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Assessment of ergosterol in cervical mucus samples

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Zásady pro vypracování

Ergosterol is located in cell membranes of fungi and protozoa and its main role is to regulate membrane fluidity it has been used as a marker for fungi infection. The aim of the project is to assess ergosterol in the auto samples of women undergoing screening for HPV infection. Materials and methods. The auto samples are treated with methanol to extract unwanted substances from cell samples. The methanol will be mixed to chloroform, at a ratio 1:2 v/v, in order to extract the lipids. The lipids extracted will be identified by HPLC-MS. Ergosterol will be identified at a molecular mass of 396 using a standard curve (Sigma-Aldrich). Since ergosterol levels depend on the amount of fungi of the sample, 10 nM will of the standard be added to each sample in order to avoid a lack of detection of the sample. Alternatively, the lipid extraction of Candida cultures will be used to assess a relationship amount of fungi and ergosterol extracted. The screening will be performed to a minimum of 20 samples with detectable ergosterol (ratio fungi infection in women is usually around 10 %). The amount of ergosterol will be matched with the microbiological analysis of the cervical mucus and the incidence of HPV infection.

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Summary

Ergosterol is a sterol, which is considered the main component of the fungal cell membrane. It occurs in the pathogenic fungus *Candida albicans*. *Candida albicans,* after infection, occupies the vaginal cervix together with vaginal commensal microbiota, in which the most prevalent is *Lactobacillus* spp. When a fungal infection disrupts the vaginal microflora, then dysbiosis occurs. Dysbiosis of the vaginal environment causes the damage of the epithelial cells in the cervix; this event, in turn, leads to increased production of immune parameters as pro-inflammatory cytokines and antimicrobial proteins. Local inflammation may result in tissue damage and the enhancement of human papillomavirus oncogenic potential. Ergosterol's presence in human papillomavirus-positive or negative cervical mucus samples was assessed using the Folch lipid extraction method. Using the liquid chromatography-mass spectrometry, we measured the ergosterol in cervical mucus samples. We conclude that ergosterol may be an excellent biomarker to assess fungal infections.

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Súhrn

Ergosterol je sterol, ktorý sa považuje za hlavnú komponentu cytoplazmatickej membrány plesní. Vyskytuje sa v patogénnej plesni *Candida albicans*. *Candida albicans* po infekcii kolonizuje krčok maternice pošvy spolu s vaginálnou komenzálnou mikrobiotou, v ktorej najzastúpenejším je *Lactobacillus*. Ak je vaginálna mikroflóra narušená plesňovou infekciou, vznikne dysbióza. Dysbióza v prostredí pošvy spôsobuje poškodenie epiteliálnych buniek v krčku maternice, toto vedie vzápetí k zvýšenej produkcii imunitných parametrov ako pro-zápalové cytokininy a antimikrobiálne proteíny. Lokálny zápal môže vyústiť do poškodenia tkaniva a zlepšiť onkogenický potenciál ľudského papilomavírusu. Prítomnosť ergosterolu vo vzorkách cervikálneho hlienu, ktoré boli pozitívne alebo negatívne na ľudský papilomavirus, boli hodnotené využitím Folchovej metódy extrakcie lipidov. S použitím kvapalinovej chromatografie spojenou s hmotnostnou spektrometriou sme boli schopní zmerať ergosterol vo vzorkách cervikálneho hlienu. Z toho vyvdzujeme, že ergosterol môže byť excelentným biomarkerom pre hodnotenie pleňovej infekcie.

Kľúčové slová	ergosterol, HPLC-MS, mikrobiota, cervikálny hlien
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DECLARATION

I declare that this bachelor thesis was written independently with the help of my supervisor, prof. Juan Bautista De Sanctis, Ph.D., and using the sources listed in the references.

In Olomouc, ……………………… ………………………………

Samuel Herceg

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ABBREVIATIONS

AA	arachidonic acid
Acetyl-CoA	acetyl coenzyme A
ALA	alpha-linolenic acid
ALS	agglutinin-like sequence
Als3	agglutinin-like sequence 3
BV	bacterial vaginosis
CC	conventional cytology
CM	cervical mucus
CST	community state types
DHA	docosahexaenoic acid
EBC	exhaled breath condensate
Ece1	extent of cell elongation 1
EMMPRIN	extracellular matrix metalloproteinase inducer
EPA	eicosapentaenoic acid
Erg1p	squalene synthase
FRT	female reproductive tract
FT-ICR	Fourier transform ion cyclotron resonance
HIV	human immunodeficiency virus
HPLC-MS	High performance liquid chromatography-mass spectrometry
HPV	human papillomavirus
Hpw1	hyphal wall protein 1
Hsp90	heat shock protein 90
LBC	liquid-based cytology
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
MMP8	matrix metalloproteinase 8

MUC4	mucin4
MUC5	mucin 5
MUC6	mucin 6
PC	phosphatidylcholine
PCR	polymerase chain reaction
SAP	secreted aspartyl protease
Sap5	secreted aspartic protease 5
ToF	time of flight
VVC	vulvovaginal candidiasis

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1 INTRODUCTION

Ergosterol is the most abundant sterol in fungi. Its main function is to regulate permeability and membrane fluidity, and frequently it is used as a target of antifungal drugs (Georgiev, 2000). It is the main component of the fungal cell membrane, for example, in *Candida albicans* (Douglas and Konopka, 2014). *C. albicans* is a fungal pathogen of the genitourinary tract which competes with the normal vaginal microbiota (Bradford and Ravel, 2017, Achkar and Fries, 2010).

The vaginal microbiota is divided into community state types (Ravel *et al.*, 2011), mainly of *Lactobacillus* spp., which protect the vaginal environment against pathogenic microorganisms (Rogosa and Sharpe, 1960, Buchta, 2018). Fungi may cause vaginal dysbiosis by waning the vaginal microflora, leading to epithelial cell damage and, consequently, inflammation (Moschen *et al.*, 2016, Kyrgiou, *et al.*, 2016). Local inflammation might lead to an enhanced susceptibility to human papillomavirus (HPV) infection (Łaniewski *et al.* 2019, Castle *et al.,* 2001, Kumari and Bhor, 2021). Therefore, it may be relevant to use ergosterol as a biomarker of fungal infection (Lafi *et al.*, 2018).

2 AIMS OF THE THESIS

This thesis aims to detect fungal ergosterol in cervical mucus samples of women patients undergoing screening for HPV infection. The analysis of ergosterol in cervical mucus will allow us to ascertain fungi infection in samples of patients that have undergone analysis for HPV infection, which may be relevant to the pathology.

3 LITERATURE REVIEW

3.1 Membrane of fungi

The primary composition of the eukaryotic cell membrane is a lipid bilayer created by two layers of phospholipids, cholesterol and proteins immersed into the bilayer. The extracellular proteins are characterised by having carbohydrate moieties attached. Among the lipid moieties, phospholipids are considered to be the most present lipids in number in the plasmatic membrane. They consist of the hydrophilic polar head and nonpolar long fatty acids chains. The chains of fatty acids differ in the number of carbon molecules and the saturation of fatty acids tails, one of the chains contains one or more double bonds, which are vital for membrane fluidity (Alberts *et al*. 2008). The most abundant phospholipid in the majority of cells is phosphatidylcholine (PC), a choline phosphate connected to glycerol and two fatty acids, saturated (position 1) and unsaturated (position 2) (Alberts *et al.* 1998). The other common lipid moiety in the cell membrane is cholesterol.

Fungi cell membrane is enriched in phospholipids, glycerophospholipids, sphingolipids, glycolipids and sterols (van der Rest *et al.* 1995). Glycolipids have carbohydrates attached to the polar head and play a vital role in the assemblage of lipids into the bilayer. A significant number of sterols are located in the cell membrane, and they may decrease membrane fluidity by binding between two external phospholipids (Alberts *et al*. 1998, Tortora *et al*. 2019). Proteins located in the membrane are involved in the transport of molecules, cell signalling or enzymatic functions. Proteins situated in the membrane are divided according to their position into peripheral (located on the one side of the membrane) and transmembrane (pass through the whole membrane) proteins (Tortora *et al*. 2019).

3.1.1 Ergosterol

Ergosterol was first isolated from the sclerotia of *Claviceps purpurea* by Charles Tanret in 1889 (Vandamme, 1989). It is the most abundant sterol located in the fungal cell membrane. However, it might be found in the cytosol, where it plays a role in the distribution of substances in the cell and the extracellular vesicles of the fungal cell (Rodrigues *et al.*, 2007, Douglas and Konopka, 2014). Ergosterol also induces signal transduction in yeasts (regulation of protein kinase activity), and it regulates parasite growth, *Trypanosoma brucei*.

