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Vaginal Secretion Epithelium Count as a Prognostic Indicator of High Abundance of Ureaplasmas in Women with a Normal Nugent Score

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Abstract

Genital tract ureaplasma infections are associated with numerous complications, ranging from inflammation, through infertility, to problematic pregnancy. In the course of ureaplasma infection, the risk of human papillomavirus infection increases. Diagnostic tests for ureaplasma infections are not always carried out, especially in women with the normal Nugent test results. The study attempts to check whether it is possible to find a prognostic indicator that could suggest a high abundance of ureaplasmas ($\geq 10^4$ CFU/ml) at the stage of the initial examination of vaginal discharge. Such a prognostic factor could qualify women for further tests to detect infections with these atypical bacteria. Six hundred twenty-seven white women with a score of 0–3 on the Nugent scale were tested, including 322 patients with a high abundance of ureaplasmas ($\geq 10^4$ CFU/ml) and 305 who tested negative for these bacteria. Ureaplasma infections were detected statistically significant in women who had few or no epithelial cells in the genital swab specimens compared to the results obtained for women with numerous or very numerous epithelial cells ($p < 0.001$). The risk of the high density of ureaplasmas was 38.7% higher with fewer or no epithelial cells than with high numbers. In patients aged 18–40 years with few or no epithelial cells, a high density of ureaplasmas ($\geq 10^4$ CFU/ml) was observed significantly more frequently ($p = 0.003$). Determining the number of epithelial cells in Gram-stained slides may be the prognostic indicator of ureaplasma infection. Testing for genital ureaplasma infection should be considered, especially in women of childbearing age (18–40 years), even if the Nugent test value is normal and pH ≤ 4.6.

K e y w o r d s: *Ureaplasma*, epithelial cells, Nugent scale, *Lactobacillus*, leukocytes, pH

Introduction

The primary method for studying the microorganisms of the lower genital tract in women is the Nugent method. It was considered the gold standard worldwide due to its low cost and ease of implementation (Schwebke et al. 1996). It was a routine test performed for every woman in Poland to initially assess the possible etiological factors of the observed genital tract symptoms. This method is based on the evaluation of Gram-stained preparations, so it only shows typical bacteria classified as Gram-positive or Gram-negative (Nugent et al. 1991). In its current form, the Nugent method does not show the presence of atypical bacteria lacking a cell wall, such as ureaplasmas. Ureaplasmas belong to the family *Mycoplasmataceae* and are the smallest and the simplest self-reproducing bacteria (Razin 1992). They are most commonly sexually transmitted and have been detected in both healthy and symptomatic people. These bacteria are believed to have low virulence and are part of the normal vaginal microbiota. Their pathogenicity has not been confirmed, and the research results on this problem are inconclusive. However, it is believed that their excessive multiplication ($\geq 10^4$ CFU/ml – considered an infection) may cause complications. Most often, ureaplasma infections are assigned a role in NGU (non-gonococcal urethritis) and male infertility (negative influence on sperm metabolism, mobility, and penetration into the ovum) (Taylor-Robinson 1986; Núñez-Calonge et al. 1998). Ascending

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infection with urinary tract ureaplasma may lead to cystitis and glomerulonephritis and nephritis with plaque deposition (McDonald et al. 1982; Dewan et al. 1997). Infection of the cervix may result in premature birth (Kundsin et al. 1996) and infections in neonates in whom respiratory symptoms are mainly observed. There are suggestions that chronic ureaplasma-induced cervicitis could damage the mucosal barrier and immune protection (Lv et al. 2019). Ureaplasmas causing inflammation of the cervix increase the production of free radicals, which damages the cervical epithelial barrier and reduces the immune clearance facilitating the penetration of other pathogens (Bıyık et al. 2020).

The aim of the study was to find potential prognostic factors indicating high number of ureaplasmas in women without clinical symptoms and with normal Nugent score. The analysis included indicators such as the number of epithelial cells, leukocytes, and lactobacilli, as well as pH measurement, which can be simultaneously assessed using the criteria of the Nugent method as it has an established place in the diagnosis of female genital dysfunction (Nugent et al. 1991; Taj et al. 2014). Such preliminary assessment could qualify patients for further research, including more expensive molecular diagnostics.

Experimental

Materials and Methods

Our analysis (No. KBET/1072.6120.191.2020; approved by the Bioethics Committee of the Jagiellonian University) had a retrospective character and covered 627 women who were examined in 2007–2014.

