PALACKY UNIVERSITY OLOMOUC

Faculty of Science

Department of Biochemistry

DIPLOMA THESIS

Olomouc 2023 **Bc. Denisa Kroupová**

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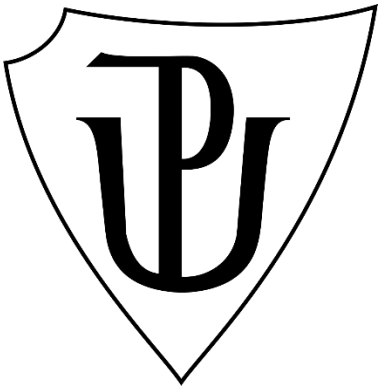

The Influence of Human Papillomavirus on Lipidomic Profile of the Cervical Mucus

DIPLOMA THESIS

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I hereby declare that I have worked on my diploma thesis independently and only used the sources listed in the references.

In Olomouc, 21.4. 2023

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Abstrakt
Rakovina děložního čípku vzniká následkem působení lidského papillomaviru (HPV). Tento vir je přenášen pohlavním stykem a jedná se o pohlavně přenosnou infekci s nejvyšší incidencí ve světě. Jelikož samotnému karcinomu předchází velmi dlouhé období působení tzv. high-risk HPV v oblasti epiteliálních buněk děložního hrdla, je zde kladen důraz na prevenci (očkování, screening). Nyní je výzkum zaměřen na objevování nových biomarkerů infekce pro jejich diagnostický potenciál a možné využití v léčbě. Výzkumy naznačují, že omega-3 mastné kyseliny a jejich bioaktivní metabolity mají protizánětlivý účinek, zatímco omega-6 mastné kyseliny a jejich metabolity rozvoj zánětu naopak podporují. Tato diplomová práce si kladla za cíl určit míru korelace u HPV pozitivního a HPV negativního vzorku a současně stanovit lipidomický profil cervicovaginálního vzorku v souvislosti se zmiňovanými mastnými kyselinami. Vzorkem byl cervikovaginální stěr epiteliálních buněk, jehož lipidová frakce byla ze vzorku izolována metodou extrakce na pevné fázi (SPE). Vzorky byly analyzovány pomocí QTrap hmotnostního spektrometru. Byl stanoven lipidomický profil 15 lipidových metabolitů. Nicméně srovnání koncentrace těchto lipidů neukázal žádný signifikantní rozdíl mezi HPV+ a HPV- vzorkem. Proto byla dále provedena věková stratifikace pacientek, která ukázala na 5 významných lipidových mediátoru zánětu – 5-HETE, 11-HETE, 15-HETE, TxB2 a PGF2a, které se různou mírou mohou podílet na rozvoji, resp. regresi tohoto zánětu v děložním hrdle. Rozdělení a srovnání vzorku podle závožnosti prekancerozního stavu rovněž neukázali žádnou signifikantní hodnotu. Potenciál mastných kyselin v prevenci zánětu se v rámci této diplomové práce nepotvrdil, nicméně v pre-analytické části došlo k chybám, které k tomuto závěru mohly vést.

Klíčová slova	Lidský papillomavirus, HPV, prekanceroza, rakovina děložního čípku, cervikovaginální stěr, lipidomické profilování, cílená lipidomika, omega-3 mastné kyseliny, omega-6 mastné kyseliny a jejich metabolity
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Abstract
Cervical cancer is caused by the Human Papillomavirus (HPV). This virus is transmitted through sexual intercourse and represents the sexually transmitted infection with the highest incidence worldwide. Since cancer itself is preceded by a long period of exposure to the so-called high-risk HPV in the cervical epithelial cells, the emphasis is placed on prevention (vaccination, screening). Currently, research is focused on discovering new biomarkers of infection for their diagnostic potential and possible use in treatment. Research indicates that omega-3 fatty acids and their bioactive metabolites have an anti-inflammatory effect, while omega-6 fatty acids and their metabolites promote the development of inflammation. This diploma thesis aims to determine the degree of correlation between HPV-positive and HPV-negative samples and to determine the lipidomic profile of samples. The sample was a cervicovaginal smear of epithelial cells. The lipid fraction was isolated by solid phase extraction (SPE). Samples were analyzed using a QTrap mass spectrometer. A lipidomic profile of metabolites was identified. Nevertheless, the comparison of these lipid concentrations showed no significant difference between HPV+ and HPV- samples. Therefore, the age stratification of the patients was carried out, which showed 5 important lipid mediators of inflammation - 5-HETE, 11-HETE, 15-HETE, TxB2, and PGF2a, which, to a certain extent, can participate in the progression or regression of this inflammation of the cervix. The division and comparison of the sample according to the severity of the precancerous condition also showed no significant value. The potential of fatty acids in the prevention of inflammation was not confirmed in this diploma thesis, however, there were mistakes in the pre-analytical phase that could have led to this conclusion.

Keywords	Human Papillomavirus, HPV, precancerous stage, cervical cancer, cervicovaginal smear, lipidomic profiling, targeted lipidomics, omega-3 fatty acids, omega-6 fatty acids, and their metabolites
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Aims of the Thesis

- Theoretical research focused on cervical cancer and human papillomavirus (HPV) – summarize the current knowledge and relevant information regarding HPV and lipid composition within the cell
- Cervicovaginal samples preparation – lipidomic analysis of cervicovaginal smear
- Mass spectrometry analysis and data acquisition
- Lipidomic profiling to determine the correlation between HPV positivity and the degree of inflammatory response

1. INTRODUCTION

The cause of cervical cancer is the virus – Human Papillomavirus (HPV). In the Czech Republic, cervical cancer is the 4th leading cause of death among women although cancer development due to HPV infection is a very rare case and most infections are cleared out within several months (Graham, 2017; Tsikouras et al., 2016). However, scientific groups should emphasize further research in this area for several following reasons.

Firstly, this virus is the most common sexually transmitted infection. Almost all sexually active adults acquire the infection at some point in their life. If they do and the infection becomes persistent and unhealed, it may develop into cancer of the cervix in women (Manini & Montomoli, 2018). Moreover, the additional factors affecting HPV persistence are not fully understood (Zang & Hu, 2021).

Then, there is no treatment against the virus itself. The care is focused on the symptoms that HPV causes.

And finally, in most cases, the human body is not capable of developing immunity after the HPV infection. If it does, antibodies specific to HPV capsid proteins are produced at a very slow pace (Hamsikova et al., 2013).

All above mentioned could be a motivation for preventive steps in the first place. Vaccination is considered as primary a prevention strategy. There are several vaccines on the Czech market. Since both sexes may be vectors but also infected, both women and men should consider getting vaccinated. The secondary prevention strategy is represented by annual screening tests. This involves Pap test and HPV DNA testing (Crosbie et al., 2013).

It seems cervical cancer is largely preventable through detection and treatment. Both vaccination and screening together have an immense potential to save lives, however, every other implication in either prevention or therapy of precancerous stages and cancer has a significant meaning. Therefore, the research has focused on discovering potential biomarkers that would have led to more effective diagnoses of precancerous lesions before the stage ends up as cervical cancer.

It has been recently shown that lipid mediators may play a crucial role in viral infections *in vivo*– some in their progression and some in their resolution. Hence, lipid mediators may have diagnostic potential in routine analysis or even represent new prevention options. The importance of identifying and quantifying lipids in biomedical research thus should not be underestimated (Tam, 2013).

This thesis aims to determine the correlation between HPV positivity and the degree of inflammatory response. Lipids of interest involve omega-3 and omega-6 fatty acids and their metabolites. These lipids are compared in HPV-positive and HPV-negative samples to provide insight into the protection or triggering of HPV infection in a cell leading to the discovery of new targets for intervention. Obtained results of this study could be a reason for further research and possible guidance for new prevention options for cervical cancer.

2. THEORETICAL PART

2.1Human Papillomavirus

Papillomavirus is a small double-stranded DNA virus from the *Papillomaviridae* family. The virus itself has approximately 55 nm in diameter and its genome has a size of almost 8 kb (Balasubramaniam et al., 2019; Van Doorslaer et al., 2017). All papillomaviruses have a non-enveloped icosahedral protein capsid composed of 360 copies of the L1 protein organized into 72 pentameric capsomeres that have one L2 at the center. L1 and L2 are both produced by the virus. L1 creates about 80 % of total viral proteins (Graham, 2017; Tumban, 2019).

HPVs make up a group of over 200 related viruses. Approximately 40 of these are transmitted during sexual intercourse via skin-to-skin contact (Burd, 2003; Della Fera et al., 2021). Up to 80 % of adult acquires the virus at some point in their life (Kyrgiou et al., 2017).

Papillomaviruses, in an obligation of a host organism, infect a huge range of hosts in a species-specific manner. HPV genomes have been isolated and characterized from reptiles, birds, marsupials, and multiple other mammalian species suggesting an evolutionary history spanning more than 300 million years (Bernard et al., 2010).

They are greatly epitheliotropic – the viral life cycle and establishment of infection are tightly linked to the differentiation of the infected epithelial cell (Burd, 2003).

The causal linkage between HPV and cervical cancer might be considered an important discover. This was accomplished by German scientist Harald zur Hausen and his group in the 1980s. The new knowledge had an enormous meaning as it allowed us to put an effort into developing effective prophylactic vaccines for HPVs. In 2008, in recognition of his discovery, zur Hausen was awarded the Nobel Prize in Physiology and Medicine (Hampton, 2008).

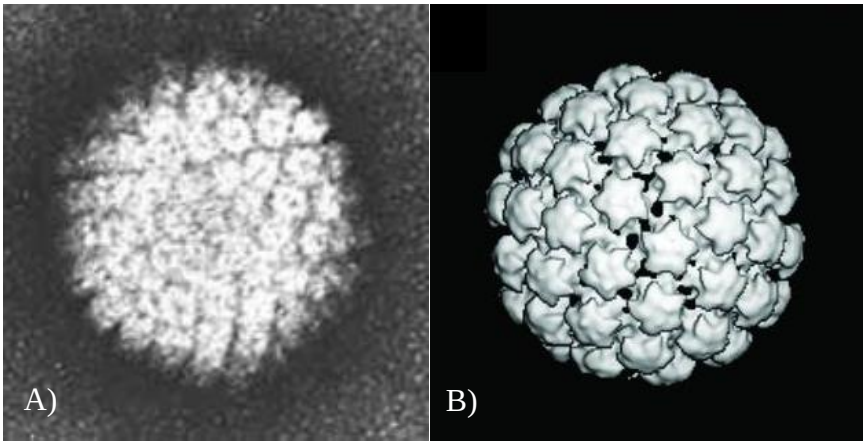


Figure 1: A) The HPV virion under the electron microscope (Kalu et al., 2018); B) Computerized reconstruction of virion (Buck et al., 2008)

2.1.1 HPV Classification

All HPVs are sorted into five groups based on DNA sequence homology of L1 gene. This gene is responsible for encoding L1 capsid protein. L1 gene region has been highly conserved and therefore used for the identification of new papillomavirus types.

To discover a new genotype of HPV, the whole genome has to be sequenced and compared with already known HPV genomes. The determining factor is L1 gene region which differs by 10 % from the other HPVs (Kocjan et al., 2015).

The evolutionary HPV genotype groups are Alpha, Beta, Gamma, Mu, Nu (Van Doorslaer et al., 2017).

HPV tends to have strict tissue specificity for establishing the infection since this tissue must support its growth and allows to multiply of viral particles – this phenomenon is called tissue tropism. While Beta, Gamma, Mu, and Nu HPVs infect cutaneous epithelium, Alpha HPVs infect both cutaneous and mucosal epithelia, preferably.

The Alpha HPV is the biggest group out of the above five mentioned. It involves 64 HPVs and approximately 40 of them are sexually transmitted and therefore could infect the anogenital region. They display themselves in the anogenital region either as genital wards or as lesions. More importantly. these alpha HPVs are then classified as low-risk HPVs (LR-HPV) and high-risk HPVs (HR-HPV) based on their oncogenic potential (see Paragraphs 2.1.1.1 and 2.1.1.2) (Stanley, 2010).

The second biggest group is Beta HPV that mainly infects cutaneous epithelia. So far, over 50 genotypes have been characterized. These HPVs are associated with non-melanoma squamous cell carcinomas (Tommasino, 2017).

Gama, Mu, and Nu only cause benign infections (Graham, 2017).

2.1.1.1 Low-risk Alpha HPV

Low-risk HPVs are involved in the development of genital warts (Hamsikova et al., 2013). These HPVs are not associated with lesion progression to invasive cancer. Mostly, they are harmless, and infection disappears spontaneously.

2.1.1.2 High-risk Alpha HPV

A specific subset of HPVs is called high-risk – they are oncogenic and lead to cancer progression. To develop into cancer, the immune system has failed in clearing the infection and it has become persistent. So, over a period of several years, together with associated changes in the infected host cells, it can cause cervical cancer.

Except for cervical cancer, this virus has been also associated with other types of cancers of the anogenital tract (penile, vulvar, vaginal, and anal cancer) and oropharyngeal cancer (Kamolratanakul & Pitisuttithum, 2021).

There are about 15 hr-HPV types. Two of these, HPV18 and especially HPV16, are the most prevalent sexually transmitted viral infection worldwide. They are also responsible for 70 % of all HPV-related cancers (Graham, 2017).

2.1.2 Genome of HPV

The genome (8.0 kb) is made of one molecule of circular, episomal DNA (Figure 2) that is divided into three main regions – early (E) and late (L) coding regions and non-coding region that is referred to as the long control region (LCR). Coding sequences contain 8 open reading frames (ORFs). All HPV types are very similar in genome organization (de Villiers et al., 2004).