Ergosterol is derived from the lanosterol pathway. It differs from cholesterol in the presence of methyl group linked to C-24 carbon and two double-bonds between 7-8 and 22-23 carbons (Dohnal *et al*., 2009, Leaver, 2018, Haubrich *et al*., 2018). Its primary function is to regulate permeability and membrane fluidity. It is a target of antifungal drugs, inhibiting the ergosterol biosynthesis enzymes (Georgiev, 2000). Ergosterol activates the inflammasomes, and it can induce pyroptosis (Rodrigues, 2018). Ergosterol is the precursor of vitamin D_2, created by ionising ergosterol (Dohnal *et al*., 2009).

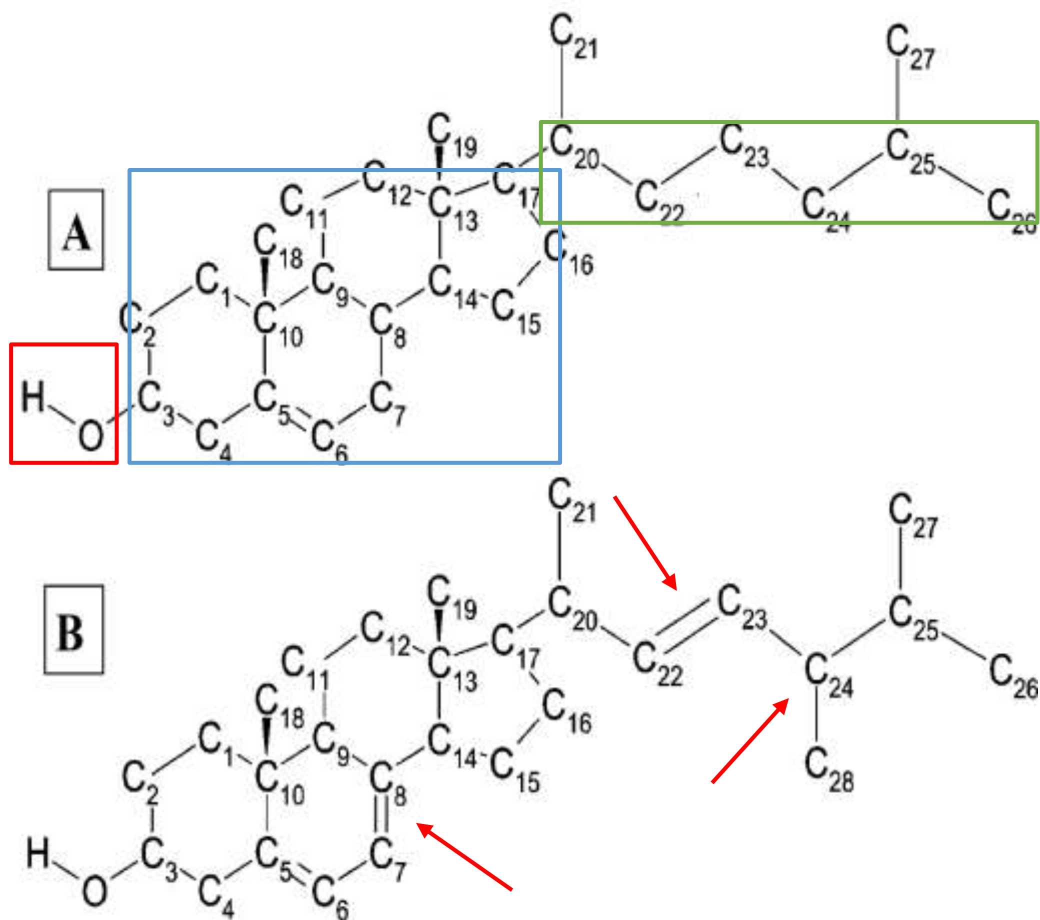

Figure 3. 1 Chemical structure of the fungal and mammalian sterols situated in plasmatic membrane **(A)** Cholesterol is a sterol synthesised by most animals, consisting of steroid ring – in a blue frame, the polar hydroxyl group on C-3 – in a red frame and isooctyl chain on C-17 – in a green frame (Nes, 2011). **(B)** Ergosterol is a fungal sterol with a similar structure to cholesterol, it differs in presence of methyl group on C-24, in double bonds between C-7 and C-8 and C-22 and C-23 – red arrows (Dohnal et al., 2009). (According to Cournia et al., 2007)

3.1.2 Metabolic pathways of ergosterol in *Candida Albicans*

Each main eukaryotic kingdom has its specific sterol-derived structure incorporated into the plasma membrane. In plants, it is sitosterol, in the plasma membrane of animal cholesterol and fungi, ergosterol. They share common early steps in their sterol biosynthetic pathways, starting with acetyl coenzyme A (Acetyl-CoA) until the formation of squalene epoxide (Bloch, 1983, Dupont *et al.,* 2012). Ergosterol biosynthesis includes approximately 30 enzymes called Erg proteins, codified by the transcription factor Upc2p in *C. albicans*. The pathway might be separated into three parts: mevalonate biosynthesis, farnesyl pyrophosphate biosynthesis and ergosterol biosynthesis (Silver *et al.* 2004, Hu *et al.*, 2017).

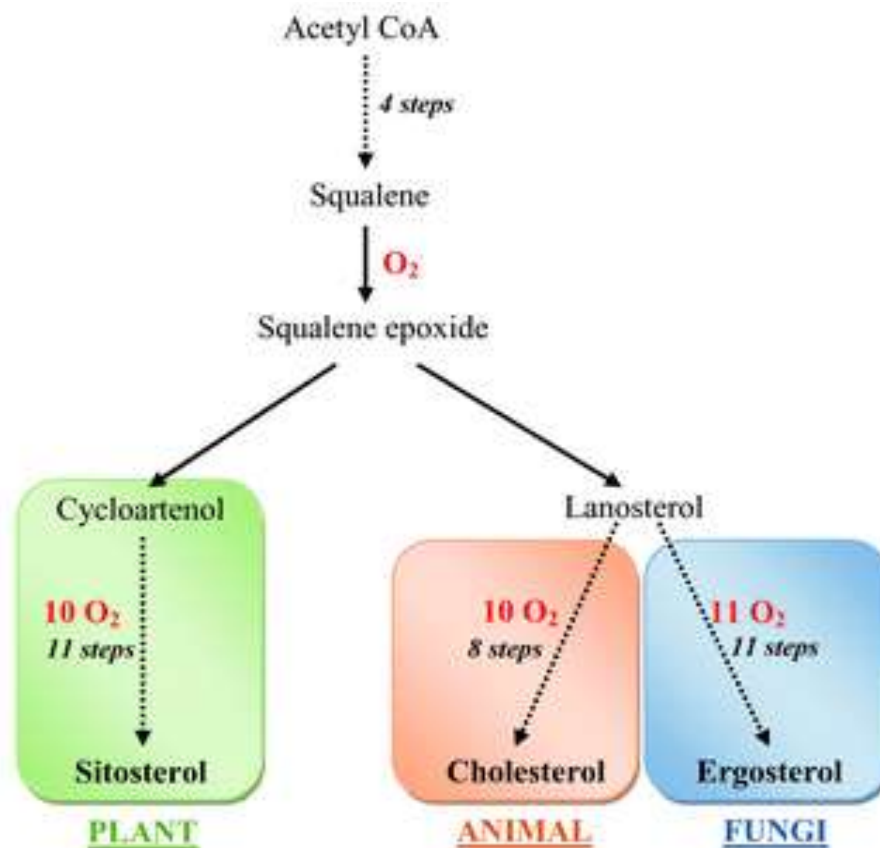

Figure 3. 2 The biosynthesis pathways of sterols. A simplified version of pathways, which as an initial compound, has an Acetyl-CoA. Erg enzymes subsequently modify Acetyl-CoA to squalene. The essential element for biosynthesis is oxygen (shown in red). The pathways separate after squalene epoxide is formed. The products cycloartenol (precursor of sitosterol) and lanosterol (precursor of cholesterol and ergosterol) are

formed. Finally, 11-steps of the pathway are required for ergosterol production (Galea and Brown, 2009, Dupont et al., 2012). (According to Dupont et al., 2012).