The authors decided not to use the term 'ureaplasma infection' in this study because the patients included in the analysis did not show clinical symptoms.

Cervical and vaginal swabs were taken from each woman using sterile polystyrene shafts with viscose swabs and placed in sterile transport tubes without a medium (Deltalab, Poland). Material from the genital tract was not collected during menstrual bleeding, from women undergoing antibiotic therapy, or within two weeks after the antibiotic therapy. Vaginal swabs were seeded for *Trichonema vaginalis*, yeast-like fungi of the genus *Candida*, *Neisseria,* streptococci, enterococci, Enterobacterales, and *Gardnerella vaginalis.* Only women with a high number of ureaplasmas ($\geq 10^4$ CFU/ml) were included. We excluded women with mycoplasma-ureaplasma co-infections and ureaplasma with all of the above. The presence of *T. vaginalis* was confirmed from direct preparations of the collected vaginal discharge. Aerobic bacteria were cultured on Columbia Agar with 5% sheep blood (Biocorp) incubated in aerobic conditions at 37°C for 24 hours. The same conditions were applied to Enterobacterales cultured on MacConkey agar (Biocorp) and enterococci cultured on Bile Esculin Azide LAB-agar (Biocorp). The presence of *G. vaginalis* was confirmed by the BD Gardnerella Selective Agar with 5% Human Blood (BD), whereas MRS Agar (Oxoid) was used for lactobacilli. *Neisseria gonorrhoeae* was detected on the Roiron agar incubated in 5% CO_2 atmospheric conditions for 24–48 hours. Schaedler medium (BD) with vitamin K and 5% sheep blood was used for strictly anaerobic bacteria. Anaerobic bacteria were cultured under strictly anaerobic conditions at 37°C for 48 hours. Clinical materials were incubated on Sabouraud agar (Biocorp) under aerobic conditions at 37°C for 24 hours to detect the genus *Candida* yeast-like fungi.

Specimens collected from the cervix wall were examined for the presence of atypical bacteria (*Mycoplasma hominis*, *Ureaplasma* spp., and *Chlamydia trachomatis*). The presence of *C. trachomatis* was confirmed by demonstrating intracytoplasmic inclusions in the bacteria grown in McCoy cells. Intracytoplasmic inclusions were detected after 48 hours by iodine staining. A Mycoplasma IST 2 kit was used for detection of mycoplasma and ureaplasma infections (BioMérieux). This test allows identification of genital mycoplasma within 48 hours and quantitative estimation of the number of bacteria ($\geq 10^4$ CFU/ml). The cervical secretions were transferred to the BioMérieux transport medium, which was then transferred to BioMérieux culture media, and liquid and solid PPLO medium according to the procedures described earlier by Hayflick (Hayflick 1965; Biernat-Sudolska et al. 2006).

The collected vaginal discharge allowed to concurrently prepare a smear on a slide and stain it with the Gram method for the Nugent method (Nugent et al. 1991). Then, the smears were evaluated by the Nugent scoring system and assigned scores of 0–10. The Nugent score for bacterial vaginosis (BV) is based on the total number of large Gram-positive rods (*Lactobacillus* morphotypes), the number of small Gram-variable and Gram-negative rods (*G. vaginalis*, *Bacteroides*, and *Prevotella* morphotypes), and curved Gram-negative rods (*Mobiluncus* morphotypes). Each morphotype was quantified per field, and a summed score was given. A score of 0–3 is representative of a normal microbiota, a score of 4–6 coresponds to disturbed or altered microbiota, and a score of 7–10 is consistent with BV microbiota. The Nugent score was evaluated by only one experienced microbiologist to prevent the inter-observer variation, using a magnification of 1,000× (an oil immersion). Two categories were adopted when assessing the number of leukocytes and epithelial cells: I – no/few (0–2), and II – numerous/very numerous ($3 \geq 4$) per slide field. When assessing the number of *Lactobacillus* morphotypes, the categories as presented

Table I
Control and study group patient characteristics considering patient age and pH value.

	Control group n = 305						Study group n = 322					
	Med	IQR	M	SD	Min	Max	Med	IQR	M	SD	Min	Max
Age	30.0	(27.0; 34.0)	30.8	6.1	18.0	63.0	31.0	(26.0; 35.0)	31.4	6.5	19.0	56.0
pH	4.6	(4.6; 4.9)	4.7	0.3	3.8	5.5	4.6	(4.6; 4.9)	4.7	0.3	4.0	5.6

Med – median, M – mean, SD – standard deviation, IQR – interquartile range, Min – minimum, Max – maximum

previously in the Nugent scoring were adopted, and there were: I – no (0), II – few (1–5), III – numerous (6–30), IV – very numerous (> 30) per field.