The early region genes encode three functional proteins E1, E2, E4, and three oncoproteins E5, E6, E7 playing important role in cancer progression**.**

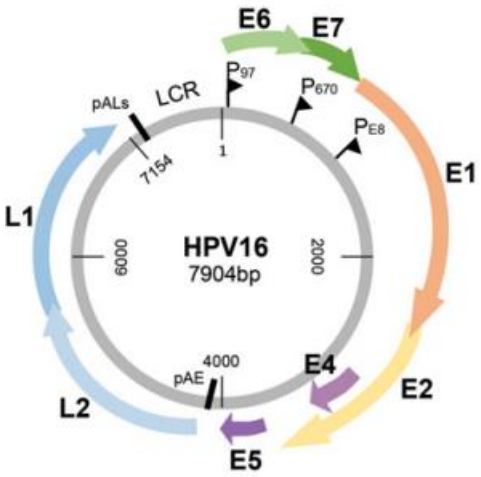


Figure 2: Diagram of genome HPV16. The genome itself is depicted with a grey circle. Individual genes are represented by colored curves above the genome. P_{97}, $P_{670,}$ and P_{E8} stand for protomers (Graham, 2017).

E1 and E2 proteins recognize the origin of replication and initiate viral DNA replication. E2 also regulates gene transcription of the virus. E4 is expressed in both early and late stages of the viral life cycle (Galván et al., 2011). Oncoprotein E5 indirectly contributes to the completion of the viral life cycle by generating the appropriate environment within the superficial cells (Ilahi & Bhatti, 2020).E6 and E7 oncoproteins promote dysregulation of tightly regulated replication of the epithelial cells by altering the p53 and pRB (retinoblastoma protein) pathways that try to prevent the unregulated growth of the epithelial cells and allow the accumulation of genetic damage. In other words, E6 and E7 oncoproteins are responsible for cancer progression (Galván et al., 2011).

The late region encodes L1 and L2 capsid proteins having control over virion assembly. The assembly process takes place in the most superficial layer of the epithelium where cells have already undergone terminal differentiation and these proteins are expressed and new virions (infectious viral particles) are released to the body (Doorbar et al., 2012).

Long control region (LCR) contains the replication origin and transcription factor-binding sites that contribute to regulating DNA replication by controlling the viral gene transcription (Graham, 2017).

Several other early ORFs have been identified – e.g., E3 or E8, but their expression is not uniformly observed throughout the *Papillomaviridae* (Harari et al., 2014).

2.1.3 Epithelial Cells of the Cervix

Epithelial cells create lines of the outer surfaces of organs and vessels, so they separate the interior of the body from the external environment. Primarily, they serve as a protective barrier to invasion by pathogens. The cells are usually stratified into several layers. These cells also make up the cervix – the lower part of the uterus (Figure 3).

They have a cylindrical shape and are connected to the vagina through the endocervical canal. The endocervical canal is lined with stratified squamous epithelium that covers ectocervix and endocervix, respectively. The transient zone between these cells is called the squamocolumnar junction (Bhatla et al., 2018).

HPV only infects the basal layer of the epithelium – these cells are closest to the interior of the body and are less mature. HPV is fully dependent on the differentiation pathway of cells to fulfill its lifecycle (Doorbar et al., 2012). As these basal cells divide and mature, they move up toward the outmost squamous-shaped cells and supply them with new cells (Crosbie et al., 2013).

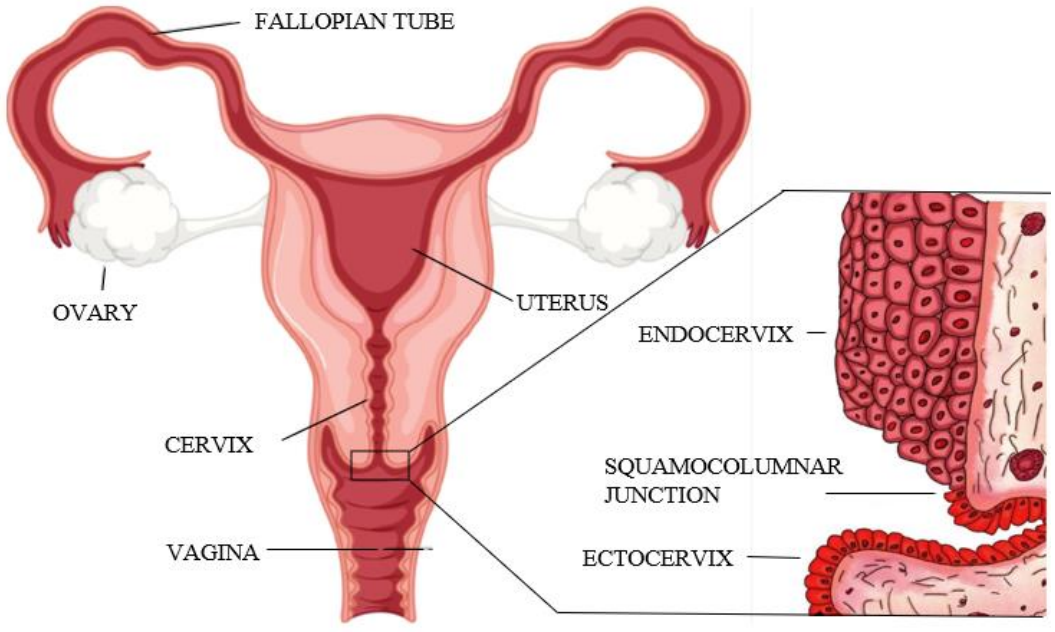


Figure 3: The scheme of a woman's reproductive system. The cervix is made of layers of epithelial cells when the most inter one is referred to as the basal epithelial layer. The HPV virions most likely use the squamocolumnar junction to get access to the basal cells to establish the infection (Lacroix et al., 2020).

HPV virions must get their way toward the basal cells. There are two possibilities – either there might be some abrasions or microcuts in the epithelium HPV acquire access to and infect the basal cells or more likely HPV enters and infect basal cells in a region between endo- and ectocervix called the squamous columnar junction (Burd, 2003; Crosbie et al., 2013).

2.1.4 The Progression of the HPV Infection

2.1.4.1 Early Stage of the HPV Infection

The HPV virion accesses the cell through the interaction of L1 capsid protein with a receptor of the basal cell located on its surface. This cellular receptor is heparin sulphate proteoglycans (HSPGs). When this initial bond is built up, the viral particle undergoes cyclophilin B-mediated conformational change which enables N-terminus of the L2 capsid protein to be exposed on its surface. A proteolytic enzyme furin or PC5/6 cleaves the N-terminus of the capsid protein. This step permits binding to the secondary L2-specific receptor - the annexin A2 heterotetramer – on the surface of the basal cell. There is an involvement of other receptors such as epidermal growth factor receptors (EGFRs), integrins (α6 integrin), laminins, syndecan-1, and vimentin (Raff et al., 2013). The type of used receptor might be reliant on the HPV type. After successful binding, the HPV virion is engulfed by the process of endocytosis (DiGiuseppe et al., 2017).

Eventually, the viral DNA is released from the capsid and transported through the tubulin-mediated pathway and via nuclear pore into the cellular nucleus or it misuses the breakdown of the nuclear membrane during the cell division to get to the nucleus. Then, the virus is finally able to initiate its transcription. HPV enters the nucleus 24 hours after the attachment on the basal cell (Everett, 2013).

2.1.4.2 E1 and E2 Proteins

After the entrance into the nucleus of dividing basal cells, viral transcription is initiated. The genes encoding E1 and E2 proteins are expressed in the first place since they are necessary for the replication of the HPV genome (Ozbun, 2002). E2 works as a transcription factor that regulates viral early promoter to direct expression of the E6 and E7 oncoproteins (Moody & Laimins, 2010). In infected basal epithelium, episomal viral DNA is replicated by the replication of the cellular genome and equally partitioned into daughter cells (McBride, 2013).

Interestingly, viral proteins are expressed at low rates in infected basal cells to avoid triggering the immune reaction (Peh et al., 2002). This is possible thanks to repression of the P97 promoter by E2 (J. A. Smith et al., 2014).

As basal cells are differentiating and amplifying, they are carrying viral genomes with them moving into the outmost epithelial layers(Oldak et al., 2004).

2.1.4.3 E6 and E7 Proteins

HPV may replicate with or without being incorporated into the DNA of basal cells through the activity of two viral genes E6 and E7. The expressed E6 and E7 oncoproteins are essential for stimulation of the cell growth, inhibition of differentiation, and induction of chromosomal instability that will result in tumorigenesis. They act cooperatively in avoiding immune detection (Middleton et al., 2003). They cause dysregulation of tightly scheduled replication of the epithelial cells by altering p53 and pRB pathways (Figure 4). These pathways prevent unregulated growth of the epithelial cells. Main tumor suppressor proteins p53 and pRB retinoblastoma are disrupted by E6 and E7 viral proteins so they cause DNA repair mechanisms and apoptosis – cell is proliferating rapidly and uncoordinated (Balasubramaniam et al., 2019).

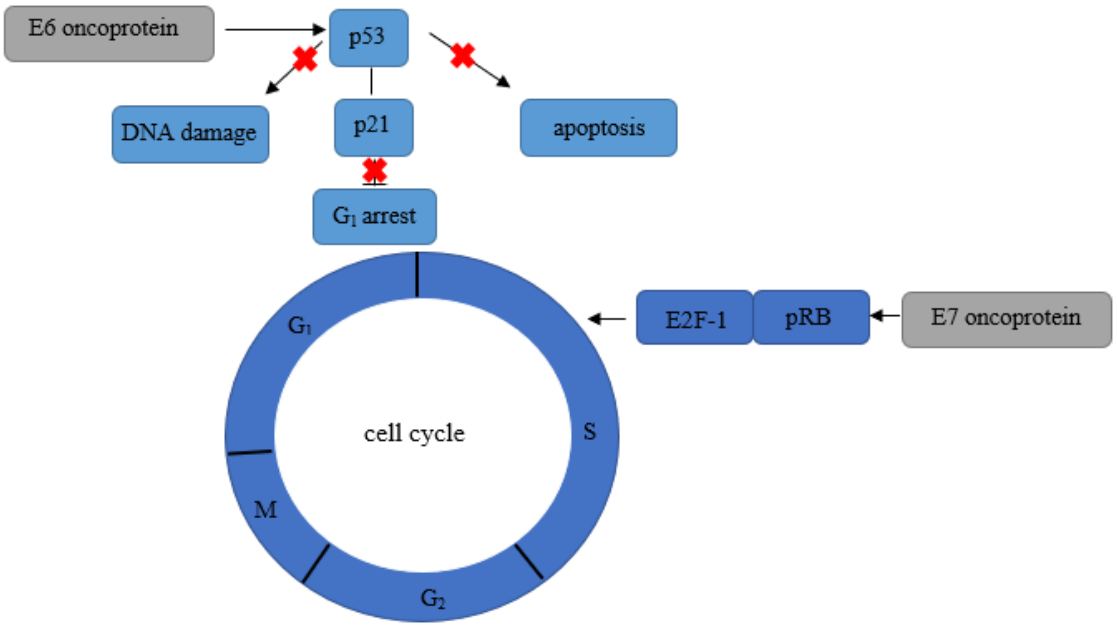


Figure 4: HPV E6 and E7 genes encode multifactorial proteins that bind primarily to cellular p53 and pRB proteins, disrupt their function, and alter cell cycle regulatory pathways leading to cellular transformation (Burd, 2003).

2.1.4.4 E4 and E5 Proteins

E4 and E5 HPV proteins have multiple tasks to preserve the convenient conditions for HPV genome amplification within the infected epithelium. Later in the virus replication cycle, E4 proteins have a role in restructuring of cytokeratin filaments to render cells fragile and more liable to release progeny virions (Q. Wang et al., 2004). E5 helps the virus evade the immune response by the repression of MHC presentation of viral peptides (Ashrafi et al., 2006).

2.1.4.5 L1 and L2 Capsid Proteins

L1 and L2 capsid proteins assembly into a virion particle takes place in the cellular nucleus as the last phase of the infectious process. This delayed production represents a good viral strategy as both proteins are highly immunogenic and could trigger the early immune reaction. L2 is synthesized before L1 does (Florin et al., 2002). L1 proteins form pentametric capsomeres in the cytoplasm and these structures are then transported into the nucleus. There, L2 is incorporated at the center of L1 protein capsomeres so the fully mature virion is formed. (Middleton et al., 2003) Virions are shed from the epithelial surface but as such they can survive only some time, so they commonly reinfect cells at sites adjacent to where they are shed (Bryan & Brown, 2000).

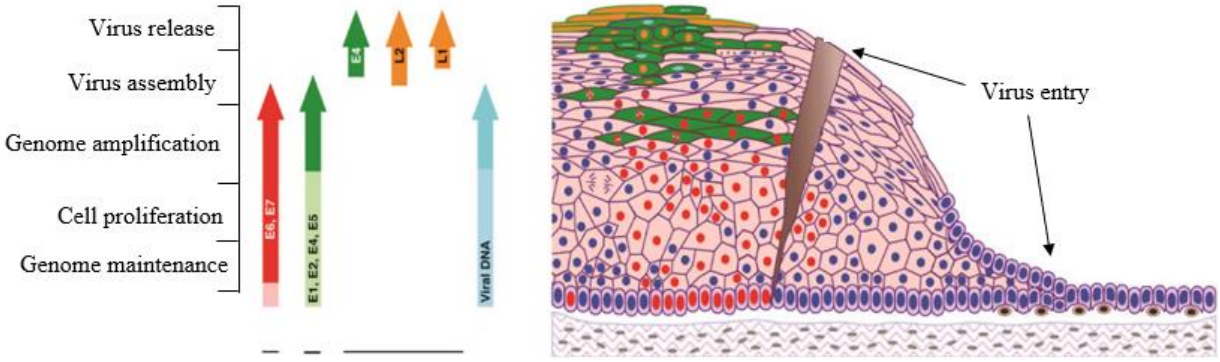


Figure 5: The progression of the HR-HPV infection. Basal cells in the cervical epithelium create one bottom layer where the HPV infection begins. These cells divide actively and supply the upper layers with new cells. Different events in the virus life cycle are triggered at different stages during this migration –in the bottom layers, the early HPV genes E6 and E7 are expressed to stimulate cells to divide themselves. In the mid-layers, proteins E1, E2, E4, and E5 necessary for genome amplification become elevated in these cells, allowing genome amplification to occur. In the upper layers of epithelium, the late genes L1, L2, and E4 are expressed. L1 and L2 proteins enclose the viral genome to form new virions (Doorbar et al., 2012).