3.2 Vaginal microbiota

The vaginal microbiome is considered to be a unique microbiota (Buchta, 2018). Besides *Lactobacillus* spp., several anaerobes are encountered in vaginal mucus (Li *et al.*, 2012). *Lactobacillus* spp. largely colonises the vaginal mucosa under the influence of sex hormones, oestrogens mainly, which stimulate the production of glycogen from epithelial cells. *Lactobacillus* degrades glycogen to lactic acid, keeping a low pH level in the vagina. This low pH leads to the formation of a barrier for preventing the possible pathogens entering the lower female reproductive tract (FRT) (Rogosa and Sharpe, 1960, Buchta, 2018).

3.2.1 Cervical mucus and its production

Cervical mucus (CM) is produced by vaginal epithelial cells, serves as the lubricant of vaginal mucosa and prevents vagina, uterus and cervix from potential microbial infections (Causey, 2007, Adnane *et al*., 2018). Goblet cells are responsible for the production of CM, which is modified on the biochemical level according to mucus and menstrual cycle (Gipson, 2001, Han, 2017). The structure and viscosity of CM are highly dependent on hormone regulation; therefore, components of CM differ during ovulation, pregnancy and menstrual cycle. CM is composed of water, lipids, carbohydrates, cholesterol, inorganic ions, mucins MUC4, 5 and 6 involved in viscosity, proteoglycans, enzymes, immunoglobulins, lactoferrin, lysosome, immunoglobulin A, antimicrobial proteins and DNA, from dead leukocytes, epithelial cells and bacteria (Gipson *et al.*, 1999, Gipson, 2001, Flori *et al.*, 2007, De Tomasi *et al*., 2018). During ovulation, CM viscosity is influenced by oestrogen. It is more elastic, less viscous and less acidic, allowing sperms to penetrate through the cervical mucus (López-Gatius *et al.*, 1993, Gipson *et al.,* 1999, Gipson, 2001, Tsiligianni *et al.*, 2011). The crucial role of CM is blocking pathogenic bacteria and prevent pathogens from entering the lower FRT (female reproductive tract), which is more vulnerable to infections than upper FTR (De Tomasi *et al*., 2018).

3.2.2 Papanicolau test

The Pap smear test was invented in 1943 by George Papanicolau. It is a sensitive diagnostic tool used in gynaecology. With a special brush inserted into the cervix, cervical cells are collected from the transition zone. Further, analysis is performed by possible two mechanisms – liquid-based cytology (LBC) and conventional cytology (CC). The difference is that in liquid-based cytology, the brush is preserved with the sample in solution. In traditional cytology, the brush cannot be analysed together with the specimen. While performing LBC, the test piece is stored in an alcoholic solution. CC method directly transfers the sample from the brush onto the slide. The Papanicolau sampling serves as a diagnostic test of endometrial cancer and the presence of viral or bacterial infectious diseases, like human papillomavirus (HPV) or human immunodeficiency virus (HIV) (Mayer and Budh, 2020).

3.2.3 Normal vaginal microbiome

The female reproductive tract is divided into two regions – lower and upper FRT. The cervical transformation zone separates these two regions. Vagina and ectocervix, belong to lower FRT and are associated with high microbial abundance, unlike upper FRT (Bekhtereva and Dorosevich, 2009, Perez-Muñoz *et al.*, 2017). Ectocervix and endocervix have cells, which form the structure of epithelia. A single layer of columnar cells mainly composes upper FRT; on the other side squamous epithelial cells form lower FRT. The division between these two cell types is secured by squamocolumnar cells. Junctions between individual cells provide stability and integrity of barrier for pathogens. Between squamous epithelial cells, in lower FRT, are less tight junctions, which cause the permeability for pathogens; however, it might provide a transport space for immunoreactive molecules as dendric cells (DeTomasi *et al.* 2019).

Each region has a specific tissue structure and immune activity (Lee *et al.,* 2015). The vaginal environment is characterised by low pH (< 4,5), vaginal microbiota and vaginal epithelium (Buchta, 2018). The secretion of immunoreactive molecules (cytokines, chemokine factors), an exhibition of specialised immune properties, and consequently strengthening a physical barrier (Boomsma *et al.,* 2009, García-Velasco and Arici, 1999).

Vaginal epithelial cells and different *Lactobacillus* spp. produce L-lactic acid. However, the D-isoform of lactic acid is produced only by *Lactobacillus* such as *Lactobacillus cripspatus* and *Lactobacillus jensenii*. One of the functions of lactic acid is

to modulate the extracellular matrix metalloproteinase inducer (EMMPRIN). When EMMPRIN is downregulated, the matrix metalloproteinase 8 (MMP8) enzyme activity decreases and consequently, the consistency of the vaginal plug changes (Rahkonen *et al.,* 2009, Witkin *et al.*, 2013). The high presence of D-lactic acid may lead to better function of this plug (Witkin and Linhares, 2016). The essential part of lactic acid production is glycogen availability, which is degraded by α-amylase abundant in vaginal mucus; the products are maltose, maltotriose and α-dextrins. It was shown that α-amylase present in the vaginal fluid has no microbial origin (Spear *et al.*, 2014).

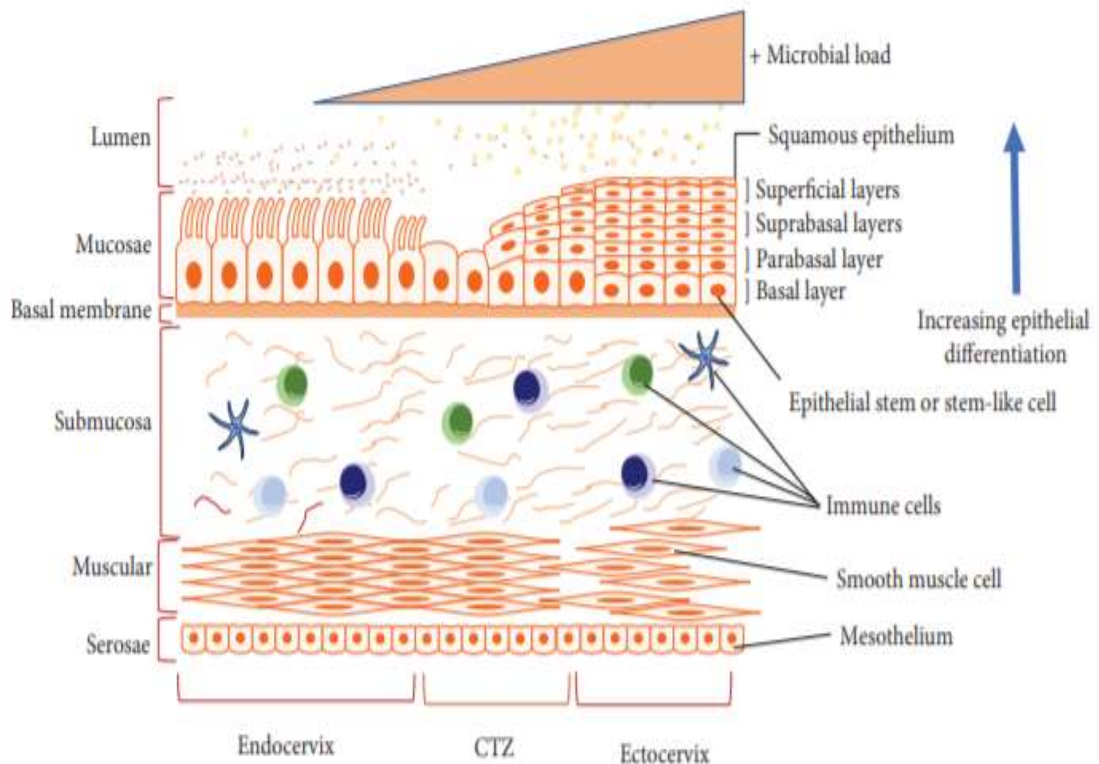

Figure 3. 3 The different cell composition in the endo and ectocervix and interaction with immune cells and microbacterial load (De Tomasi et al., 2018).

3.2.3.1 Five community state types group

The vaginal environment contains several bacterial species, not only *Lactobacillus* spp. All bacterial species in the vaginal environment are divided into community state types (CST); they have similar abundance and microbial phylotype. Each group consists of several species, which operate together. This CST helps us to understand how the vaginal compartment functions (Ravel *et al.*, 2011, Human

Microbiome Project Consortium, 2012, Van de Wijgert *et al.*, 2014). The microflora is slightly different during menopause, ovulation or pregnancy (Farage and Maibach, 2005).