The pH of the vaginal discharge was assessed immediately after collection. It was measured with the PEHANON colored indicator strips (Macherey-Nagel GmbH & Co. KG) and ranged from 3.8 to 5.5. The values of ≤ 4.6 were considered correct.

The analysis covered adult white women who visited the Microbiological Diagnostics Laboratory of the Department of Microbiology of the Jagiellonian University Medical College. The women came for a prophylactic examination of the vaginal microbiota before a planned pregnancy (without subjective symptoms of infection in the genital tract) or due to symptoms such as vaginal discharge, irritation, and itching.

Specimens with a score of 0–3 on the Nugent scale were selected for our analysis. The control group (n = 305) included women who did not demonstrate the presence of infection with ureaplasma. The study group (n = 322) included women with the high number of ureaplasmas (≥ 10^4 CFU/ml). In both the control group and the study group to determine the influence of age on the assessed prognostic indicators, the subjects were divided into four age groups: I: 18–30 years; II: 31–40; III: 41–50; IV: over 50. In the study group, the number of patients in the 1st group was n = 160; II: n = 137; III: n = 20; IV: n = 5, and in the control group, respectively, I: n = 173; II: n = 110; III: n = 20; IV: n = 2.

Control and study group patient characteristics are presented in Table I. The age and pH values were expressed as the median and interquartile range (IQR). Intergroup differences were analyzed using Fisher's exact and Pearson's chi-square test. A one-way logistic regression model was used to estimate the risk of the high density of ureaplasmas (≥ 10^4 CFU/ml). Statistical analysis was performed using IBM SPSS Statistics 26. The significance level for all statistical tests was set at $p < 0.05$.

Results

To determine the risk of high abundance of ureaplasmas (≥ 10^4 CFU/ml) based on the presence of the prognostic indicators studied by us, we conducted analyses covering both a single indicator and several indicators in various combinations with one another.

The high density of ureaplasmas versus the pH value. A high number of ureaplasmas (≥ 10^4 CFU/ml) were observed in 53.6% of women with pH ≤ 4.6 and in 48.6% of women with pH > 4.6. These differences were not statistically significant ($p = 0.242$, Pearson's chi-square test).

The high abundance of ureaplasmas and the number of bacteria of the genus *Lactobacillus*. A high number of ureaplasmas (≥ 10^4 CFU/ml) occurred in 52% of women in whom *Lactobacillus* bacteria were numerous and very numerous. In contrast, those infections were found in 44% of women with few or any bacteria. These differences were not statistically significant ($p = 0.349$; Pearson's chi-square test).

The high density of ureaplasmas and the number of epithelial cells. In our studies, the high number of ureaplasmas (≥ 10^4 CFU/ml) was confirmed more often in women who had few or no epithelial cells in the specimens from the genital swabs. Compared to the results obtained for women with numerous or very numerous epithelial cells, these results were statistically significant (58.1% vs. 36.4%, $p < 0.001$, Pearson's chi-square test).

Using a one-way logistic regression model, it was shown that in women with the Nugent score 0–3 (n = 627), the risk of a high abundance of ureaplasmas (≥ 10^4 CFU/ml) is 38.7% higher with few / no epithelial cells than with numerous/very numerous epithelial cells (OR 1.38, CI 1.14–1.68, $p = 0.001$).

The high density of ureaplasmas versus the presence and number of leukocytes. In our study, the high number of ureaplasmas (≥ 10^4 CFU/ml) were observed in 52.8% of patients with low or no leukocytes in the vaginal smear and in 44.0% of women with numerous or very numerous leukocytes. There were no statistically significant differences between these groups ($p = 0.135$, Pearson's chi-square test). The comparison of the control group and the study group, taking into account individual prognostic indicators, is shown in Fig. 1.