The papillomavirus lifecycle takes 2–3 weeks. This time is necessary for a cervical cell to migrate from the basal to the outmost layers of the epithelium, mature, undergo senescence, and die (Graham, 2017).

2.1.4.6 Pathway of Tumorogenesis from Premalignant Stages

HPV infection has a smart way of immune invasion that facilitates staying undetected for a long period of time (Schiffman et al., 2007). The virus avoids causing cell death, necrosis, or a viraemic phase that would activate an inflammatory response. Viral antigens might be detected only in a superficial layer of epithelial cells destined for shedding out (Frazer, 2009). The key change required for progression to malignancy is increased expression of the viral oncoproteins E6 and E7 in infected epithelial cells. E6 and E7 oncoproteins are very efficient at blocking negative regulators of the cell cycle which support the accumulation of genetic damage (Crosbie et al., 2013).

2.1.5 Risk Factors of HPV-associated Cancers

The fact that most people get HPV infection as young adults suggests that the decades-long process of cancer progression and the rareness of this progress must be influenced by secondary multifactorial events cooperating with persistent HR-HPV infection that leads to cancer establishment. There are certain risk factors contributing to the severity of HPV-associated disease followed by cervical cancer development (Graham, 2017).

Certainly, immune status (immunocompromised and immunosuppressed individuals) and high-risk human papilloma type seem to be the clear risk factors for progression of the HPV infection. The rest implicated factors are highly probable but need further research (Crosbie et al., 2013). Several studies have reported that smoking, early age of first intercourse and certain sexual behavior as high number of sexual partners (Balasubramaniam et al., 2019) recent oral contraceptive use, and *Chlamydia trachomatis* infection have also been implicated (Crosbie et al., 2013). Other additional factors such as alcohol or diet have not been established (Burd, 2003).

However, more recently, the evidence that the vaginal microbiome can have either a protective or stimulatory effect on cervical disease progression has begun to be at the forefront of research (Kyrgiou et al., 2017). They indicate that the specific composition of the bacterial population might have different effects on anogenital HPV persistence (Groves & Coleman, 2015). In addition, the composition of vaginal microbiota might be a population-level risk factor for HPV that has largely been overlooked (Borgogna et al., 2020).

2.1.6 HPV-associated Diseases

It has already been stated that HR-HPVs are tumor-promoting agents. The progression of cancer, however, is not a good outcome for HPV. Since tumor cells regress the viral replicative life cycle and progeny virions cannot be produced. It is rather a rare event (Graham, 2017).

On the other hand, HPV-associated diseases or cervical diseases are very common because every case of cervical cancer is foregone by the cervical disease which results directly from HVP infection (Berman & Schiller, 2017), and HPV infection acquires almost all sexually active adults (Dunne et al., 2007).

The cervical disease is categorized either into a three-stage system – "cervical intraepithelial neoplasia" (CIN) or a two-stage system – "squamous intraepithelial lesion" (SIL). CIN is distinguished into CIN1, CIN2, and CIN3 according to the severity of cancerous cell progression (Figure 6). CIN1 equals low-grade (L) SIL, CIN2 or CIN3 to high-grade (H) SIL. Progress from CIN1 to 3 (or LSIL to HSIL) is clearly recognizable under the microscope (Doorbar et al., 2015).

CIN1 is associated with transient HPV infection that has a very low-grade histologic change. Also, infection with multiple HPV types is typical for CIN1. Approximately 80–90% of cases of CIN1 regress. Mostly, the repetition of cytology test is recommended (Martin & O'Leary, 2011).

CIN2 or CIN3 in most cases represents a persistent infection. This results in precancerous disease, but even for CIN3, a lower percentage of high-grade lesions spontaneously clear (40% for CIN 2 and 33% for CIN 3), and a higher percentage progress to cancer if not treated (5% for CIN 2 and more than 12% for CIN 3). Treatment of these lesions prevents the progression to invasive cancer (Crosbie et al., 2013).

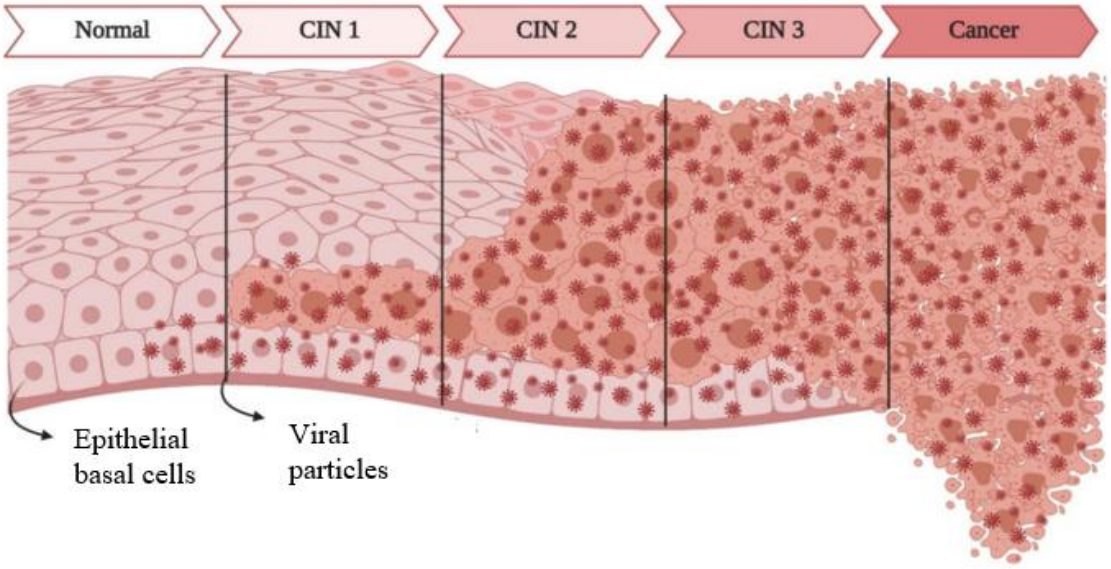


Figure 6: Cervical intraepithelial neoplasia – CIN1, 2, and 3 represent three grades of precancerous lesions due to persistent infection and up-regulation of E6 and E7 oncoprotein expression. CIN may lead to cervical cancer formation (Causin et al., 2021)

Sometimes, "atypical cells of undermined significance" (ASC-US) is termed when reporting equivocal results (Burd, 2003).

2.1.7 HPV-associated Cancers

HPV infection has been related to a few other cancers such as vaginal, vulvar, anal, oropharyngeal, and penile. They are associated with high-risk types of HPV infection and the malignant progression is preceded by HPV-associated disease (e.g., vulvar intraepithelial neoplasia, VIN or anal intraepithelial neoplasia, AIN). It is important to state that even those types of cancers are caused due to HPV sexually transmitted nature (Serrano et al., 2015).

The numbers of HPV infection are high – 84 % of VIN and 74 % of vaginal cancer has been caused by HR-HPV infection (Graham, 2017).

As has been found out, penile cancer is an exception as it could be associated with low-risk types HPV 6 and 11 (Anic & Giuliano, 2011).

Anal cancer affects women and homosexual men. This cancer is significantly connected to HIV-positive patients (C.-C. J. Wang et al., 2017).

2.1.8 HPV Screening Tests

In the Czech Republic, cervical cancer is the second most frequent malignant disease right after breast cancer. Approximately 1,000 women are diagnosed each year, and one

woman dies of it every day. But this can be partly avoided by well-timed prevention in a form of well-available screening tests.

According to Decree No. 70/2012 Coll of the Ministry of Health of the Czech Republic, every woman in the Czech Republic aged 15 and older is entitled to a preventive gynecological examination, including cytological examination, at one-year intervals (Sehnal & Sláma, 2020).

Screening tests allow to start with immediate treatment of precancerous stages. Currently, there are two types of preventive examination of the cervix: the Papanikolaou test (Pap test) and the HPV test.

Pap test is a cytological test when smear collection is done. Cells are scraped from the surface of the cervix (squamous epithelial cells) and then examined under the microscope for abnormally shaped cells or abnormally dividing cells – so-called koilocytes. They possess a compressed nucleus with a perinuclear vacuole (Figure 7).

However, the sensitivity of this method is limited and allows detection of around 50% to 70% of abnormal koilocytes (Tachezy & Rob, 2007).

Every abnormal finding or unclear result in Pap test needs a follow-up test. This leads to the use of HPV testing (Sehnal & Sláma, 2020).

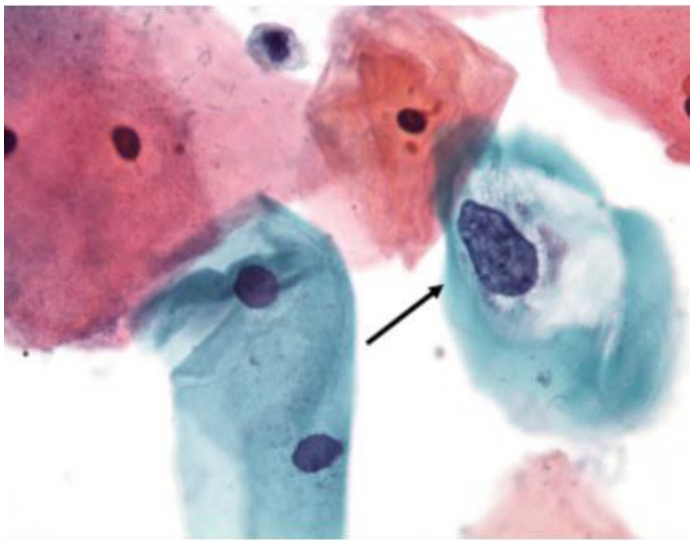


Figure 7: Koilocytes are mature squamous cells with recognizable nuclear enlargement and cytoplasmic alterations caused by the productive HPV infection. Binucleation is also frequent. Papanicolaou stain, original magnification 1000 × (Paintal & Nayar, 2014).

HPV test is based on a molecular technique for the detection of present viral DNA or RNA and thus, the presence of HPV infection in the patient´s body. This testing is considered a remarkable diagnostic tool for the identification of HPV.

Several methods of molecular assays that are available for HPV detection in tissues and tissue samples. An example could be southern transfer hybridization (STH), dot blot hybridization (DB) and *in situ* hybridization (ISH), polymerase chain reaction (PCR), or E6/E7 mRNA is detected by reverse transcriptase PCR by nucleic acid sequence-based amplification (NASBA) (Zaravinos et al., 2009).

The use of both Pap test and follow-up HPV test to screen for cervical cancer is called co-testing.

In the Czech Republic, HPV testing is performed to stratify patients with unclear results of a PAP smear result. From January 1st, 2021 HPV DNA test is paid for as a part of prevention for women at the age of 35 and 45 and is reimbursed by all Czech health insurance companies (Sehnal & Sláma, 2020).

Colposcopy does not belong among the preventive examination, however, women should be referred for this when further detailed examination of the cervix, vulva, and, vagina is needed. The examination is performed with a colposcope, a microscope with magnification, and strong illumination. An instrument called a speculum is placed into the vagina to help see the cervix. First, a small amount of iodine on a swab is applied to see any abnormal cells on the surface layer of the cervix. Then, a biopsy is taken for further testing (Burness et al., 2020).

2.1.8.1 HPV Genotyping Screening

In general, viruses are classified based on their serotype - surface antigens allowing the classification of the viruses into subspecies. HPV is an exception as it is classified by its genotype (see Paragraph 2.1.1) (Stanley, 2010).

The HPV genotyping enables to identify of the exact causal HPV genotypes and, in connection with this, determines whether it has carcinogenic potential (Venetianer et al., 2020). On the other hand, HPV DNA testing, a gold standard in the HPV screening, just indicates the presence of the HPV DNA in the epithelium (Zaravinos et al., 2009).

Nowadays, different genotyping commercial kits are available (Shilling et al., 2020) – e.g., PapilloCeck® is based on the identification of a fragment of the E1 HPV gene using a DNA microarray. Additional HPV genotyping kits have been compared and listed by Arbyn *et al.* (Arbyn et al., 2021).

In medicine, HPV genotyping is considered as a secondary testing method after Pap cytology test and HPV test – and thus is not part of annual screening at gynecologist. More likely, genotyping is used in clinical studies and it used to have crucial meaning in vaccination development (Venetianer et al., 2020).

2.1.9 Vaccination

Since the linkage between the causative agent (HPV) and cervical cancer has been discovered, it allowed to start working on vaccination representing a fundamental preventive strategy against the HPV infection (Crosbie et al., 2013).

To date, three prophylactic vaccines – Gardasil, Gardasil 9, and Cervarix – have been introduced on the Czech market to precede infection with multiple types of HPV (Sehnal et al., 2022). They all are recommended to both boys and girls since both may be vectors of this infection. Additionally, boys are endangered by cancer of anus, penis, or oropharynx caused by HPV.

All vaccines have been shown incredibly high immunogenicity. The immunogen responsible for the immune response and protection is represented by virus-like particles (VLPs). These are put together from non-infectious recombinant L1 capsid protein specific for the type of HPV and lack viral DNA so the vaccine cannot induce the infection. However, it stimulates the production of neutralizing antibodies by B-lymphocytes (Pinto et al., 2018). The task of these antibodies is blocking the human papillomavirus virions ahead of their accessing the proliferating basal cells (Crosbie et al., 2013).