With the usage of 16S rRNA (ribosomal ribonucleic acid) gene sequencing, bacterial microorganisms that occupy the FRT were quickly ascertain. In vaginal microbiota forms at least five types of CST. The prevalence of Lactobacillus spp. dominates four CST, namely CST-I, CST-II, CST-III and CST-V. CST-I is connected with *Lactobacillus crispatus*, CST-II with *Lactobacillus gasseri*, CST-III with *Lactobacillus iners,* and *Lactobacillus jensenii* dominates CST-V. CST-IV contains *Lactobacillus* spp. and the mixture of anaerobic bacteria, including *Gardnerella*, *Atopobium*, *Mobiluncus*, *Prevotella*, and *Dialister, Megasphaera*, *Peptoniphilus*, *Sneathia*, *Eggerthella*, *Aerococcus*, *Finegoldia* etc. Then, CST-IV is divided into two subtypes CST-IVA and CST-IVB, both associated with bacterial vaginosis BV-like (bacterial vaginosis) microbiota. CST-IVA is characterised by the presence of *L. inners* together with *Corynobaterium*, *Finegoldia* and *Streptococcus*. CST-IVB type is connected with *Atopobium*, *Prevotella*, *Parvimonas*, *Sneathia*, *Gardnerella*, *Mobiluncus* and *Peptoniphilus* (Ravel *et al*., 2011, Gajer *et al.*, 2012). Besides the presence of different microbial organisms, CST-IVA and IVB differ in Nugent scores (Ma and Li, 2017). Nugent scores is a gram staining scoring technique used to diagnose bacterial vaginosis (Nugent *et al.*, 1991). CST-IVB is associated mainly with high Nugent scores, whereas CST-IVA is connected with low Nugent scores (Ma and Li, 2017).

The vaginal microbiome differs among ethnic groups. In Asian and white women, the vagina is mainly occupied by *Lactobacillus* spp.; however, the prevalence of *Lactobacillus* spp. is statistically lower in women of Hispanic and Afro-American ethnicity. The differences can be caused by internal factors such as the composition or quantity of vaginal secretions, ligands on epithelial cells or external factors as personal hygiene, birth control, sexual behaviour etc. (Jacques Ravel *et al*., 2011).

Table 3. 1 The characterisation of vaginal microbiota – the community state types and bacteria, which are classified into particular CST from I, II, III and V mainly dominated by *Lactobacillus* spp. and group IV divided into CST-IVA and CST-IVB, including facultative anaerobic bacteria (Ravel et al., 2011, Gajer et al., 2012). (according to Buchta, 2018)

	Community state types (CST)					
	Group I	**Group II**	**Group III**	**Group IV-A**	**Group IV-B**	**Group V**
Bacteria	*L. crispatus*	*L. gasseri*	*L. iners*	*Anaerococcus, Corynebacterium, Finegoldia, Streptococcus*	*Atopobium, Prevotella, Paryimonas, Sneathia, Gardnerell, Mobiluncus, Peptoniphilus*	*L. jensenii*

3.3. Fungal diseases of the vagina and cervix

Candida albicans is considered to be a fungal pathogen of the genitourinary tract. Alongside bacteria, fungi frequently occupy the lower FRT and create a mycobiome – fungal species living in a specific environment (Bradford and Ravel, 2017, Achkar and Fries, 2010). *Candida* spp. is the most frequent fungal pathogen that causes infectious diseases in the genital and urinary tract. It is a dimorphic fungus because it can exist in yeast and mould (Achkar and Fries, 2010). Among vaginal mycobiota, *Candida* is one of the most common causes of vaginal infection. Vulvovaginal candidiasis (VVC) is the result of *Candida* spp. infection. *Candida albicans* is dominant and most abundant species among *Candida* spp., non-*C. albicans* species include *C. krussei, C. tropicalis, C. glabrata* etc. (Sobel, 1985 Bradford and Ravel, 2016). Some key factors essential for spreading Candida are environment, defects of the immune response, instabilities in epithelial integrity, and disharmony in the microbial environment (Iliev and Underhill, 2012). The dimorphic characteristic of *Candida* causes pathogenesis (Jacobsen *et al.*, 2013). Hyphal form adheres to epithelial cells and triggers a mucosal

invasion generating symptomatic manifestations (Kimura and Pearsall, 1980, Sobel *et al.*, 1981, Sobel, 1989. *Candida* prevalence is closely dependent on the abundance of *Lactobacillus* spp.; when there is a small number of *Lactobacillus*, the organism is more susceptible to an increase of VVC (Bradford and Ravel, 2017). Bacterial organisms negatively influence the formation of the fungal biofilm by inhibiting the growth and gene suppression (Park *et al.*, 2014). The genes that are upregulated during biofilm formation are hyphal wall protein 1 (Hwp1) and agglutinin-like sequence 3 (Als3) adhesins, and the extent of cell elongation 1 (Ece1) and secreted aspartic protease 5 (Sap5) connected with hyphal elongation. The transcription of these genes is significantly down-regulated when *Candida* is co-cultivated with living bacteria (Birse *et al.*, 1993, Staab *et al.*, 1999, Hoyer, 2001, Naglik *et al.*, 2003, Park *et al.,* 2014).

Symptomatic colonisation of non-*C. albicans* species is associated with an inflammatory reaction. VVC is usually triggered by sexual intercourse or spreading from the gastrointestinal tract. Frequent sexual activity, oral sex, the high level of oestrogen caused by oral contraceptive etc. are vital features associated with VVC (Foxman, 1990, Eckert *et al.*, 1998, Cetin *et al.*, 2007). Antibiotics contribute to the development of VVC since they alter the typical microbiota. Common symptoms of VVC include soreness, irritation, vulvar pruritus, burning, thick vaginal discharge (Anderson *et al.*, 2004, Achkar and Fries, 2010).

Vulvovaginal candidiasis is treated according to the severity of the disease. Several oral and topical drugs are available to treat fungal infections, azoles, polyenes, allylamines fluconazole, clotrimazole, amphotericin B, etc. The therapeutic target is mainly the ergosterol biosynthesis and its inhibition (Achkar and Fries, 2010, Noël, 2012, Lv *et al*., 2016). The specific inhibition depends on the compound used. Azoles prevent the activity of lanosterol-14α demethylase; the polyenes decrease the amount of ergosterol on fungi membrane, and allylamines inhibit the enzyme squalene synthase (Erg1p). The disruption of ergosterol is fatal for the fungi (Lv *et al.*, 2016).

3.3.1 *Candida albicans* and mechanism of pathogenicity

Candida albicans range of virulence is broad, and there are several factors responsible for it. These factors are hyphal-yeast transition, the expression of invasins and adhesins, thigmotropism, biofilm formation and the production of hydrolytic enzymes (Mayer *et al.*, 2013).

The hyphal-yeast transition is the result of the dimorphism of *Candida albicans* (Jacobsen *et al.*, 2012). This dimorphism is dependent on environmental factors such as pH level – yeast form grows at a lower pH level (< 6), as it is in the case of the hyphal form (pH > 7) (Odds, 1988). The hyphal form is more invasive than the yeast form (Berman and Sudbery, 2002).

An essential characteristic of pathogenicity is contact sensing (thigmotropism); the yeast contacts the cell surface. The formation of yeast is changed into hyphal growth. Thigomotropism is closely connected with the construction of biofilm, which is another pathogenicity factor. After contact with the surface (biotic/abiotic), biofilm formation is triggered. The biofilm occurrence was recorded on the epithelial cells (biotic), catheters and dentures (abiotic) (Kumamoto, 2008, Mayer *et al.*, 2013). The biofilm formation process consists of adherence of yeast to the cell, further the proliferation, formation of hyphal cell in the upper section of biofilm, increase of extracellular matrix and subsequent dispersion of yeast from the biofilm (Uppuluri *et al.*, 2010, Finkel and Mitchell, 2011). Heat shock proteins regulate the dispersion of bacteria; heat shock protein 90 (Hsp90) protein is one of the most important proteins of the group (Robbins *et al.*, 2011).

Hydrolases contribute to the penetration of *C. albicans* into the host cells. Hyphens produce various types of hydrolases such as proteases, phospholipases and lipases (Wächtler *et al.*, 2012, Mayer *et al.*, 2013). Secreted aspartyl protease (SAP) family is secreted in the invasion of the mucosal layer during VVC (Bradford and Ravel, 2017).

The production of adhesins and invasins by the *Candida* spp. is critical for pathogenesis since the adherence to host cells (Mayer *et al.*, 2013). Proteins such as agglutinin-like sequence (ALS) proteins and Hwp1 etc. belong to the group of adhesins. Als3 plays an essential role during vaginal infection. *Candida* can invade host cells via active penetration and endocytosis. Invasins, SSa1 heat shock protein and Als3 trigger endocytosis by binding to host E-cadherin.