Incidence of high abundance of ureaplasmas versus different combinations of prognostic indicators. The statistically significant results were obtained only in three cases when assessing the frequency of the high

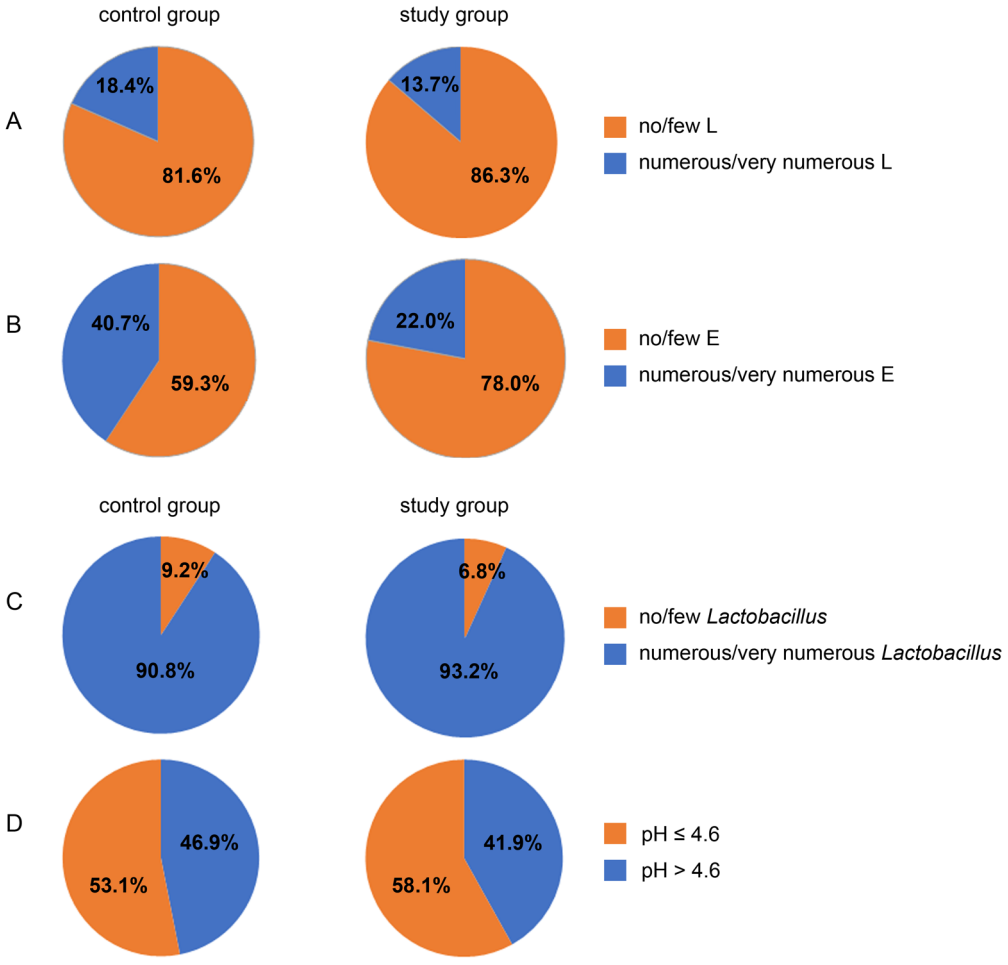

Fig. 1. Comparison between the control group and the study group, taking into account individual diagnostic indicator tested in the study: A) leukocytes – L, B) epithelial cells – E

number of ureaplasmas ($\geq 10^4$ CFU/ml), with various combinations of the analyzed indicators. Our analysis showed that high density of these atypical bacteria occurs significantly more often when i) leukocytes and epithelia were few or absent (57.8% vs. 32.2%, $p < 0.001$, Pearson's chi-square test); ii) in the presence of few/no epithelia and pH ≤ 4.6 (60.6% vs. 39.0%, $p < 0.001$, Pearson's chi-square test); iii) when there were no leukocytes and epithelial cells, or there were few of these cells, and the pH value was below 4.6 (60.6% vs. 39.5%, $p = 0.022$, Pearson's chi-square test). A comparison of the percentage of women in the control and study groups, taking into account the combined prognostic indicators for which statistical significance was determined, is presented in Fig. 2. Using a one-way logistic regression model, it was shown that the risk of high abundance of ureaplasmas ($\geq 10^4$ CFU/ml) is 37.0% higher in the case when, simultaneously, leukocytes and epithelia were almost absent or absent (n = 450; OR 1.37, CI 1.12–1.67, $p = 0.002$). Our analysis also showed that the risk of the high number of ureaplasmas is 53.9% higher in women with a pH value below 4.6 who have few or no epithelial cells (n = 364 OR 1.54, CI 1.20–1.97, $p = 0.001$). With the simultaneous presence of three indicators: scarce or absent leukocytes and epithelial cells and a pH ≤ 4.6, the risk of the high density of ureaplasmas ($\geq 10^4$ CFU/ml) is 54.1% higher (n = 287; OR 1.54, CI 1.19–1.98, $p = 0.001$), compared to when the pH value is above 4.6, and the epithelial cells and leukocytes are abundant.