Nonavalent Gardasil 9 (Merck & Co, Whitehouse Station, NJ, USA) vaccine makes for preventing infection with 9 HPV types (6, 11, 16, 18, 31, 33, 45, 52, 58), quadrivalent Gardasil (Merck & Co, Whitehouse Station, NJ, USA) contributes to preventing infection with 4 HPV types (6, 11, 16 and 18) and bivalent Cervarix (GSK, Rixensart, Belgium) helps preventing infection with HPV types 16 and 18.

Gardasil and Gardasil 9 have 3 doses vaccination scheme when the second and third is given in 2 or 6 months, respectively, after the first dose. Cervarix also requires 3 doses in 0, 1 and 6 months (Rosalik et al., 2021). Recently it was approved to be given with only 2 injections at 0 and 6 months in girls and boys younger than 15 years (Kamolratanakul & Pitisuttithum, 2021).

Despite of vaccination implementation and its high efficacy, annual screening tests are still recommended. In the future, it may allow intervals between screening to be prolonged (Crosbie et al., 2013).

To provide complete information on HPV vaccination, nowadays, there has been an effort to develop therapeutic vaccines which would help with the healing process. They focus on E6 and E7 oncoproteins responsible for the initial changes in the epithelium (A. Yang et al., 2016).

2.1.10 Potential Biomarker of Precancerous Lesions and Cervical Cancer

The research about biomarkers of different molecular nature is essentially important. With the activation and suppression of biological processes, these biological molecules might vary, not only in quantity, but also regarding their shape, interactions, location, and activity (B. J. Smith et al., 2021).

These molecules have great potential to become molecular indicators of cancer risk assessment, screening and early detection of cancer, accurate diagnosis, patient prognosis, prediction of response to therapy, and cancer monitoring.

To be introduced to screening programs and clinical applications as such, biomarkers must accomplish several criteria – e.g., they have to be measurable in an obtained sample. The type, and amount of sample is also very important. The non-invasive sampling is preferred. Biomarkers are represented by proteins, nucleic acids, or lipids. (Sarhadi & Armengol, 2022).

Regarding cervical cancer, biomarkers such as p16/Ki67 dual immunostaining, methylation of viral DNA, E6/E7 mRNA detection, and others have been proposed as new possibilities in a strategy of cervical cancer diagnostics (Sehnal & Sláma, 2020).

2.1.10.1 HPV Protein Biomarker: p16/Ki-67

P16 is a protein of a cell that is upregulated during HPV infection. It serves as an HPV independent biomarker of cervical cancer. p16 immunostaining protocols have been developed for cervical cytology and histology. When a patient is p16-positive, she is referred to colposcopy immediately (Wentzensen et al., 2016).

Ki-67 represents a cell proliferation biomarker. The staining assay was expanded by this other protein to be more objective – now, finding one cell positive for p16/Ki-67 dual immunostaining is considered a positive result (Wentzensen et al., 2012).

2.1.10.2 HPV Protein Biomarker: E6 and E7 oncoproteins

The aforementioned E6 and E7 oncoproteins have a crucial role in HPV infection development. Their expression is higher in cervical precancers compared to transient HPV infections. A lateral flow assay was developed to detect the E6 oncoprotein from the most important carcinogenic types, HPV16, 18, and 45 (Wentzensen et al., 2016).

2.1.10.3 HPV Epigenetic Biomarker: Host DNA Methylation

Increased methylation of host genes seems to be a biomarker that enables to distinguish between acute HPV infection, precancer and cancer. Three methylation markers, CADM1, MAL, and miR-124-2 have been evaluated extensively in cervical cancer screening studies (De Strooper et al., 2014). Molecular testing for methylation markers offers several advantages – molecular tests are not subjective, may offer higher throughput, and can be conducted from a variety of specimen types, including self-sampling specimens. This offers the possibility of conducting primary screening from a self-collected specimen (Wentzensen et al., 2016).

Gene methylation is related to the HPV genome as well. A characteristic pattern was observed with increased methylation particularly in the E2, L2, and L1 regions. Individual CpG sites from these regions showed a good discrimination between HPV infection and precancer, suggesting that HPV methylation (Arbyn et al., 2014). The exact role of viral DNA methylation remains unclear (Galván et al., 2011).

2.2 Lipidomics

Lipids are organic compounds representing very important part of the cells, tissues, and organs in living organisms. They hold a function in vital cellular processes such as energy metabolism, signaling, and are also significant constituents of cell membrane structure (Kvasnička et al., 2023). An imbalance in their homeostasis can lead to serious condition such as chronic inflammation (Züllig et al., 2020). Therefore, lipid-focused research might provide a piece of information about dysregulation considering the pathogenesis of many diseases. With clinical data involved, this research may be seen as an efficient tool in the discovery of new useful biomarkers (Kvasnička et al., 2023).

Lipids assembly in a cell, a complete lipid profile, is referred to as a lipidome (Züllig et al., 2020).

Lipidomics is a relatively new field of study with its twenty years long history but to date, it has been referred to as one of the fastest-expanding research field of the last decade proved by submitted publications on a lipidomic topic (Züllig et al., 2020). This

progress is possible due to mass spectrometry-based technologies to identify and profile small molecules (Züllig et al., 2020).

2.2.1 The Potential Lipidome of the Cervical Cancer

Recently, several studies have been published suggesting the vaginal microenvironment has been an important factor in both progression and regression of HPV infection and the further development of cervical cancer. The vaginal microenvironment consists of the microbially- and host-produced metabolites and microbiota made up of commensal bacteria in the vagina. All three factors together have the ability to influence the clinical status of the patient (Borgogna et al., 2020; Kyrgiou et al., 2017).

Modifications in the lipidome have been established in almost all types of cancer which proves cancer being a metabolic disease (Wishart et al., 2016). It was possible to find some in cell lines *in vitro* but also at the level of the tumor, surrounding tissues, and in peripheral biofluids. However, the impact of HPV infection on lipidome composition still remains relatively unexplored (Wolrab et al., 2019).

Ilhan *et al.* studied the linkage between metabolite alternations, vaginal microbiome, and cervical cancer. They reported metabolic profiles that distinguished HPV-positive from HPV-negative women. They described high abundancies of lipid metabolites – specifically plasmalogens, sphingomyelins, phosphatidylcholines, and long-chain polyunsaturated fatty acids in the cervical cancer patients. These results could be explained by an increase in cell proliferation and cell membrane synthesis through activation of oncogenic pathways in the tumour microenvironment (Ilhan et al., 2019).

Borgogna *et al.* have observed the differences in the vaginal metabolome of HPV-positive and HPV-negative women in the matter of several metabolites, including biogenic amines, glutathione, and lipid-related metabolites. They found out that lyso- and monacylglycero- lipids were significantly different between high-risk HPV-positive and low-risk HPV-positive women. Additionally, higher biogenic amine and phospholipid concentrations were observed in HPV+ women compared with HPV- women. However, they emphasize the importance of further research on metabolite differences caused by HPV status (Borgogna et al., 2020).

2.2.2 Polyunsaturated Fatty Acids and their Metabolites

Essential polyunsaturated fatty acids (PUFA) – omega-3 and omega-6 – need to be obtained from diet since human body does not possess enzymes to synthesize them *de novo* or to convert omega-3 into omega-6 and vice versa (Ponnampalam et al., 2021)

PUFAs are important components of the cell membrane. They give it fluidity and enable membrane transport. Moreover, they are part of many biologically important substances – eicosanoids, prostaglandins, leukotrienes, or thromboxanes. These represent intracellular signaling lipid mediators that influence blood clotting, pain, or inflammation.

The maintenance of balanced omega-6/omega-3 ratio is tremendously important. This is because omega-3 and omega-6 and their derivates are characterized by opposing effect on human body. Whereas omega-6 appears to have a pro-inflammatory effect, omega-3 gives rise to a class of anti-inflammatory/pro-resolution lipid mediators. The failure to resolve inflammation might lead to chronic disease or tissue damage (Serhan et al., 2008).

2.2.2.1 Omega-3 Fatty Acids

Omega-3 fatty acids involves α-linolenic acid (ALA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Their metabolic pathway is depicted in Figure 8. ALA can be found in plants, whereas DHA and EPA are found in algae and fish.

DHA- and EPA-derived metabolites promote the resolution of inflammation. These molecules seem to stop further infiltration of immune cells and thus stimulate the return to homeostasis. After the rapid release of lipid precursors from the plasma membrane, various enzymes in a complex network metabolize them to produce a large range of lipid metabolites (Tam, 2013). The ALA metabolites 13-HOTrE and 13-HDoHE effects the anti-inflammatory and pro-resolution processes (Groeger et al., 2010). Furthermore, 18-HEPE, 17- HDoHE, and 14-HDoHE can be metabolized into resolvins, protectins, and maresins – so-called anti-inflammatory specialized pro-resolving mediators (SPMs) (Serhan et al., 2009). These lipid mediators can prevent further infiltration of immune cells to the site of infection and prompt the phagocytosis of apoptotic immune and epithelial cells. Their involvement in inflammation allows faster establishment of homeostasis (Serhan et al., 2011).

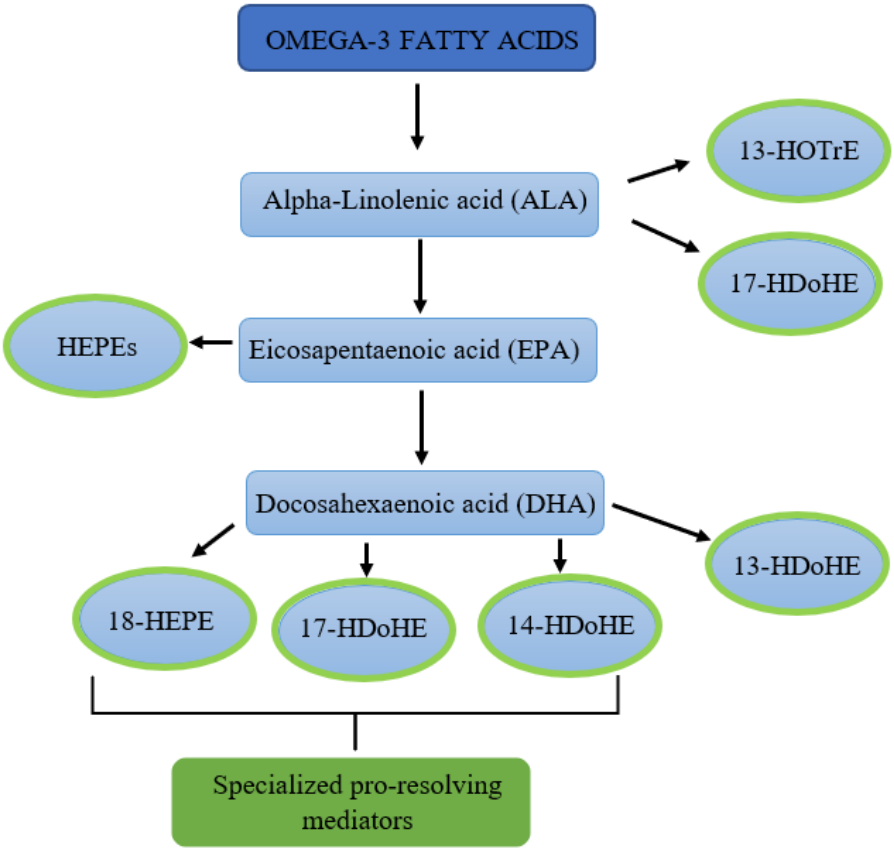


Figure 8: Simplified omega-3 metabolism pathways. Green circle stands for the anti-inflammatory/pro-resolution activity of the lipid mediators (Tam, 2013).

2.2.2.2 Omega-6 Fatty Acids

Omega-6 fatty acids – linoleic acid (LA) and arachidonic acid (AA) – contribute significantly to the fatty acids present in membrane phospholipids of cells involved in inflammation. LA can be converted to AA (Figure 9).

LA can be metabolized into hydroxylated linoleic acids 9-HODE and 13-HODE. They act as an antagonist since 9-HODE is ascribed a pro-inflammatory role, while 13-HODE plays a pro-resolution role (Schulze-Tanzil et al., 2002).

AA is metabolized into inflammatory eicosanoid lipid mediators including prostaglandins and leukotrienes. They play a role in the regulation of a wide variety of physiological as well as pathophysiological inflammatory responses (R. Yang et al., 2011). There are three major metabolic pathways for the enzymatic biogenesis of AA – the cyclooxygenase pathway (COX), lipoxygenase pathway (LOX), and cytochrome P450 pathway. The cyclooxygenase pathway (COX-1 and COX-2) produces prostaglandins ($PGF_{2\alpha}$ and PGE_2) and thromboxanes (TXA_2, TXB_2) (Funk, 2001). Although many prostaglandins have pro-inflammatory activity because of their

vasomodulatory effects, PGE_2 – the most abundant prostaglandin in a human body – seems to be involved in both pro- and anti-inflammatory reactions. It expands arterial dilation, increases microvascular permeability, and induces pain (Funk, 2001). On the other hand, the anti-inflammatory activity is represented by up-regulating cAMP and inducing the secretion of interleukin 10 (IL-10) and anti-inflammatory cytokines (Sha et al., 2012).

The lipoxygenase pathway (5-LOX, 12-LOX, and 15-LOX) then produces leukotrienes (LTB_4), and numerous hydroperoxyl (HPETEs), hydroxy fatty acids (HETEs), hepoxilins and lipoxins. Interestingly, HETE lipid mediators being in the same group of metabolic products have opposing effects. 5-HETE represents pro-inflammatory lipid mediator produced by the 5-LOX metabolic pathway. In contrast to that, 12- and 15-HETE possess anti-inflammatory activities and are derived from 12- and 15-LOX (Krönke et al., 2009, p. 15).