Active penetration is associated with hyphal formation (Mayer *et al., 2013,* Bradford and Ravel*, 2017).*

3.4 Mass spectrophotometry

Mass spectrometry (MS) is a qualitative, destructive, analytical tool widely used in various scientific fields such as proteomics, lipidomics, biochemistry, pharmaceutics etc. The ability to identify the analyte directly from the sample is considered to be very useful and perspective (Urban, 2016). This analytical technique is used because of its high selectivity, which may be influenced by external factors related to the sample or the instrument itself. Factors, which specifically affect the selectivity of the instrument are, for instance, the contamination of components, effectivity to detect the analyte or transmission efficiency etc. On the other hand, the sample-based factors occur as well, such as the concentration of an analyte, limited signal stability, analyte degradation, interference during detection, or sample preparation (Hoffman and Stroobant, 2007, Urban, 2016, Gross, 2017*)*.

The basic of the method is to produce ions from organic or inorganic compounds, for example, by electron ionisation. The ion is further fragmented either to radical and even ion or new molecule and odd ion. These ions are then separated according to their mass-charge ratio, and subsequently, their abundance is detected (Hoffman and Stroobant, 2007, Gross*,* 2017*)*. Identifying the sample may differ; a target or non-target identifying the structure of interest.. On the other hand, in non-target analysis, analytes are identified using reference libraries of electron ionisation mass spectra (Milman, 2015). The mass-charge ratio and the number of ions produce a mass spectra, which frequently forms the base graph. The highest peak is called a base peak, according to which a final compound is determined. The bar graph is divided into two axes, where the y-axis represents the abundance of ions, and the x-axis interprets the mass-charge ratio (Hoffman and Stroobant, 2007). The instrument is suitable for the analysis of various compounds, which are divided into gases, volatile and semivolatile compounds based on the polarity of the molecule, molecular mass and volatility (Milman, 2015).

MS consists of mainly three steps: ionisation, separation, and detection; therefore, the spectrometer comprises ionising apparatus, vacuum chamber with magnetic field and detector (McDonald *et al*., 2011, *Gross, 2017).* The Ionizing apparatus can ionise the neutral molecule to an ion form. Several ionising techniques are known, electron, chemical, field ionisation suitable for gas-phase ionisation; electrospray, atmospheric pressure chemical ionisation and atmospheric pressure photoionisation used for the liquid phase. Finally, solid-phase uses ionising techniques as matrix-assisted laser desorption, plasma desorption etc. *(*Hoffman and Vincent Stroobant, 2007). Another

essential component of the mass spectrometer is a mass analyser, which function is the separation of the ions according to the mass-charge value. Four main types of mass analysers are manufactured: quadrupole, time of Flight (ToF), quadrupole ion trap, and Fourier transform ion cyclotron resonance (FT-ICR). Quadrupole analyser consists of four electrical rods – two with negative and two with a positive charge. Rods are during analysis withering the polarity; therefore, ions are changing the movement path – each ion has its unique trajectory. ToF accelerates ions on the same kinetic energy and differentiates them according to the mass-charge ratio (El-Aneed *et al.,* 2009, Arevato *et al.*, 2019). Basic features on which detectors depend are ion velocity, molecular mass and charge. Faraday cup measures the charge, when ion hits the surface of the detector and is neutralised. Electron multipliers or electron-optical ion detectors use for analysis of the kinetic energy of the ion, which hits the surface of the detector; they generate secondary electrons amplifying to produce a signal (Hoffman and Stroobant, 2007).

4 MATERIAL AND METHODS

4.1 Chemicals and reagents

- Acetic acid analytical grade (Merck Sigma-Aldrich, Cat. No. 64-19-7)
- Arachidonic acid standard (Merck Sigma-Aldrich, Cat. No. 23401)
- Chloroform (Merck Sigma-Aldrich, Cat. No. 288306)
- Ceramides standards C14, C17, C18:1, C20, C24, C24:1 (Merck Sigma-Aldrich, Cat. No. 860514; 860517; 860519; 860520; 860524; 860525; 860700)
- Ceramides standards with deuterium C16, C18, C18:1, C24, C24:1 (Merck Sigma-Aldrich, Cat. No. 860676; 860677; 860747; 860678; 860679)
- Dichloromethane for HPLC (Merck Sigma-Aldrich, Cat. No. 2770997)
- Docosahexaenoic acid standard (Merck Sigma-Aldrich, Cat. No. D2534)
- Eicosapentaenoic acid standard (Merck Sigma-Aldrich, Cat. No. 44864)
- Ergosterol standard (Merck Sigma-Aldrich, Cat. No. 57-87-4)
- Fluorescent probe Myco P (custom-made, Generi Biotech)
- Forward primer Myco 1 (custom-made, Generi Biotech)
- Hexane G.R. (Merck Sigma-Aldrich, Cat. No. 296090)
- Linolenic acid standard (Merck Sigma-Aldrich, Cat. No. 62160)
- Methanol (Merck Sigma-Aldrich, Cat. No. 322415)
- Reverse primer Myco 2 (custom-made, Generi Biotech)
- Ultrapure Ethanol for MALDI/TOF*
- Ultrapure water purified by Mili-Q (Millipore)
- Taq DNA polymerase (Thermo Fisher Scientific, Cat. No. AB 0301B,)

Forward primer Myco 1 sequence: 5´ GCTGTGTGCCTAATACATGCAT 3´ 22 bp
Reverse primer Myco 2 sequence: 5´ CACCATCTGTCATTCTGTTAACCT 3´ 24 bp
Probe Myco P sequence: 5´ ATCCGCACAAGCGGTGGAGC 3´ 20 bp

4.2 List of solutions

- Lysis buffer: 1 ml 10 mM Tris-HCl (pH 8,3), 1 ml 50 mM KCl, 1 ml 1% TRITON-X 100, bring to the final volume of 100 ml by adding 100 ml deionised water

- Proteinase K solution: 40 mg proteinase K lyophilised powder, 2 ml 10% SDS, 40 µl 1 M EDTA (pH 8,0), bring to a final volume of 20 ml by adding deionised water Aliquots were prepared
- Lysis buffer with proteinase K: 1,045 ml of lysis buffer and 55 µl of proteinase K solution were mixed, and aliquots of 100 µl were prepared
- Myco P probe stock solution: lyophilised Myco P probe was diluted in DEPC-treated water to a final concentration of 0,1 mM
- Myco P probe working solution: stock solution of Myco P probe was diluted in DEPC-treated water to a final concentration of 1,25 µM
- Primers Myco 1 and Myco 2 stock solution: lyophilised Myco 1 and Myco 2 primers were diluted in DEPC-treated water to a final concentration of 0,1 mM
- Primers Myco 1 and Myco 2 working solution: stock solution of Myco 1 and Myco 2 primers was diluted in DEPC-treated water to a final concentration of 10 µM
- 10 mM dNTPs: 100 µl 100 mM dATP, 100 µl 100 mM dTTP, 100 µl 100 mM dGTP, 100 µl 100 mM dCTP were diluted in 600 µl of DEPC-treated water

4.3 List of equipments

- Benchtop Centrifuge 5810R (Eppendorf)
- Box Mars Safety class 2 flowbox (Trigon-plus)
- Centrifuge 5430 (Eppendorf)
- Dionex UltiMate 3000 LC-system (Thermo Fisher Scientific)
- Dry Block Thermostat (Biosan)
- Laboratory fume cupboard (Merci®)
- LightCycler® 480 Instrument (Roche)
- Milli-Q (Milipore)
- Security Guard 4 × 2.0 mm C18 guard pre-column (Phenomenex).
- Shaker Genius 3 (IKA Vortex)
- Vortex (Labnet international)
- QTRAP® 5500 LC-MS/MS System (AB Sciex)

4.4 Biological material

Swabs obtained human vaginal fluid samples between February 2013 and June 2016 from Czech. The samples were from women aged 17–88 (median age 33) regardless of the cytomorphology findings and histopathology (Jaworek et al. 2019 research). All the samples (2,198 cervical swabs and 217 self-sampled cervicovaginal swabs) were stored in cobas PCR Cell Collection Media (Roche Diagnostics, Risch-Rotkreuz, Switzerland).

Kidney epithelial Vero (ATCC® CCL-81™) cell lines were purchased from ATCC (Manassas, VA, USA) cultured in medium. A totally 4 cell lines were infected by *Mycolplasma* spp. and 4 were noninfected.

Candida albicans culture cultivated on slant agar.