Incidence of the high density of ureaplasmas versus age. In the age groups I and II, the high number of ureaplasmas were significantly more frequent in patients with few or no epithelial cells (respectively, 55.5% vs. 33.6%, $p < 0.001$ and 61.6% vs. 40.0%, $p = 0.003$, Pearson's chi-square test). In the case of other indicators, analyzed separately or in various combinations, no statistically significant differences were found in individual age groups.

The age-standardized logistic regression model showed that the risk of the high density of ureaplasmas was 121.3% higher (OR 2.21, CI 1.58–3.10, $p < 0.001$) in the presence of only a few or absence of epithelial cells. The risk of high abundance of ureaplasmas decreases with the patient's age, by 1.5% with each subsequent year (OR 0.98, CI 0.97–0.99, $p = 0.001$).

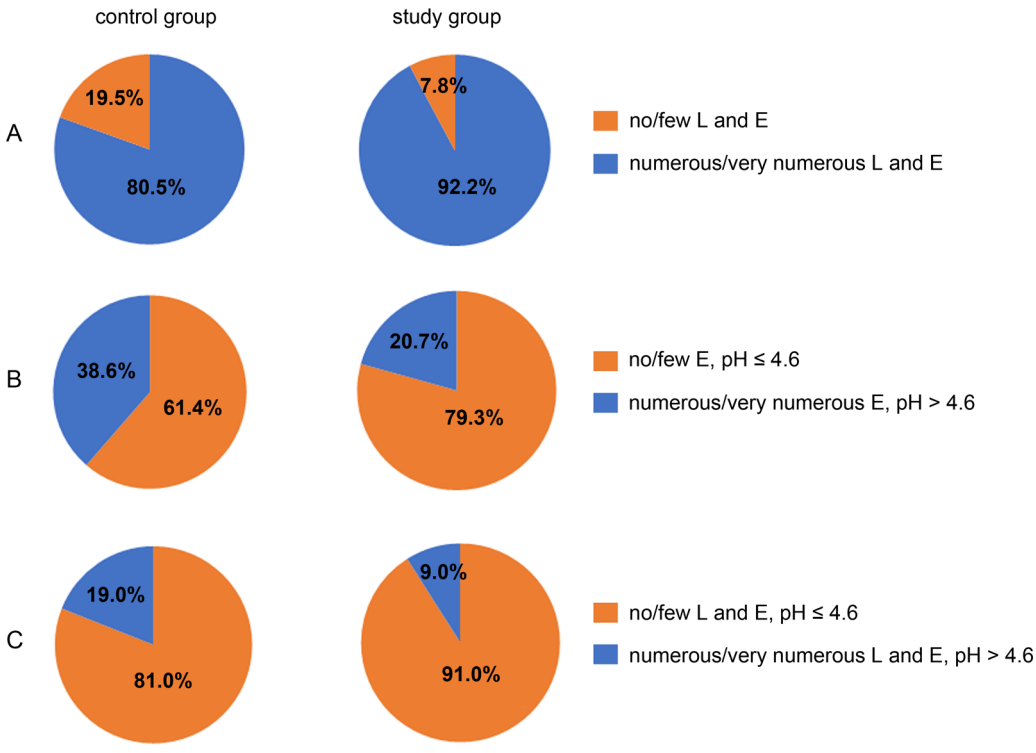

Fig. 2. Comparison of the control and study groups, taking into account the combination of the diagnostic indicators tested: A) leukocytes – L and epithelial cells – E, $p < 0.001$; B) epithelial