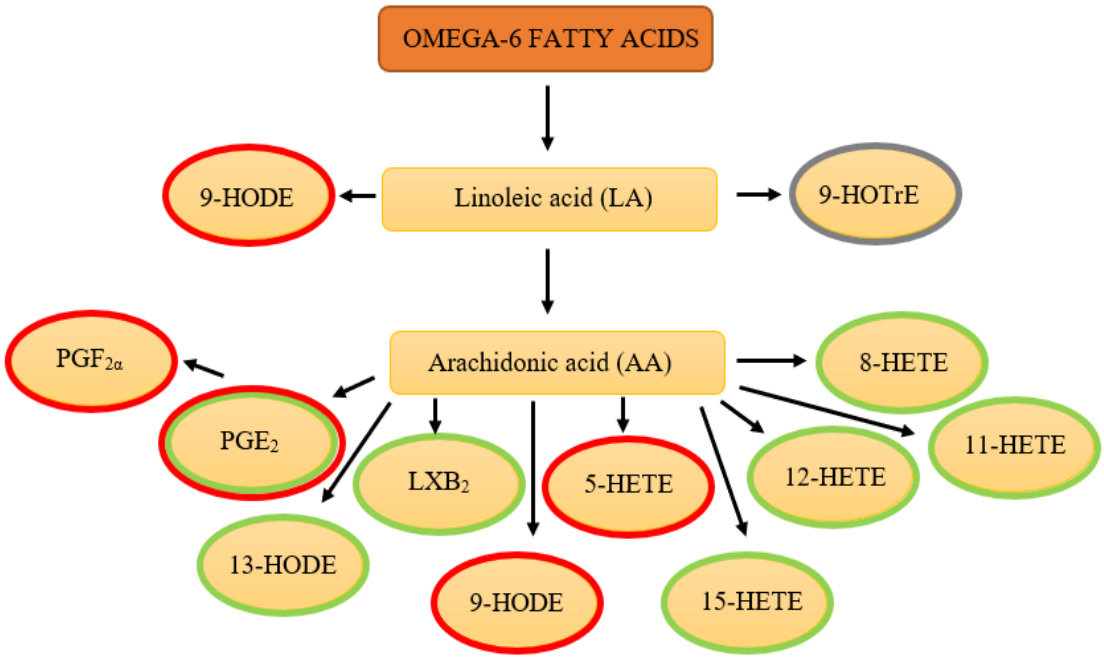


Figure 9: Simplified omega-6 metabolism pathways. Green circle stands for anti-inflammatory/pro-resolution activity and red for the pro-inflammation activity of the lipid mediators. The grey circle symbolizes an unknown function (Tam, 2013).

Finally, the cytochrome P450 pathway produces epoxides and corresponding dihydroxy metabolites of arachidonic acids (EETs and diHETrEs). EET and diHETrE have anti-inflammatory effects (Holtzman, 1992).

2.2.3 Lipidomic Analysis

Analyzing lipids might be a challenge because of their chemical complexity and branched metabolic pathways. Many factors must be considered that may affect the acquired lipid profile and its reproducibility.

Lipids in the samples may be analyzed using different approaches. However, certain steps in the lipidomic workflow must be followed in the pre-analytical, analytical, and post-analytical phases. The lipidomic workflow (Figure10) includes the design of the experiment, sample collection, sample preparation (including homogenization, extraction, and derivatization, if needed), quality control, the analysis itself (chromatography-mass spectrometry), data processing, statistical evaluation and validation (Züllig et al., 2020).

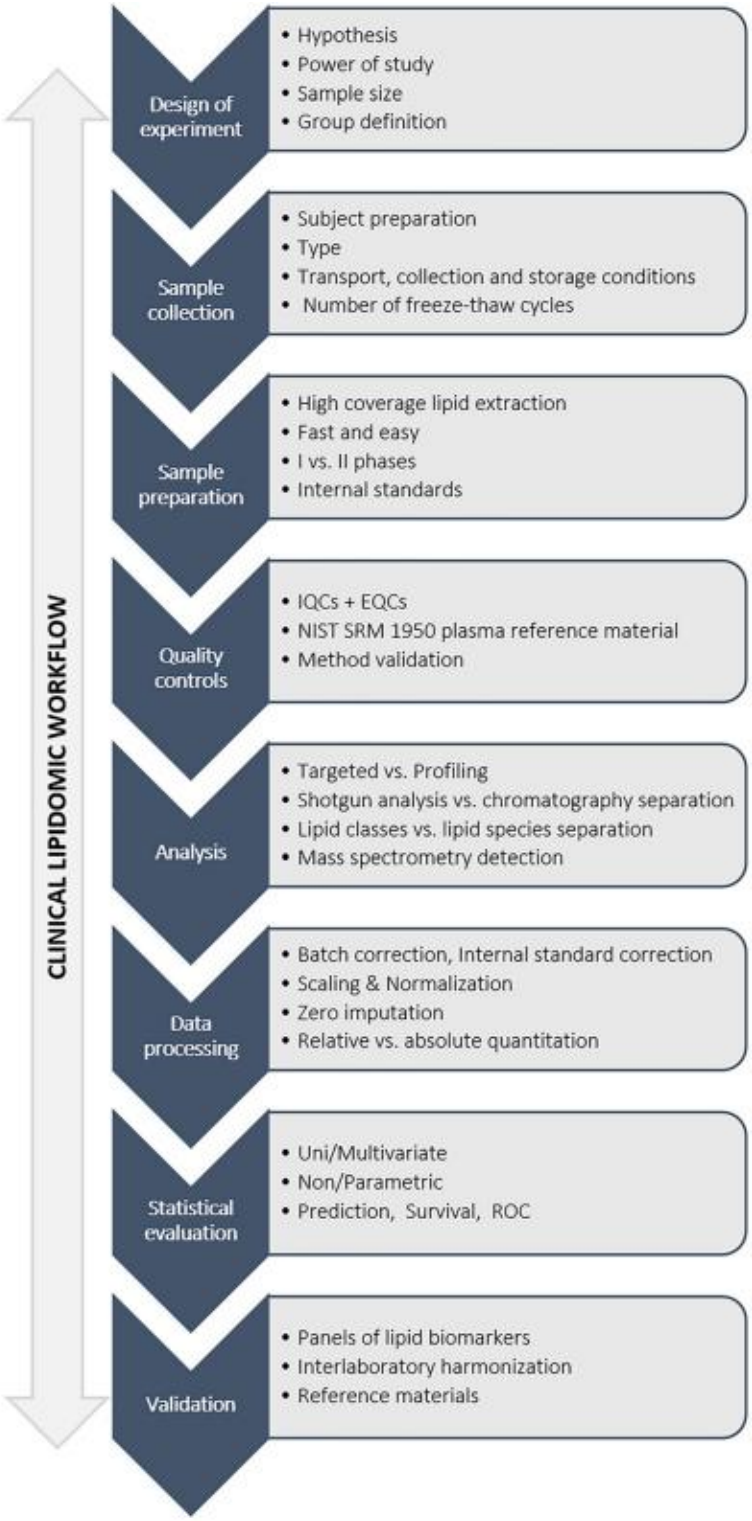


Figure 10: Overview of lipid analysis used in clinics. IQCs, internal quality controls; EQCs, external quality controls; ROC, receiver operating characteristics (Kvasnička et al., 2023).

2.2.3.1 Lipidomic Profiling

The goal of lipidomic profiling lies in the knowledge and selection of individual lipids – biomarkers differentiating between a cohort of healthy individuals and patients (Kvasnička et al., 2023).

Cervicovaginal lipidome determined by targeted lipidomic profiling may have the potential to reveal the linkage between the composition of lipids and the progression of the human papillomavirus infection. Lipid composition may vary when comparing healthy HPV-negative (HPV-) and HPV-positive (HPV+) patients (Ilhan et al., 2019).

2.2.3.2 Targeted versus Untargeted Lipidomics

There are two applied approaches in lipidomics for the research of small molecules – untargeted and targeted lipidomics.

Targeted approach involves selecting specific lipids so there is a need for preceding knowledge of the molecule of interest – this involves its structure, exact mass, and how it behaves during MS fragmentation (Carrasco-Pancorbo et al., 2009). It strives to quantify lipids within a sample and achieves high sensitivity and specificity (Kvasnička et al., 2023).

On the other hand, untargeted lipidomics does not choose certain molecules but searches for all lipids in a sample with an application of high-resolution MS instruments (Orbitrap, Quadrupole, TOF). The unknown exact masses are then compared with available database in order to characterize the molecules (Carrasco-Pancorbo et al., 2009).

2.2.3.3 Mass Spectrometry of Lipids

Mass spectrometry (MS) has been an extensively used analytical tool nowadays. It possesses the ability to accurately measure a large quantity of lipid molecules to determine the lipidomic profile. Moreover, it achieves high selectivity and sensitivity (Kvasnička et al., 2023).

The electrospray ionization (ESI) technology allows for volatilizing lipids from an aqueous sample with no need of prior derivatization. Lipid species are extracted from samples using solid phase extraction (SPE) to elevate sensitivity and specificity and deuterated analogs of analytes are added as internal standards (Deems et al., 2007).

High-performance liquid chromatography (HPLC) and collision-induced decomposition, when combined with ESI-MS and multiple reaction monitoring (MRM), can isolate and identify individual lipid species by matching their MRM signal and LC retention time with those of the pure standards. Quantification is achieved using the stable

isotope dilution method and comparison with quantitation standards (Hall & Murphy, 1998).

3 EXPERIMENTAL PART

3.1 Sample Collection and Processing

The cervicovaginal smear samples were obtained within the HPVpro study. This study had been approved by The Ethics Committee (Protocol no. 97/13).

Samples were collected by the gynecologists at University Hospital, Olomouc, and in a gynecological ward in the Moravian-Silesian region.

The inclusion criterium was the age 30-64. Participants must not be pregnant, have a history of CIN or cervical cancer, an increased risk of bleeding, cervical conization, or hysterectomy. Samples were provided with clinical data of a patient and HPV status (HPV-negative or HPV-positive) determined based on HPV genotyping.

Sample collection was performed using The Cervex-Brush (Rovers Medical Devices B.V., Oss, Netherlands). After collection, the samples were stored at RT in ThinPrep® Pap Test PreservCyt® solution based on 50% methanol (Hologic, Inc. Marlborough, USA). All samples were tested with 3 HPV detection methods: The digene® HC2, cobas 4800, and QIAscreen. The positive samples proceeded to HPV genotyping with the PapilloCheck kit.

I was working with a subset of 74 cervicovaginal smear samples of healthy HPV-negative controls [n= 37] and HR-HPV-positive samples [n=37].

Clinical data for each sample are summarized in Excel sheet in the Additional File 1.

The samples had a volume of 2 mL overall. I prepare two aliquots, each of 1 mL, so measurements in two different laboratories could undergo. PUFAs were measured at The Leiden University Medical Center (LUMC) in Leiden, The Netherlands. The other 1 mL has been stored at -80°C for the future measurement of ceramides and ergosterol at The Institute of Molecular and Translational Medicine (IMTM) in Olomouc, The Czech Republic.

3.2 Instruments

- LC system (Sil-40C X3; Shimadzu)
- Mass spectrometer (QTrap 6500; Sciex)
- VWR Mixer mini vortex
- Centrifuge (Eppendorf 5415 R)
- Nitrogen evaporator (Biotage, Turbovap Lv, Sweden AB)
- Ultrasonicator (VWR Ultra sonic cleaner, part of Avantor)

- Fume hood

3.3 Chemicals and Solutions

- Methanol, LC-MS grade (MeOH; Honeywell)
- Water, LC-MS grade (Honeywell)
- Water, HPLC grade (Biosolve)
- 10% formic acid in HPLC grade water (VWR)
- N-hexane (VWR)
- 99% methyl formate, spectrophotometric grade (Honeywell)
- Acetic acid (Honeywell-Reidel de Haën)
- Mobile phase A
    - 0.01% acetic acid in water, LC-MS grade (Honeywell-Reidel de Haën)
- Mobile phase B
    - 0.01% acetic acid (Honeywell-Reidel de Haën) in MeOH, LC-MS grade (Merck)
- Internal standard solution (IS):
    - PGE2-d4 in MeOH (Prostaglandin E2-d4; 50ng/mL; Cayman)
    - LTB4-d4 in MeOH (Leukotriene B4-d4; 50ng/mL; Cayman)
    - 15-HETE-d4 in MeOH (15-Hydroxyeicosatetraenoic Acid-d4; 50 ng/mL; Cayman)
    - DHA-d5 in MeOH (Docosahexaenoic acid-d5; 500ng/mL; Cayman)
    - 8-iso-PGF2α-d4 in MeOH (8-iso-prostaglandin-2α-d4; 100ng/mL; Cayman)
    - 14(15)EET-d11 in MeOH (14(15)-epoxy-eicosatrienoic acid; 50ng/mL; Cayman)
- Low lipid mediators mix in MeOH for calibration line
- High lipid mediators mix in MeOH for calibration line
- PUFA2 lipid mediators mix in MeOH for calibration line
- PUFA3 lipid mediators mix in MeOH for calibration line
- System suitability test (SST)
- Extension mix (EM)

3.4 Equipment and Consumables

- 1,5 mL Eppendorf tubes (Eppendorf)

- 5 mL Eppendorf tubes (Eppendorf)
- SPE cartridges (SepPak C18 200 mg; 3 mL; Waters)
- SPE vacuum manifold (Waters)
- 7 mL glass vial (PYREX®)
- Glass autosampler vials and cups (Agilent)
- Glass autosampler inserts (Agilent)
- Pipette tips and repetition pipette tips (Mettler Toledo, Rainin, Bioclean Ultra)
- Pasteur pipettes

3.5 Workflow

3.5.1 Transport of the Samples

When transporting to LUMC, Leiden, The Netherlands, samples were in a polystyrene box on dry ice. Nevertheless, dry ice melted during the transport, so samples were undefined time (approximately hours) at room temperature.

3.5.2 Quality Control Samples

Quality control sample (QC) represented pooled samples of a certain volume of all cervicovaginal smear samples. Out of 74 original samples, 40 µL was added to the pooled sample so it could be aliquoted into QC samples.