Exhaled breath condensate (EBC) samples obtained by 23 volunteers, aged from 20 to 23 years old, who have not overcome COVID-19; volunteers consisted of 9 males and 14 females. Volunteers exhaled for 10 min via a tube into Turbo 14 exhaled breath collector, EBC condensed at –5 °C and samples were stored in Falcon tubes at –80 °C. Kamila Baslarová collected EBC samples as part of her thesis.

4.5 Experimental and evaluation procedures

4.5.1 Ergosterol standard curve preparation

The stock solution of ergosterol was prepared from the standard 10 mg/ml from dissolved in chloroform and stored in the dark (ergosterol may be degraded by light and is toxic). Calibration series consisted of 12 concentrations from 100 μg/ml to 1 fg/ml. The stock solution was diluted 100x by adding 1 μl of ergosterol standard to 99 μl of ethanol. Following concentrations for calibration series were prepared by 10-fold serial dilution, adding 90 μl of ethanol to 10 μl solution with higher concentration. After preparation, the calibration series were measured by LC-MS/MS.

4.5.2 Hexane and dichloromethane extraction of ergosterol from *Candida albicans* culture

Candida albicans was transferred using a laboratory spatula to a 15-ml Falcon tube, and chloroform was added to 15-ml Falcon tubes. Three sets of extractions were done. The samples were extracted with 400 μl of chloroform. In three tubes, methanol

was added in the other three hexane, and the last ones, dichloromethane, was added (100 or 200 µl). After thoroughly mixing the samples, 400 µl of water was added, and the samples were re-extracted. The lipid fraction that contains either hexane, dichloromethane or chloroform was carefully taken with a pipette, dryed under nitrogen, and resuspended in methanol for HPLC/MS analysis..

4.5.3 Real-Time PCR for detection of ergosterol in *Mycoplasma* spp.

The cells were cultivated for two weeks without any antibiotics in the culture medium. The scraper harvested the cells to avoid repeated washing during enzymatic harvesting. The cell suspension, containing approximately $2{,}5\times10^5$–5×10^5 cells, was collected. The sample was spined down at 2000 rpm for 5 min at laboratory temperature. The supernatant was removed, and it was added 100 µl of lysis buffer with proteinase K, and further vortexed. The sample was transferred to the 1,5-ml microcentrifuge tube and incubated at 37 °C overnight. Proteinase K was inactivated by incubating the sample for another 10 min at 95 °C. The DNA sample was stored at –20 °C for subsequent PCR, which was done according to the protocol described in book Laboratory techniques in cellular and molecular medicine by Drábek *et al.*, 2021. The stock solutions of Myco P probe and primers Myco 1 and Myco 2 were prepared. PCR master reaction mix was prepared according to Table 4.1. 1 µl of sample DNA from 1,5-ml microcentrifuge tube was transferred to intended wells, positive isolated DNA from (*Mycoplasma* positive cells) and negative (DEPC-treated water) control was included. 8-tube strips were centrifuged at 2000 rpm for 2 min. Real-time PCR instrument was programmed with parameters in Table 4.2.

Table 4. 1 The components of PCR master reaction mix for detection of *Mycoplasma* spp.

Reagent	Concertation	Volume/well [µl]
DEPC-treated water		10,7
Taq Reaction buffer	10X	2,5
$MgCl_2$ solution	50 mM	2,0
Myco 1 Forward Primer	10 µM	2,0
Myco 2 Reverse Primer	10 µM	2,0
Myco P probe	1,25 µM	4,0
dNTPs	10 mM	0,5
Taq DNA polymerase	5 U/µl	0,3
Sample DNA – added separately to wells		1,0
Total		25

Table 4. 2 The PCR profile of reaction for detection of *Mycoplasma* spp.

Step		Temperature [°C]	Time
Initial denaturation		95	15 min
40 cycles	**Denaturation**	95	45 sec
	Annealing	55	1:40 min
Hold		4	forever

4.5.4 Ergosterol extraction from vaginal fluid samples by Folch lipid extraction method

One ml of the vaginal fluid samples extracted and stored in the Cobas stabilising solution was mixed with 1 ml of chloroform/methanol mixture with a 2:1 ratio (v/v). The sample was vortexed vigorously for 5 minutes. Then 1 ml of cold (4 ^{o} C) distilled water was added into samples, vortexed again vigorously to extract all the lipids. Subsequently, the samples were centrifuged for 10 min at 1000 rpm. Three phases were created: the top aqueous fraction, the bottom chloroform fraction and an interface containing proteins and

glycans. The bottom phase with chloroform transferred into 1.5-ml Eppendorf tube and dried by the nitrogen stream. Afterwards, the lipids were dissolved in 100 µl of ethanol, and 100 µl of solution were transferred into vials. The automatic sampler collected and injected the sample into the apparatus, LC-MS/MS, to perform the analysis.

The samples were also extracted to detect fatty acids, alpha-linolenic acid, arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid as part of David Celý thesis. The aim was to ascertain if ergosterol could alter the levels of the aforementioned fatty acids in cervical mucus.

4.5.5 Lipid extraction of exhaled breath condensate using Folch method

The extraction mixture was prepared from methanol and chloroform as described previously. Afterwards, the dried phase of lipids was dissolved in 100 µl of ethanol, and 100 µl of solution were transferred into vials. The samples were analysed by LC-MS/MS. The species of interest were ceramides (Kamila Baslarová thesis) and ergosterol.

4.5.6 Analysis of ergosterol by LC-MS/MS

LC-MS/MS was carried out using a QTrap 5500 mass spectrometer (AB Sciex) coupled to a Dionex UltiMate 3000 LC-system as described by Veltman et al., 2021. The separation column was Kinetex 2,1 × 50 mm C18, guarded with a Security Guard 4 × 2.0 mm C18 guard pre-column (Phenomenex). The mobile phases were MilliQ water (A) and methanol (B), both with 0.01% acetic acid, with the following gradient: first minute 20% B, increase to 35% B in 3 min and further increase to 99% B in 15 min and 100% B in 17,1. The 100% B was held for 0,9 min, then the gradient was decreased back to 20% B in 1 min, and the column was equilibrated at 20% B for another minute. The total run time was 20 min. MS was operated under the following conditions: the collision gas flow was set to medium; the drying temperature was 400°C, the needle voltage was −4,500 V, the curtain gas was 30 psi, ion source gas 1 was 40 psi, and the ion source gas 2 was 30 psi. The lipid standards were purchased from Sigma Aldrich and Avanti Polar Lipids. LC-MS/MS Analysis was performed by Mgr. Tomas Ozdian, PhD. and supervised and analysed by prof. Juan Bautista De Sanctis, PhD.

5 RESULTS

5.1 Preparation of standard curve for ergosterol assay

The standard curve for ergosterol was prepared using multiple dilutions as described in Chapter 4.5.1. Figure 5.1 represents the standard curve of ergosterol. The slope of the standard curve was determined to 2.92×10^{-15}. The Pearson correlation coefficient was determined to R = 0.993 (Fig. 5.1).

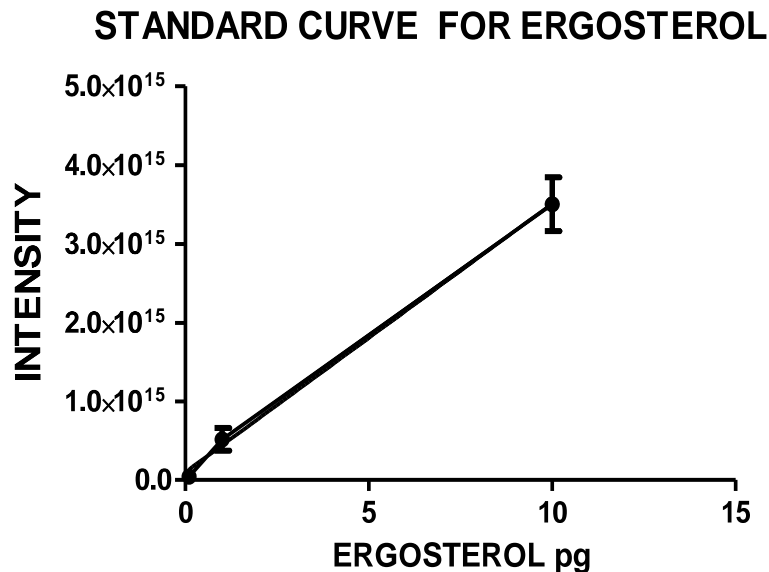

Figure 5. 1 Standard curve for ergosterol assay. Data are shown as mean ± SD, n = 5.