Discussion

Our many years of experience in the diagnosis of genital tract infections were the starting point for the initial assessment of the dependence of ureaplasma infections on the results of the Nugent scale. The results not presented in this study showed that ureaplasma infections were significantly more common in women with a score of 0–3 on the Nugent scale (n = 768, $p < 0.001$, Pearson's chi-square test). Our observations suggested that the results obtained with this method do not correlate with the high density of ureaplasmas. Therefore, our present research attempted to find prognostic indicators assessed in parallel with the commonly used Nugent method, which could indirectly indicate infection with these bacteria. The indicators selected for our analysis included the number of *Lactobacillus*, the number of epithelia and leukocytes, and the pH value in determining the risk of ureaplasma infection. Epithelial cells and leukocytes are visible on microbiological slides after Gram staining but are not assessed in the Nugent method. The diagnostic value of leukocytes as a prognostic indicator indirectly suggestive of ureaplasma infection was demonstrated by Okodo et al. (2017). These researchers, analyzing preparations made with the Papanicolaou technique, showed a statistically significant relationship between the *Ureaplasma urealyticum* infection and the presence of secondary changes in the cells of the cervical squamous epithelium called cannonballs. Out of the morphotypes assessed by the Nugent method, we chose the number of *Lactobacillus*, noting the considerable impact of this type of bacteria on the vaginal microbiota, which is clearly emphasized in the literature (Tachedjian et al. 2017; Witkin and Linhares 2017).

It has long been known that numerous antibacterial agents produced by lactobacilli, including bacteriocins, H_2O_2, or lactic acid, can inhibit the excessive multiplication of various pathogens. In our study, however, no dependence of the high number of ureaplasmas on the number of lactobacilli was observed. Some *Lactobacillus* species, regardless of their abundance, may be more active than others in keeping *Ureaplasma* spp. below the level considered being an infection. This, however, requires further research (Daniele et al. 2011).

Lactic acid, produced by *Lactobacillus*, plays a unique role in the vagina, favoring the domination of microorganisms with low pathogenic potential. Some *Lactobacillus* species produce only the D-isomer of lactic acid, which has a weaker protective effect (Amabebe and Anumba 2018). For example, *Lactobacillus iners* that dominate the vaginal microbiota is usually associated with dysbiosis. It appears to be less stable and more susceptible to alteration (since this species does not produce lactic acid). Similarly, *Lactobacillus jensenii* has weaker protective properties (it only produces D-lactic acid). On the other hand, *Lactobacillus crispatus* is beneficial as this species produces both D- and

L-lactic acid, associated with increased stability of the vaginal microbiota (developing dysbiosis is less probable), thus producing lower risk of BV. Perhaps routine determination of the number and species of *Lactobacillus* would be beneficial as it could indicate the risk of BV (Virtanen et al. 2019) and the possibility of various infections, including genital mycoplasmas.

Another indicator selected here was the number of epithelia. It is known that ureaplasmas strongly adhere to various cells, including epithelial cells (Razin 1992; 1999). *G. vaginalis* is a microorganism with strong adhesive properties. Its adherence to the epithelium is observed in Gram-stained preparations as characteristics indicator cells. To clearly assess the impact of high number of ureaplasmas ($\geq 10^4$ CFU/ml) on vaginal epithelial cells, the analysis did not include women infected with *G. vaginalis*. By adhering to the epithelium, ureaplasmas can damage the cells, and the irritating ammonia produced by them can influence the development of the inflammation. We expected that such actions could affect the number of exfoliated epithelial cells and leukocytes found in Gram-stained slides. The abundance of these cells could be a possible marker of an ongoing ureaplasma infection. In our analysis, a higher incidence of ureaplasma infections was observed only in the absence or with low numbers of epithelia. This relationship is also visible when the pH value is within the normal range. The beneficial effects of lactic acid on the cervix and vaginal epithelial cells (increased survival of vaginal epithelial cells, facilitated repair of damaged DNA) were demonstrated (Wagner et al. 2015; Amabebe and Anumba 2019). As a result, vaginal epithelial cells protected with lactic acid probably peel to a lesser extent, hence perhaps in Gram-stained preparations, their small number was observed with a large number of *Lactobacillus* bacteria.