Since quality control is a mixture of every original cervicovaginal sample, it should have shown the average value of every measured analyte and thus ensured repeatability.

Before starting the measurement, two QC samples were measured as pilot samples so the intensities of the lipid mediators could be checked to indicate if the concentration of cervicovaginal smear samples were accurate. After quality control measurement (volume of 200 µL), it was decided that 500 µL of each cervicovaginal sample would have been used for the MS measurement.

3.5.3 Internal Standard (IS) Preparation

Isotope -labeled (deuterium-labeled) internal standards are generally added to the samples for quantification. IS has the same retention time as analytes of interest but the mass is different due to a bigger mass of deuterium. This helps with ion suppression and enables correcting the area of the LC spectra when quantifying.

In-house IS (50/100/500 ng/mL) was prepared to be added to samples including cervicovaginal samples, QC, calibration line samples, and SST. The composition of IS is attached in Additional File 2.

3.5.4 Calibration Lines Preparation

We used two calibration lines – CLR and CLP. CLR represents an external calibration line for the quantification of non-polyunsaturated fatty acids in samples. This line consists of 7 samples, each sample contains specific lipid mediators mentioned in Additional file 1.

On the other hand, CLP is a calibration line of 7 samples that enables quantifying polyunsaturated fatty acids. Its compounds are also shown in Additional File 2.

3.5.5 System Suitability Test

System suitability test (SST; 0.5 ng/mL) is a mixture of standards used as reference when running analysis.

3.5.6 Extension Mix

Extension mix (EM) contains 4 additional lipid mediators missing in SST - 13,14-dihydro-15-keto-PGE2, 12-HHTrE, RvD5, and 12-oxo-LTB4.

3.5.7 Solid Phase Extraction

Samples were stored at -80°C until ready for the SPE working procedure. In preparation for the solid phase extraction, a ratio of methanol: sample volume (3:1) was added. Then, 3 μL of in-house IS was added. After a brief vortexing, the samples were incubated for 20 minutes at -20°C to induce protein precipitation. Thereafter, they were centrifuged for 10 minutes at 4°C, 13.2 krpm. 2.75 mL of HPLC-grade water was added to samples for a purpose of diluting methanol in the sample to 15-25 %. Samples were acidified with 10% formic acid to get pH 3,5. Acidification is performed to increase the affinity for the C18 matrix in the cartridge. In the end, 8 μL of 10% formic acid was added to get pH 3,5. Samples were vortex. The SPE chamber was prepared. C18 cartridges were placed on the top of the SPE glass chamber, and several steps had to follow to activate the C18 cartridges (conditioning of C18 cartridges with 3 mL MeOH, equilibrating of C18 cartridges with 3mL HPLC-grade water). After these steps, it was possible to apply the samples. The vacuum in a chamber helped to set the rate flow – “dropwise”. After that, the columns were washed with 3 mL HPLC-grade water. Samples were then washed with 3 mL n-hexane afterward. After the latter step, all ventils had to be closed and samples

were eluted with 3 mL methyl formate and captured to 7 mL glass vials. The samples then were dried under a stream of nitrogen (40°C, approximately 40 minutes). Dried samples were reconstituted in 150 µL 40% methanol. Then, 60 µL methanol was added to each sample. They needed to be vortex after that and sonicate for a minute. Afterward, it was possible to add 90 µL LC-MS grade water. Samples were transferred from 7 mL glass vials to Eppendorf and centrifuged at 4°C for 10 minutes, 13.2 krpm. Samples were then transferred to glass inserts of the MS autosampler´s vials. Samples were stored at -80°C until the next day when the samples started to be measured.

3.5.8 Mass Spectrometry Analysis

Before starting the measurement, cervicovaginal samples and QC samples were randomized (*RANDOM.ORG - True Random Number Service*, n.d.) to avoid the batch effect. They were then submitted via Analyst software 1.7.2.

Analysis was performed on the HPLC system (Shimadzu LC system) coupled to QTrap 6500 (Sciex) equipped with an electrospray ionization (ESI) source operating in negative mode. The HPLC system consisted of the autosampler (Shimadzu SIL-30AC), pumps (2x Shimadzu LC-30AD) and column oven (Shimadzu CTO-20AC). There were used mobile phases A (LC-MS grade water + 0,01% acetic acid) and mobile phase B (MeOH + 0,01% acetic acid) at a flow rate of 400 µL/min. Before each measurement, the system was equilibrated for 1 minute. Chromatographic separation was achieved under gradient conditions (Table 1) using a Phenomenex Kinetex C18 column with C8 precolumn in a column oven (50°C). The temperature of the autosampler was maintained at 6°C. The injection volume was 40 µL.

Mass spectrometry instrument parameters were as follows: set needle voltage at -4500V, drying temperature at 450°; ion source gas 1 at 40 psi; ion source gas 2 at 30 psi; nebulizer gas (N_2) at 30 psi; the entrance potential set at -10V and the collision gas flow "medium".

Table 1: A gradient program – 11.5-minute method using a flow rate of 0.4 mL/min, and injection volume of 40 µL.

Time (min):	0.0	1.0	1.1	2.0	4.0	7.0	7.1	9.0	9.5	11.5
Mobile phase B (%):	30	30	45	53,5	55.5	90	100	100	30	30

3.5.9 Data Analysis

Data integration was performed using SCIEX OS software. The dataset in the software contains 76 possibly identified lipid mediators. Results were manually curated to ensure that data were accurate and to remove any system artifacts and misassignments.

Each analyte was identified by its mass transition and relative retention time (RRT) for lipid metabolite analysis – data with inaccurate RRT, width, and area of the peak were excluded during the integration and the integration notes of these 76 lipid mediators were recorded to the prepared Excel sheet. Excel sheets of original cervicovaginal samples and integrated data after MS analysis were submitted in an internal LUMC program based on R language (Reemst et al., 2022). The final dataset consisted of the concentration of 15 lipid metabolites. The result of the whole process of integration was again an Excel sheet with relative retention time, area ratio, and calculated concentration for every metabolite. If the value of concentration was below the limit of detection and thus was missing, no imputation of value was performed. The concentration data proceeded for subsequent statistical analysis (Additional File 3).

Data analysis was performed using Excel (Microsoft Office) and MetaboAnalyst (5.0) (Pang et al., 2021).

4 RESULTS

The protocol was set to identify 76 fatty acid mediators overall. The final lipid profile consisted of 15 fatty acid metabolites analyzed in HPV+ and HPV- samples (Table 2).

Table 2: Lipidomic profile of HPV samples. Their pro-/anti-inflammatory effect here was listed based on information found in the literature (Tam, 2013).

Lipid metabolite:	PUFA:	Pro-/anti-inflammatory effect:
DHA	Omega-3	Anti-inflammatory
EPA	Omega-3	Anti-inflammatory
DPAn-3	Omega-3	Anti-inflammatory
8- HETE	Omega-6	Anti-inflammatory
11-HETE	Omega-6	Anti-inflammatory
12-HETE	Omega-6	Anti-inflammatory
15-HETE	Omega-6	Anti-inflammatory
AdA	Omega-6	Anti-inflammatory
5-HETE	Omega-6	Pro-inflammatory
TxB2	Omega-6	Pro-inflammatory
20-OH LTB4	Omega-6	Pro-inflammatory
PGF2α	Omega-6	Pro-inflammatory
PGE2	Omega-6	Pro-/anti-inflammatory
AA	Omega-6	Pro-inflammatory/anti-inflammatory
LA	Omega-6	Pro-inflammatory/anti-inflammatory

The Student's two-sided two-sample t-test was used to perform a pairwise comparison of the means of metabolites among the groups of HPV- samples (negative controls) and HPV+ samples (positive patients). The results were plotted into box plots (Figure 11). The vertical axis gave information about the calculated concentration [ng/mL] from the MS analysis. No significant metabolites ($p<0,05$) were observed within the resulting 15 lipid metabolites. This result indicated both HP+ and HPV- samples did not differ in the matter of present metabolites.

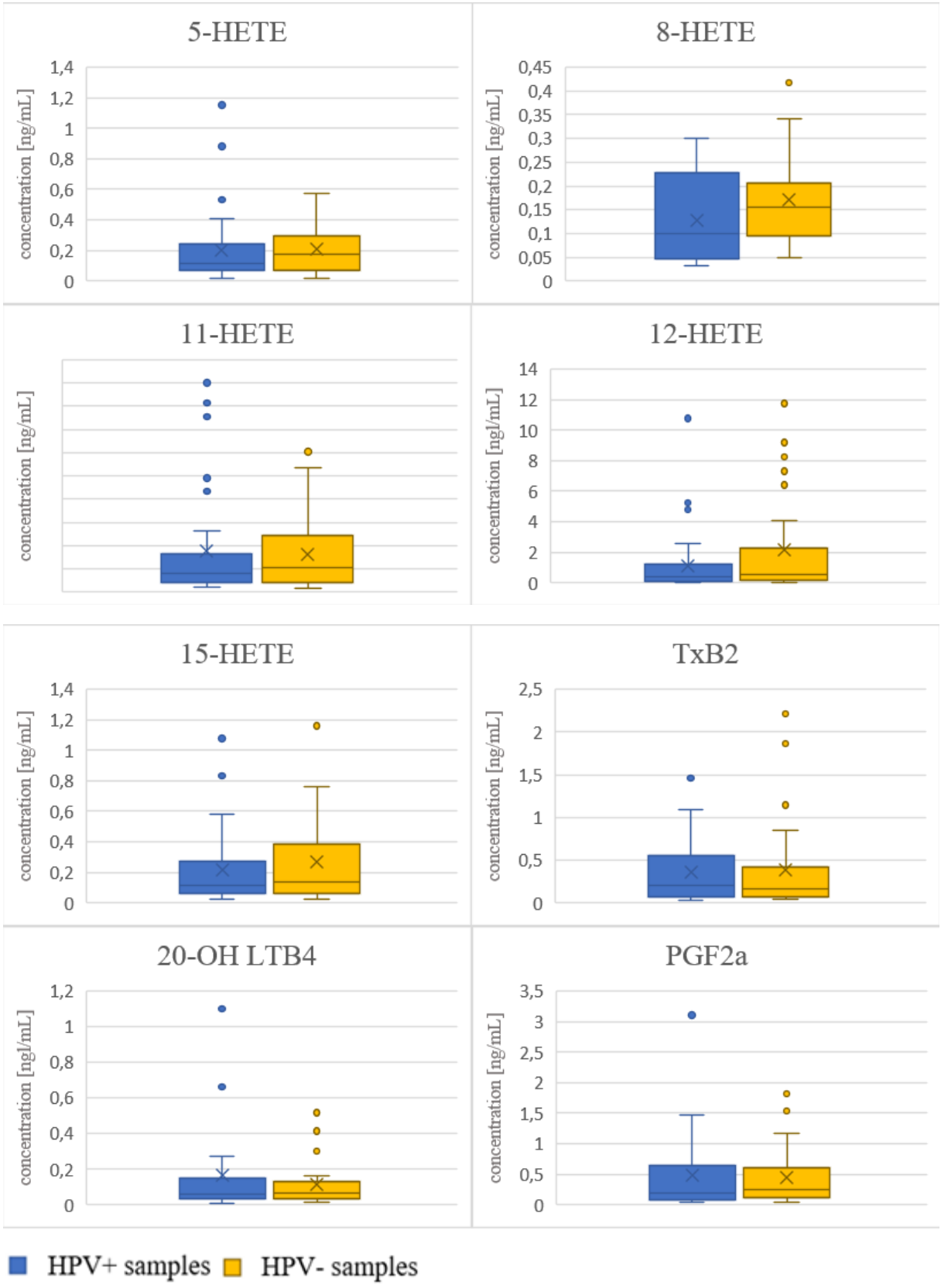


Figure 11: Each box plot compares HPV+ and HPV- samples of one metabolite out of lipidomic profile determinated in cervicovaginal smears. Blue box plot stands for HPV+ samples, yellow for HPV- samples.

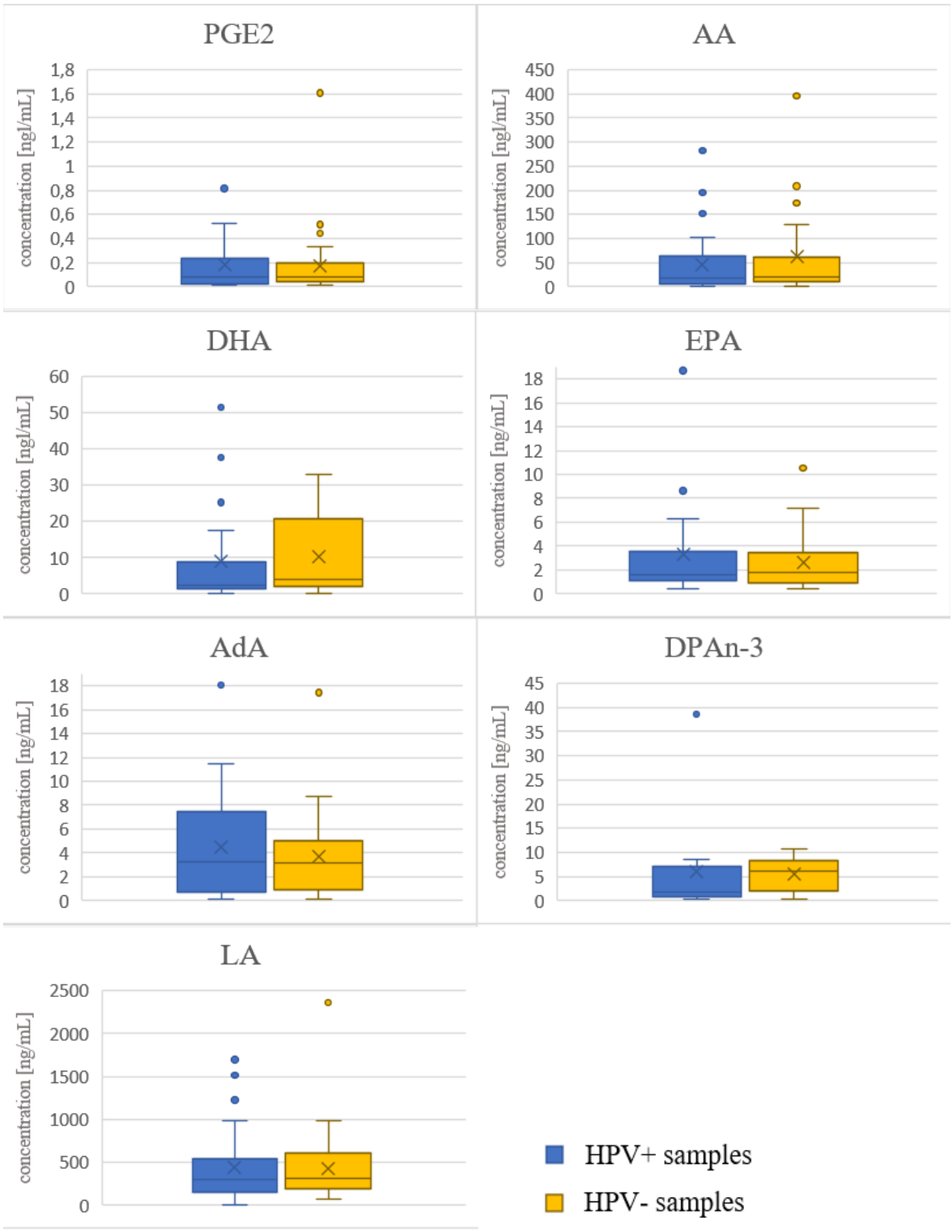


Figure 11: continued.