In order to determine the presence of ergosterol in biological samples, several experiments were performed: 1) we analysed the presence of ergosterol using 85 serum samples extracted using the Folch method, 2) we determined the presence of ergosterol in *Candida albicans* agar culture using three extraction methods and 3) we determined the presence of ergosterol in cell samples infected with *Mycoplasma* spp. using Folch method.

No presence of ergosterol was found in serum samples, and it was considered a negative control.

5.2 Lipid extraction of *Candida albicans* with hexane, dichloromethane and Folch method

To determine, which method is suitable for ergosterol extraction, three extraction methods were used. *C. albicans* cultivated on slant agar was used. Lipids were extracted

using hexane, dichloromethane, and the Folch method as described in Chapter 4.5.2. The Folch lipid extraction method was the most suitable for assessing ergosterol (Tab. 5.1).

Table 5. 1 Extraction of ergosterol with hexane, dichloromethane, and methanol (Folch method) from *C. albicans* culture cultivated on slant agar.

Folch extraction [pg]	Hexane extraction [pg]	Dichloromethane extraction [pg]
2.6	1.6	2.1
3.2	0.8	2.2
2.5	1.1	1.2
2.3	0.9	1.1

5.3 *Mycoplasma* spp. RT-PCR detection

Vero (ATCC® CCL-81™) cell lines were infected by *Mycoplasma* spp., verified by RT-PCR as described in Chapter 4.5.3. Lipids were extracted using the Folch method, and the ergosterol amount was detected by HLPC-MS. The values were obtained using the standard curve equation (Tab. 5.2).

Table 5. 2 Vero (ATCC® CCL-81™) cell lines contaminated by *Mycoplasma* spp., verified by RT-PCR, and measured the amount of ergosterol.

Contaminated cell lines [pg]	RT-PCR	Non-contaminated cell lines [pg]	RT-PCR
3.2	+	0	-
4.3	+	0	-
4.2	+	0	-
3.1	+	0	-

Legend: + (positive), **-** (negative)

5.4 Assessment of ergosterol in HPV tested vaginal fluid after Folch lipid extraction

The presence of ergosterol in vaginal fluid samples used for HPV testing were measured. The samples were extracted with methanol as part of the molecular extraction required for HPV detection. Serum samples were used as a negative control. The vaginal

fluid samples with no detectable ergosterol were considered negative. The detection of positiveness was the presence of ergosterol, SD not above 10 %, in at least three readings from the sextuplicate analysis

The total amount of samples was 60. Half of the women were HPV positive, and another half HPV negative. In 15 HPV negative samples, ergosterol was not detectable, and in 15 it was detected (Fig. 5.3). In HPV positive samples, ergosterol was not detectable in 10 samples, and it was detected in 20 samples (Fig 5.2). The difference between groups is significant using the Chi-squared test with Yate's correction (p = 0.02).

The average value of ergosterol amount in HPV negative samples was 0.432949 pg. The average value of ergosterol amount in HPV positive samples was 0.856991 pg. The T-test result was 0.0202 (Fig 5.2).

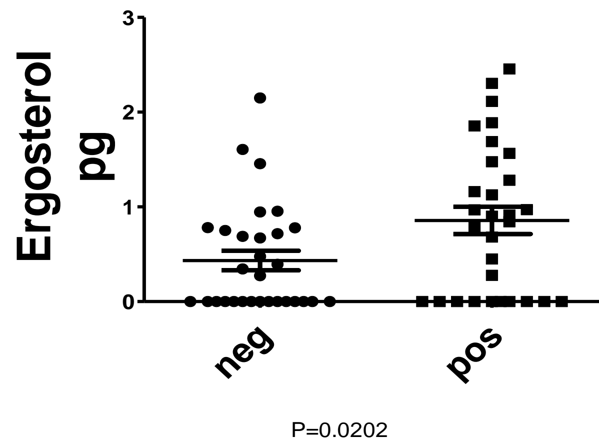

Figure 5. 2 Amount of ergosterol in negative and positive HPV tested vaginal fluid. p = 0.0202.

Figure 5.3 illustrates the two HPV negative and positive samples' results, which had only detectable ergosterol, values above 0.

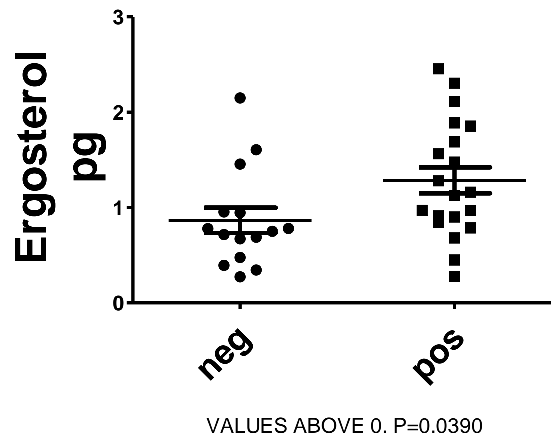

Figure 5. 3 Amount of ergosterol in negative and positive HPV tested vaginal fluid, with detectable ergosterol – values above 0. p = 0.0390, SD = 0.566201.

5.5 Fatty acid and Ergosterol detection in HPV positive and negative samples

The values of fatty acids were measured in the same samples, David Celý's experiments. The four types of fatty acids assessed were alpha-linolenic (ALA), eicosapentaenoic (EPA), docosahexaenoic (DHA), and arachidonic (AA) acids. Then we compared the differences in fatty acid composition in samples with detected ergosterol compared to the negative ones. The results are shown in Table 5.3 and Figure 5.4. The T-tests results, rounded to the nearest thousandths, for HPV positive samples were for ALA (0.387), EPA (0.666), DHA (0.989) and AA (0.130), and in HPV negative samples for ALA (0.004), EPA (0.257), DHA (0.250) and AA (0.018*).* In both groups, ALA values significantly differ between samples with and without ergosterol, as shown in Figure 5.4 and Table 5.3 show.

Table 5. 3 The amount of fatty acids in HPV positive/negative vaginal fluid samples, with and without the presence of ergosterol. Data are rounded to the nearest thousandths and shown as mean ± SD, n = 10.

	HPV positive		**HPV negative**	
	With ergosterol	**No ergosterol**	**With ergosterol**	**No ergosterol**
	Amount of fatty acids [pg]			
ALA	1.975 ± 0.457	1.671 ± 0.308	2.112 ± 0.352	2.505 ± 0.328
EPA	1.070 ± 0.492	1.150 ± 0.460	1.409 ± 0.446	1.568 ± 0.289
DHA	1.220 ± 0.338	1.221 ± 0.322	1.939 ± 0.586	2.166 ± 0.465
AA	2.255 ± 0.189	2.160 ± 0.139	0.707 ± 0.240	0.509 ± 0.189

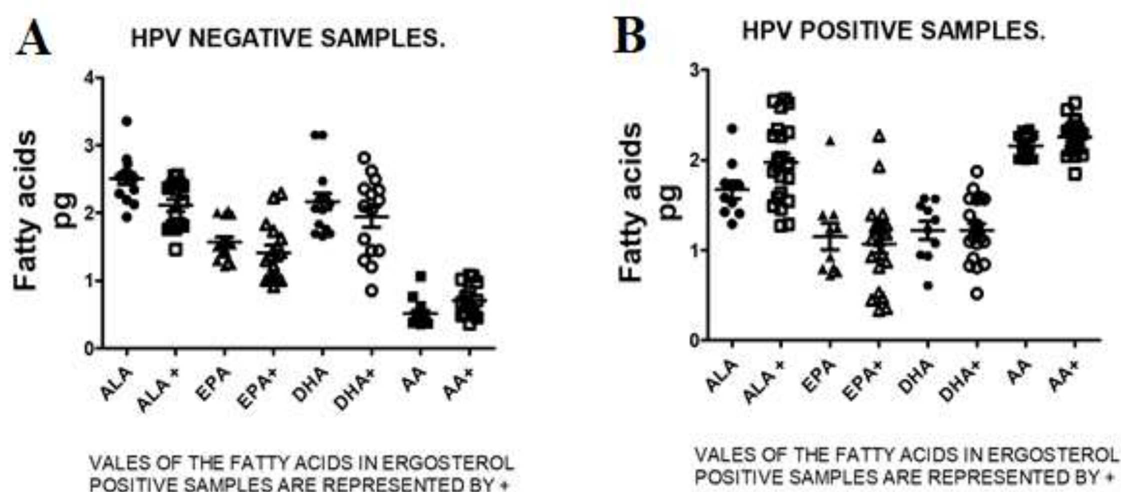

Figure 5. 4 Graphical representation of fatty acids amount in vaginal fluid samples (B) HPV positive and (A) HPV negative tested samples, with and without the presence of ergosterol. The values of alpha-linolenic acid in ergosterol positive samples are represented by +. $p < 0.05$ (HPV negative), $p < 0.001$ (HPV positive).