Our analysis showed no dependence of high density of ureaplasmas ($\geq 10^4$ CFU/ml) on the number of leukocytes. Our observations regarding the number of leukocytes are in line with the previous literature reports. Lactic acid produced by lactobacilli supports antimicrobial defense through multiple mechanisms without causing immune-mediated inflammation. The vaginal microbiome dominated by *L. crispatus*, *L. gasseri*, and *L. jensenii* is potentially associated with a lower pro-inflammatory response (Smith and Ravel 2017; Witkin and Linhares 2017). This anti-inflammatory effect was previously observed in the presence of a large number of lactobacilli, especially at low pH, which enhances lactic acid activity (Hearps et al. 2017; Tachedjia et al. 2017; Amabebe and Anumba 2018). In a study of urinary tract infections, Moi et al. (2017) observed a weaker inflammatory response with ureaplasma than *C. trachomatis* and *M. genitalium*. Other researchers, such as Geisler et al. (2003), indicated that assessing the number of leukocytes in vaginal and cervical infections has a low positive and negative predictive value and moderate sensitivity and specificity. We also included pH measurements in our analysis, as it is known from the literature that the low pH of the vaginal discharge is most often associated with normal vaginal microbiota. Nevertheless, reports indicate that vaginal acidity may be more critical for the proper functioning of the vaginal mucosa than for inhibiting potential pathogens or regulating the normal microbiota. Measuring the pH alone in the screening of vaginal microbiota seems insufficient as it is known that *Candida* infections can occur at low pH values (Linhares et al. 2011). Different species of *Lactobacillus* lower the pH of the vaginal discharge to a varying extent, and in women with a score of 0–3 on the Nugent scale, a differentiation in pH was found, with an upward trend closer to the limit of the range (O'Hanlon et al. 2019). The products of ureaplasma metabolism alkalize the environment, which may neutralize the acidity resulting from the presence of *Lactobacillus*. With a huge population of *Lactobacillus* spp., the acidity of vaginal secretions is not always disturbed. There is no apparent shift of the measurement results towards alkaline values. As a result, this may have resulted in the pH values being within the normal range despite the ureaplasma infection. In our research, only the pH value combined with the determination of the number of epithelia and leukocytes gained a significant diagnostic significance.

The results obtained here turned out to be statistically significant in women of reproductive age, who are usually the most sexually active. Due to the prospect of motherhood, women from this group most often report for vaginal microbiota tests. Therefore, the assessment of epithelial cells would be particularly important for this group of women due to the consequences of ureaplasma vertical infection in their children. It would also be valuable due to the relationship between ureaplasma ($\geq 10^4$/ml) and possible damage to the epithelium in the genital system, which creates good conditions for the penetration of other sexually transmitted pathogens which have a negative impact on women health (Lv et al. 2019; Ye et al. 2018).

In our study, statistically significant results were obtained when the number of epithelia was analyzed as a single prognostic indicator as well as together with other analyzed indicators. Therefore, it seems to us that vaginal secretion epithelial cell count has the greatest importance and can be considered a prognostic indicator of the presence of high density of ureaplasmas.

The use of molecular methods that are currently widely available might have some impact on the results presented. However, in the years 2007–2014 in which the studies covered by our analysis were performed, the methods of bacterial cultivation on microbiological

media were routinely used in Poland. In those years, culture methods were considered the gold standard in microbiological diagnostics and, in many countries, they have been the most important until now. Our study only involved women from the white ethnic group, which of course, limits the possibility of extending the conclusions to the entire female population. A weakness of our work, resulting from its retrospective nature, was also the lack of data that could be obtained from the patients included in the analysis, concerning e.g., hygienic behavior, number of sexual partners, the possibility of using lubricants, hormonal contraception, or the day of the menstrual cycle on which the vaginal discharge was collected. Such data could have influenced the results of our analysis.

Conclusions

Our analysis showed that even the introduction of additional prognostic indicators expanding the Nugent scale did not allow the presence of the high number of ureaplasmas to be clearly demonstrated. The relatively best prognostic indicator of the ongoing high density of ureaplasmas ($\geq 10^4$ CFU/ml) in women with normal Nugent scores was the determination of the number of epithelial cells. Our analysis also shows that testing for genital ureaplasma should be considered, especially in women of childbearing age (18–40 years), even if the Nugent test value is normal and the pH is ≤ 4.6.

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Ethical statement

This study was approved by the Bioethics Committee of the Jagiellonian University (No. KBET/1072.6120.191.2020).

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Author contributions

Conceptualization, M. B-S.; Methodology, M. B-S. and K. T-Ć.; Formal Analysis, M. B-S., K. T-Ć. and P.G.; Investigation, P.G.; Resources, M. B-S.; Data Curation, M. B-S.; Writing – Original Draft Preparation, M. B-S. and K. T-Ć.; Writing – Review & Editing, M. B-S. and K. T-Ć.; Visualization, M. B-S. and K. T-Ć.; Supervision, M. B-S. and K. T-Ć.; Project Administration, K. T-Ć.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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