This result of t-test was confirmed by unsupervised Principal Component Analysis (PCA; Figure 12). PCA plot was performed to identified correlation between HPV+ and HPV- samples regarding 15 identified metabolites. Pooled samples were used for normalization of HPV samples. Principal components (PC1 22 % and PC2 15 %) demonstrated the greatest spread of data which better enable pattern recognition. Since all HPV samples clustered together on PC1 and PC2 and there was no observed distinction between samples. This confirms that HPV+ and HPV- samples do not correlate. The pooled samples were made of all 74 samples so naturally, they cluster together in the middle region of the plot.

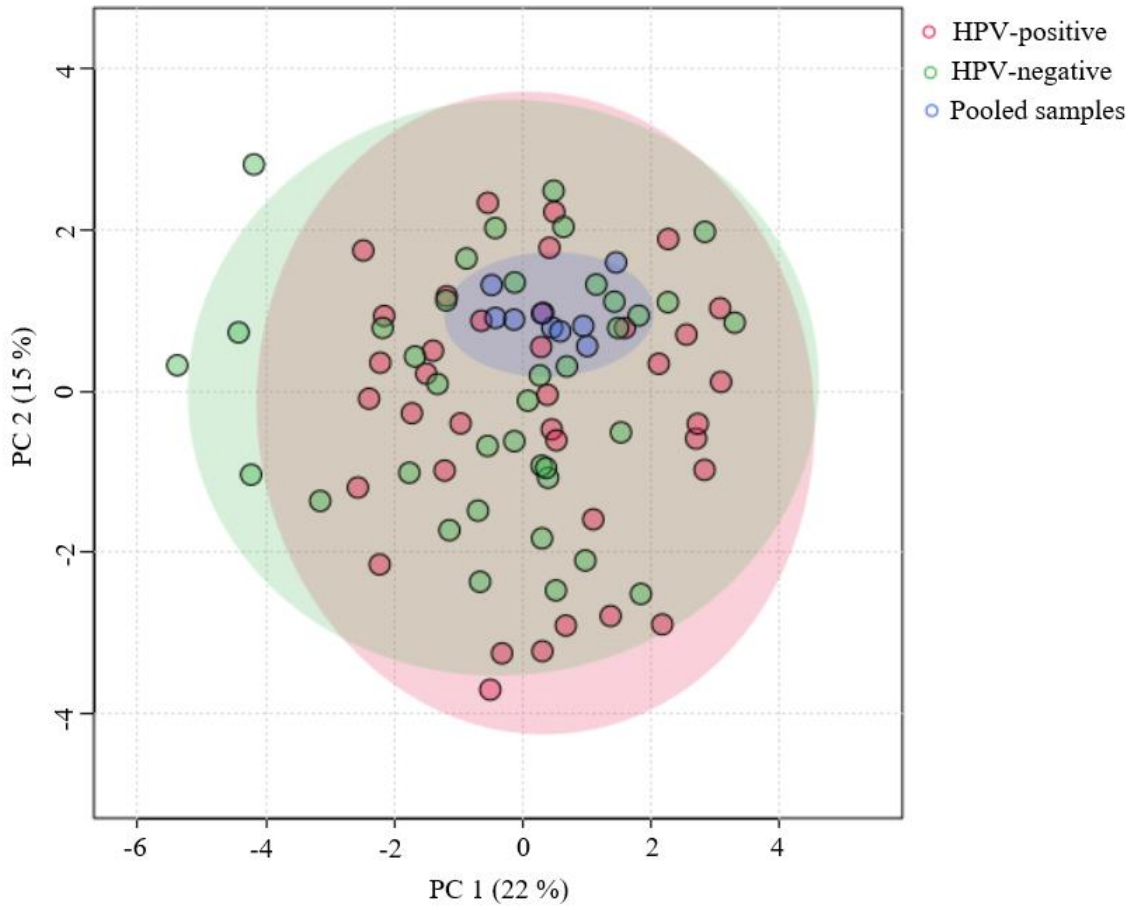


Figure 12: PCA of HPV+ and HPV- samples plotted with Metaboanalyst web. Sample normalization by a pooled sample. Data were subsequently log-transformed.

Supervised Partial Least-Squares Discriminant Analysis (PLS-DA) was used to cluster and more importantly classified HPV samples (Figure 13). Samples normalization performed by a pooled sample from group (group PQN). The PLS-DA confirms the previous result of PCA that there is no correlation observed between HPV samples.

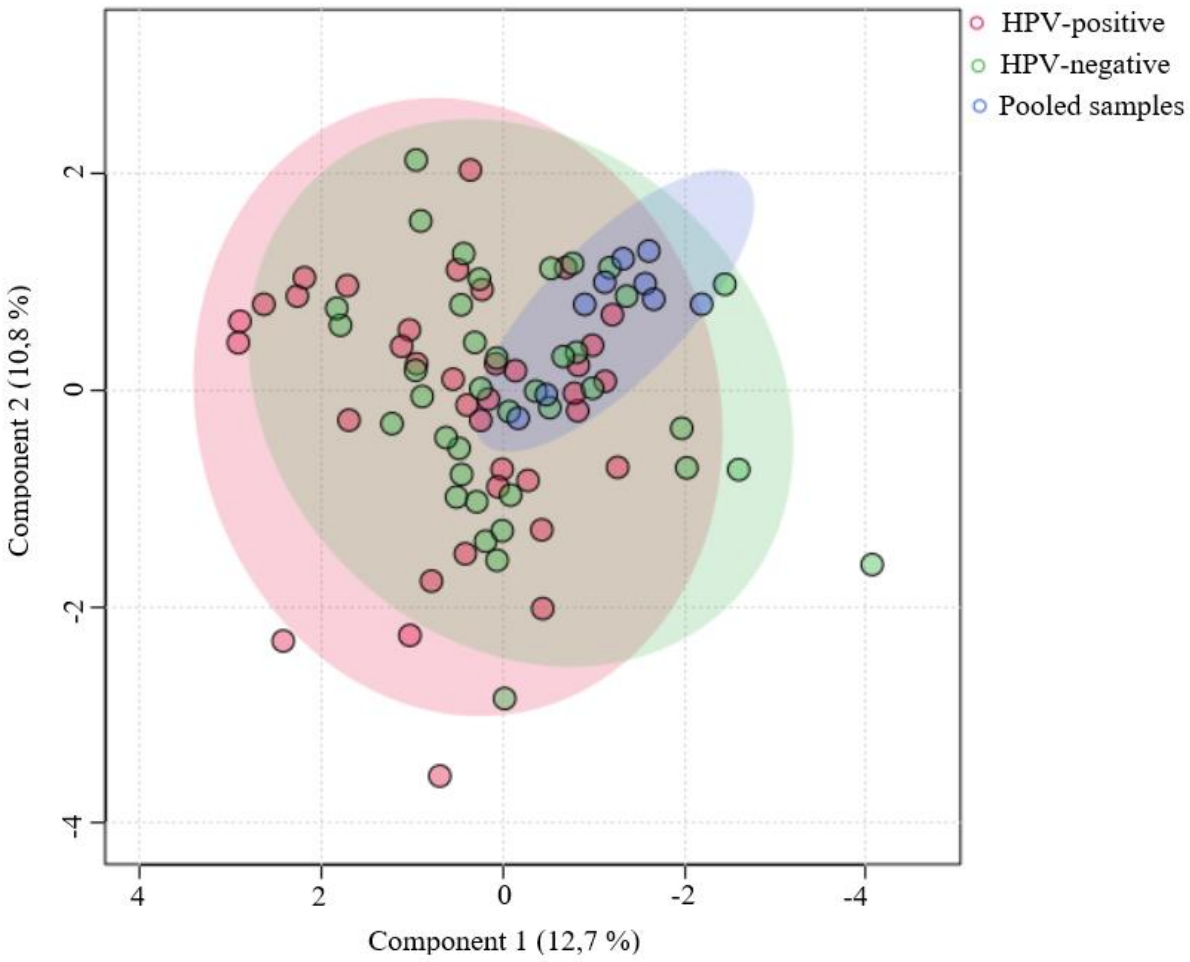


Figure 13: PLS-DA graph of HPV+ and HPV- samples plotted with Metaboanalyst. Sample normalization by a pooled sample. Data were subsequently log-transformed.

Since there was no distinction between the groups of HPV+ and HPV- samples and clinical data were at a disposal, the age stratification was performed (Figure 14). The HPVpro Study involved patients aged 29 – 59 years, so they were divided into six groups (29 – 33; 34 – 38; 39 – 43; 44 – 48; 49 – 53 and 54 – 59 years old individuals). The group of 44 – 48 years old creates the borderline of women in reproductive age.

One graph stands for one lipid metabolite. Within a graph, all age groups are clustered together based on their HPV status. All graphs were plotted based on calculated concentration values showed on a vertical axis. The same age groups for HPV+ and HP- samples were compared if they indicated any significant difference ($p<0,05$). The p-value showed significance of five metabolites – namely 5-HETE, 11-HETE, 15-HETE, TxB2, and PGF2a – that were significant in two age groups – 39 – 43 and 49 – 53.

Looking at 5-HETE, there was a significant difference in the age group 49 – 53 years old. This metabolite belongs to a group of pro-inflammatory omega-6 lipid metabolites. It was found, as expected, significantly higher in HPV+ samples in comparison with HPV-.

11-HETE has anti-inflammatory function in the human body. It was detected to be significant in HPV+ samples of the age group 39 – 43 years old.

15- HETE was identified to be significant in the age group of 39 – 43 years old individuals. It possesses anti-inflammatory activity, so it was supposed to be significantly higher in HPV- samples. Nevertheless, in measured cervicovaginal samples, it was detected in HPV+ samples.

TxB2 represents pro-inflammatory lipid metabolite. The higher concentration was found in HPV+ samples regarding age group 39 – 43 years old. On the other hand, in the age group 49 – 59 years old, higher concentration of this metabolite was detected in the HPV- samples.

PGF2a, a pro-inflammatory lipid mediator, was determined significantly higher in HPV+ samples in age group 39 – 43 years old individuals.

Interestingly, 8-HETE was not spotted in HPV- samples at all. The same applied to 12-HETE. PGE2 was detected in considerable concentration only in HPV+ samples for the youngest age group (29 – 33 years old).

DHA and EPA have the central role in omega-3 fatty acid metabolism pathways as fatty acid metabolites are derived from them. So the fluctuating concentrations might be understandable (Deschutter et al., 2019).

Otherwise, the box plots did not show any increasing or decreasing trend that could be able to highlight. It was expected that metabolite could increase or decrease with the age so e.g., the patient would be more prone to HPV inflammation then.