5.6 Ceramides in exhaled breath condensate samples. Presence of ergosterol

Lipids were extracted by Folch method from exhaled breath condensate samples from 9 males and 14 females, as described in Chapter 4.5.5. The detectable amount of ergosterol was measured in 15 cases (6 males and 9 females).

The detectable ergosterol samples differed in the picomoles of ceramides C24:1 and C26:0. A significantly lower amount of C26:0 ceramide was observed detectable in

positive ergosterol samples. On the contrary, C24:1 ceramides values were higher than in samples without detectable ergosterol (Fig. 5.5).

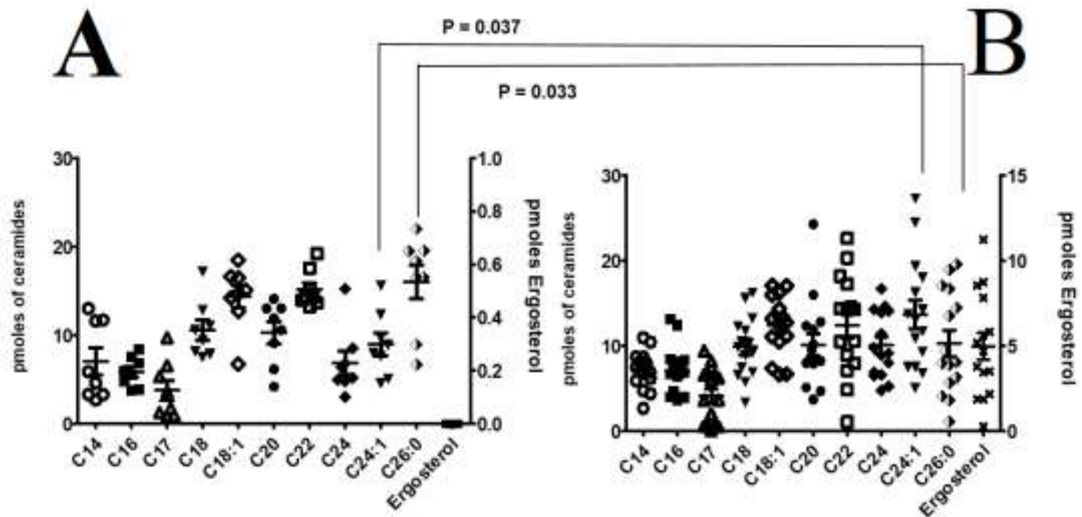

Figure 5. 5 Ceramide values in (A) ergosterol undetectable and (B) ergosterol detectable samples of exhaled breath condensate. The different ceramide amount in A and B. Data are shown as ± SD. p = 0.037 (C24:1), p = 0.033 (C26:0).

6 DISCUSSION

Human papillomavirus (HPV) is considered the causal agent of carcinoma and cervical dysplasia (Walboomers et al., 1999, Godoy-Vitorino *et al.*, 2018). The high-risk HPV oncogenic serotypes are 16, 18, 31, 33 (Godoy-Vitorino *et al.*, 2018). Two HPV types (16 and 18) cause 70% of cervical cancers and pre-cancerous cervical lesions and cancers of the anus, vulva, vagina, penis and oropharynx. The widespread of HPV might be influenced by local vaginal microbiota (WHO, 2020).

Malignant transformation occurs in the cervix, specifically in the squamocolumnar cells located between the endo and ectocervix (Marsh, 1956, Herfs *et al.*, 2012). This liability is caused by the fact that there is a thinner layer of the cells readily exposed to the virus (Läsehe *et al.*, 2021). Injuries of the cervical mucosa, frequently caused by sexual activity or pathogenic microbiota, impair the local remodelling of the mucosal epithelia and the proper protective immune response. The lack of appropriate immune vigilance facilitates the binding of external keratinocytes to the mucosa. These foreign cells carry HPV into the damaged epithelial cells and infect them (Timmons *et al.*, 2010, Läsehe *et al.*, 2021). HPV incorporates its genome into the host's DNA, which serves as an oncogene (Vinokurova *et al.*, 2008, McBride and Warburton, 2017). These include gene segments such as E6 and E7, which negatively modify the cell cycle regulators (Läsehe *et al.*, 2021) or decrease the expression of Toll-like receptor 9 (Ghittoni *et al.*, 2010).

Published observations support the assumption that the vaginal microbiome may play an essential role in cancer-induced HPV. The healthy vaginal microbiota mainly contains *Lactobacillus* spp., which provides a low pH level to protect the vagina and cervix from pathogenic agents. BV-like associated bacterial infection destroys epithelial cells and produces abnormal regulators of immune signalling, influencing the production of immune parameters such as pro-inflammatory cytokines (interleukin-6, tumour necrosis factor etc.), antimicrobial proteins and chemokines responsible for immune cells recruitment. Local inflammation may result in tissue damage, enhancing the oncogenic potential of HPV (Moschen *et al.*, 2016, Kyrgiou, *et al.*, 2016). Bacterial dysbiosis may lead to dysregulated immune function and prolonged inflammation which can cause an increased persistence of HPV infection, the transformation of the virus, viral integration, coinfection with pathogenic bacteria etc. (Łaniewski *et al.* 2019, Castle *et al.,* 2001, Kumari and Bhor, 2021). Most of the cervicovaginal infections are associated with

Gardenella vaginalis and *Atopobium vaginae*, bacteria or the fungus *Candida albicans* (Godoy-Vitorino *et al.*, 2018). It is plausible that *Candida albicans* decrease *Lactobacillus* spp. present in the vaginal environment, which could lead to BV-like mycobiota. Our findings support the hypothesis of a higher prevalence of fungi infection in HPV positive samples. However, this prevalence has to be confirmed in a higher number of samples.

Metabolomic is a widely used tool for cancer detection since it reflects the metabolic changes involved in carcinogenesis and differs in normal cells. Metabolome may be affected by host, environment, microbiome or HPV infection. Genital inflammation is associated with amino acids metabolic products such as glutamine or various lipid groups (Ilhan *et al.*, 2019). Data of Ilhan *et al.*, 2019 strengthen the linkage between genital inflammation, cancer and lipids. An increased presence of fatty acids may show abnormal metabolic production during cell transformation and their use as precursors for cell proliferation, cytokine production, abnormal gene expression, etc. (Wallner *et al.*, 2018). Interestingly, our data show a significant difference in alpha-linoleic acid levels between positive and negative samples with ergosterol. More experiments are required to understand the mechanism.

In other results involving the importance of ergosterol in biological samples, the metabolite was detected in 15 out of 23 samples of exhaled breath condensate. This result may indicate commensal or pathogenic fungi in the airways of the subjects. Fungi may alter local and peripheral immune responses that can lead to disease progression (Peleg *et al.* 2010). Fungal glycans interact with epithelial cells in airways and mediate allergic reactions or induce inflammation, as observed in many subtypes of chronic rhinosinusitis (Roy and Klein, 2013). Fungi such as *Aspergillus*, *Candida*, *Penicillium* (Ponikau *et al.* 1999) may be responsible for the sinusitis. Fungi can also promote the growth of bacteria, increase their virulence or modify their morphology (Zhang *et al.,* 2017).

In this thesis we have been able to show the importance of ergosterol detection in several biological samples. Even though further studies are required to ascertain the association of the metabolite to pathological events, the results provide important information to consider ergosterol as an important biomarker to detect pathogenic infection.

7 CONCLUSIONS

This thesis was focused on the assessment of ergosterol in the cervical mucus of patients. Samples were extracted with the Folch lipid extraction method with chloroform/methanol with ratio 2:1 (v/v) and subsequently analysed using HPLC-MS. We measured a detectable amount of ergosterol in infected Vero cell lines, cervical mucus samples, which were HPV positive or negative, and in the samples of exhaled breath condensate.

Vero cell lines were infected with Mycoplasma spp. We were able to detect ergosterol in picograms. In 60 cervical mucus samples, 20 samples that were HPV positive contained higher values of ergosterol than in 15 HPV negative samples. Ergosterol was not detected in 25 cervical mucus samples. In 15 out of 23 samples of exhaled breath condensate, we were able to detect ergosterol in picomoles values. These results indicate the promising possibility of usage ergosterol, detected with HPLC-MS, as a potential biomarker of fungal infection of the vagina and airways, which may be relevant for the study of different diseases.

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