined the miRNA expression of miR-20b, -24, -26a, -29b, -99a, -100, -147, -212 and -515-3p along with RNU48 and U6 as the references using TaqMan MicroRNA Assays (Life Technologies of Thermo Fisher Scientific Inc.). The expression of miR-20b showed 2.4-times increase in the median values measured in CIN3 samples compared with normal tissues that proved to be statistically different ($p<0.0002$, Wilcoxon Matched Pairs Test). Additionally, miR-212 was elevated 1.6-times and miR-515 showed a 4-times reduction in CIN3 as compared with normal tissue; however these differences did not reach the set value for statistical significance ($p<0.06$ and $p<0.07$, respectively). When investigating miRNA expression only in the samples known to be HPV-positive at present, miR-20b showed 1.6-times elevation whereas miR-24 and miR-515 were 1.3- and 8-times reduced in CIN3 as compared with normal tissue, which differences proved to be statistically significant ($p<0.03$, $p<0.05$ and $p<0.02$, respectively) (*for details see Appendix 2*).

Our investigation joins the line of studies aimed to find molecular alterations being characteristic of precancerous cervical neoplasias that are comparable to or perform better than histological classification. Regardless of the limitation of the study (the small sample size), our data suggest that the three miRNAs (miR-20b, miR-24 and miR-515) found differently expressed in CIN3 samples may have a potential in regard to the characterization of cervical neoplasias: miR-20b is already a known altered miRNA not only in cervical cancer but also in precancerous cervical neoplasia [Li et al. Med Oncol 2015. 32:510, Cheung et al. Cell Cycle 2012. 11:2876], miR-24 has been found to be associated with $HPV16^{+}$-positivity as a most abundant miRNA. Yet, so far no data have been published on miR-515 regarding cervical neoplasias, which is a novelty of our study (*Szekerczés et al. manuscript in preparation*).