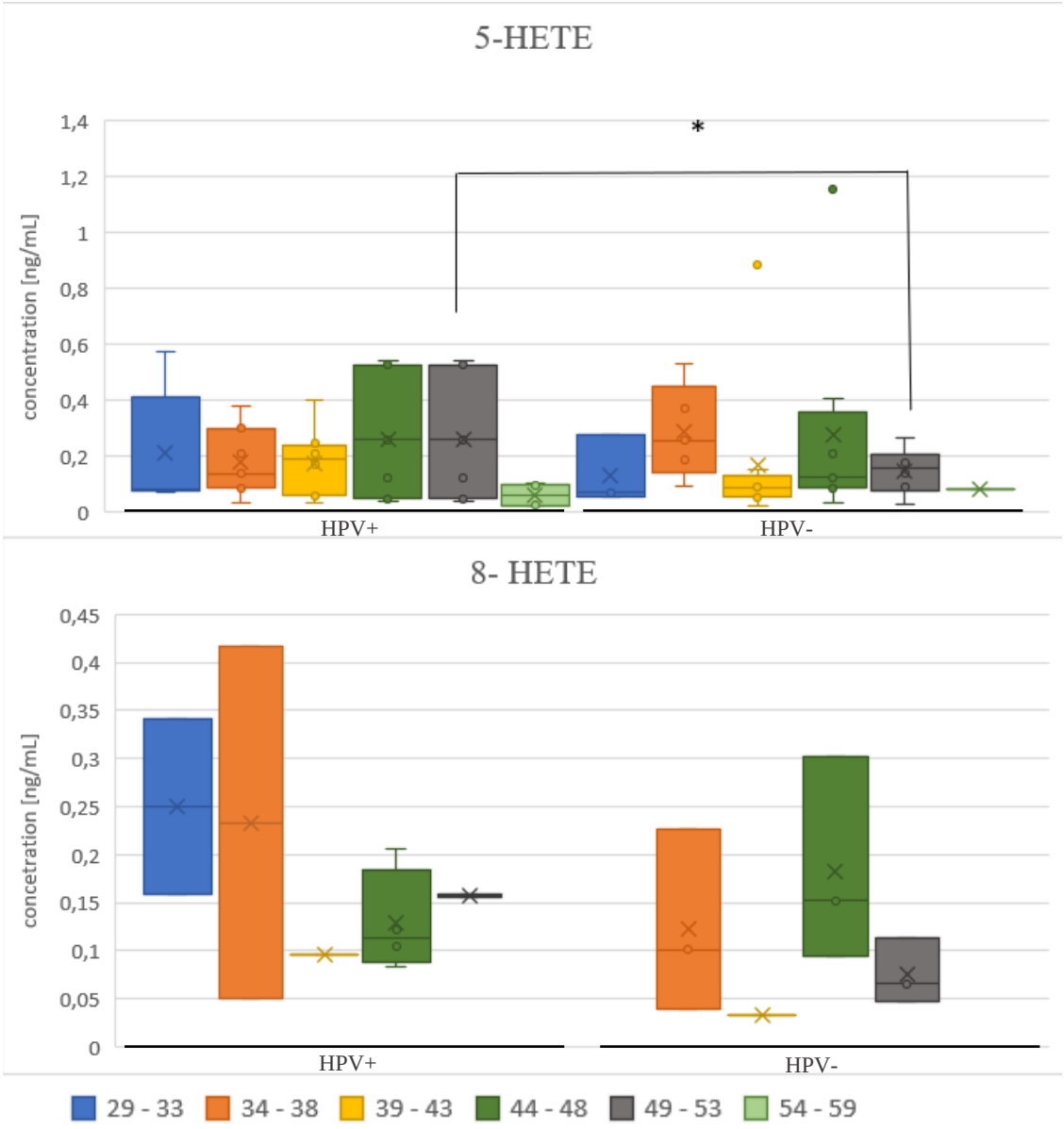
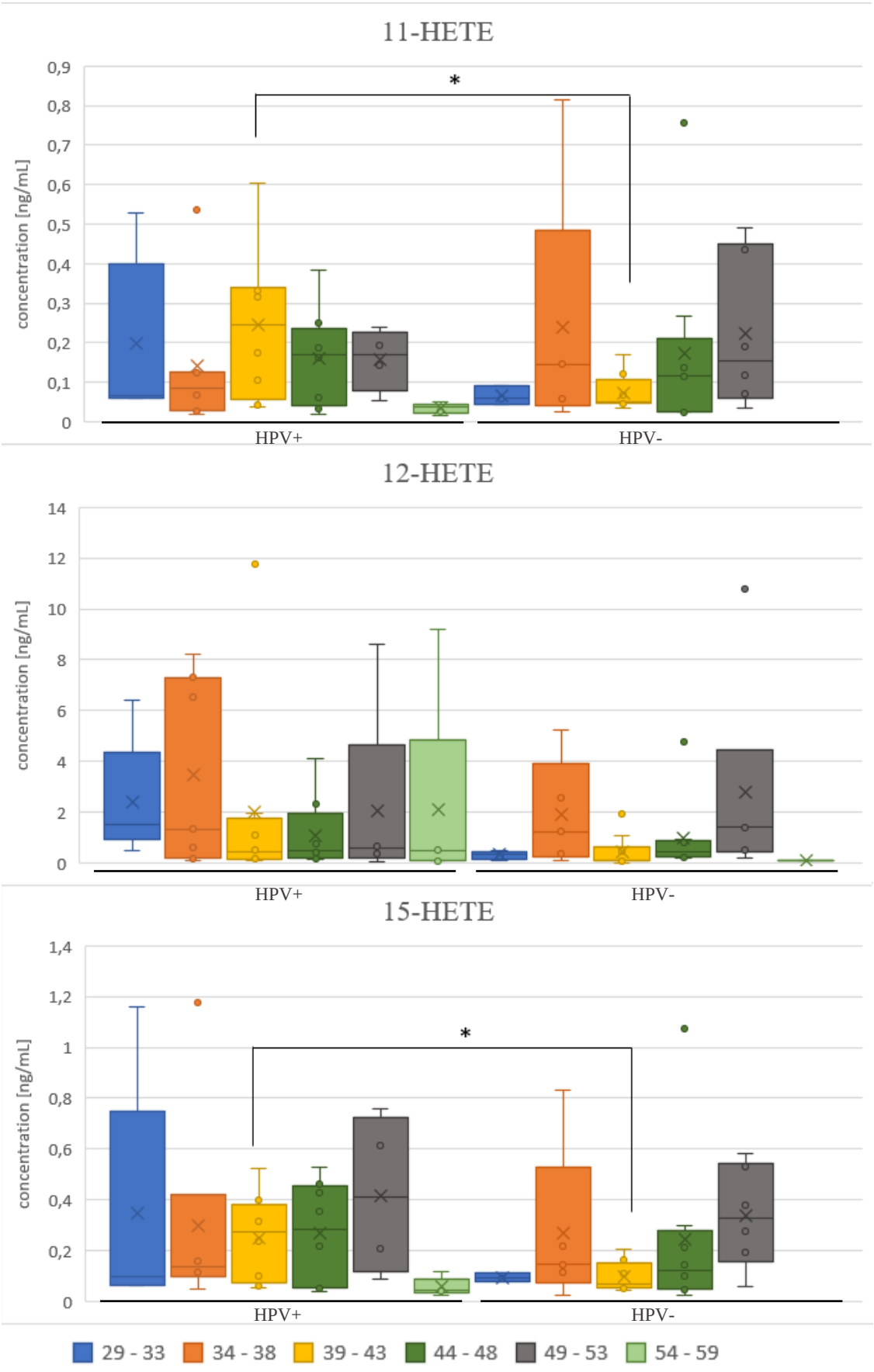


Figure 14: The box plots compare the age groups (29 – 33; 34 – 38; 39 – 43; 44 – 48; 49 – 53; 54 – 59 years old) of HPV+ and HPV- samples. The significant values are marked *.



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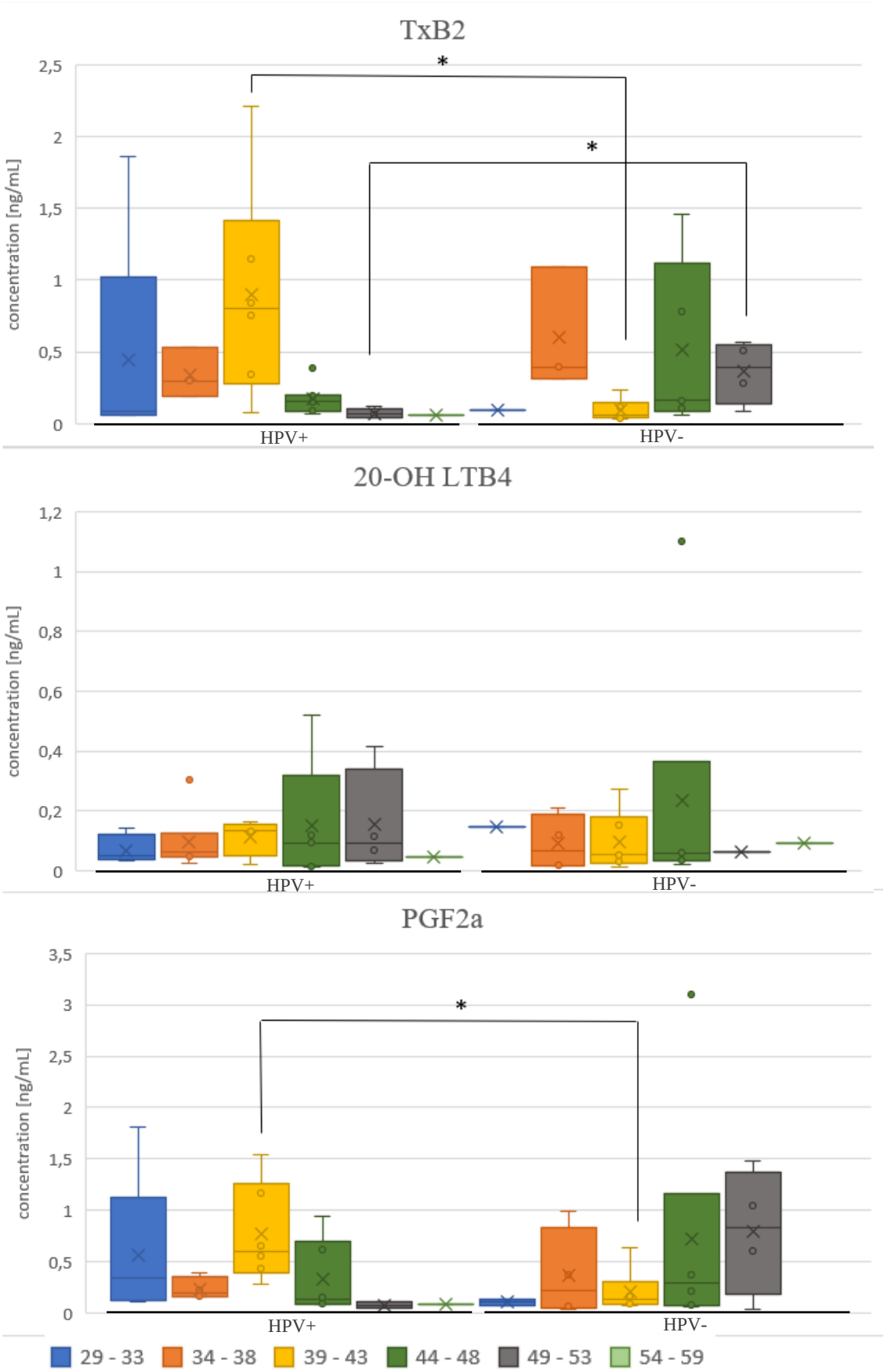

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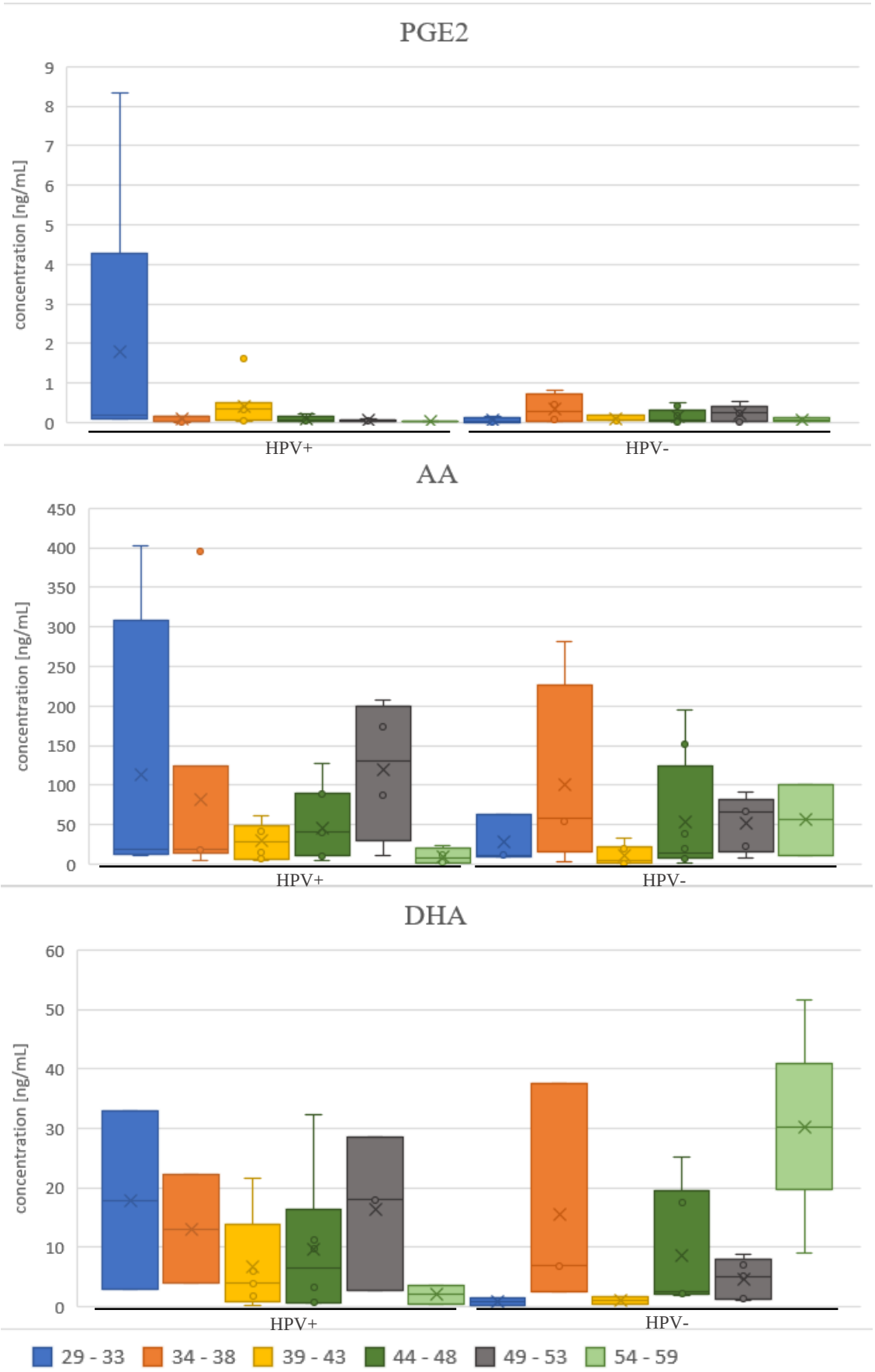

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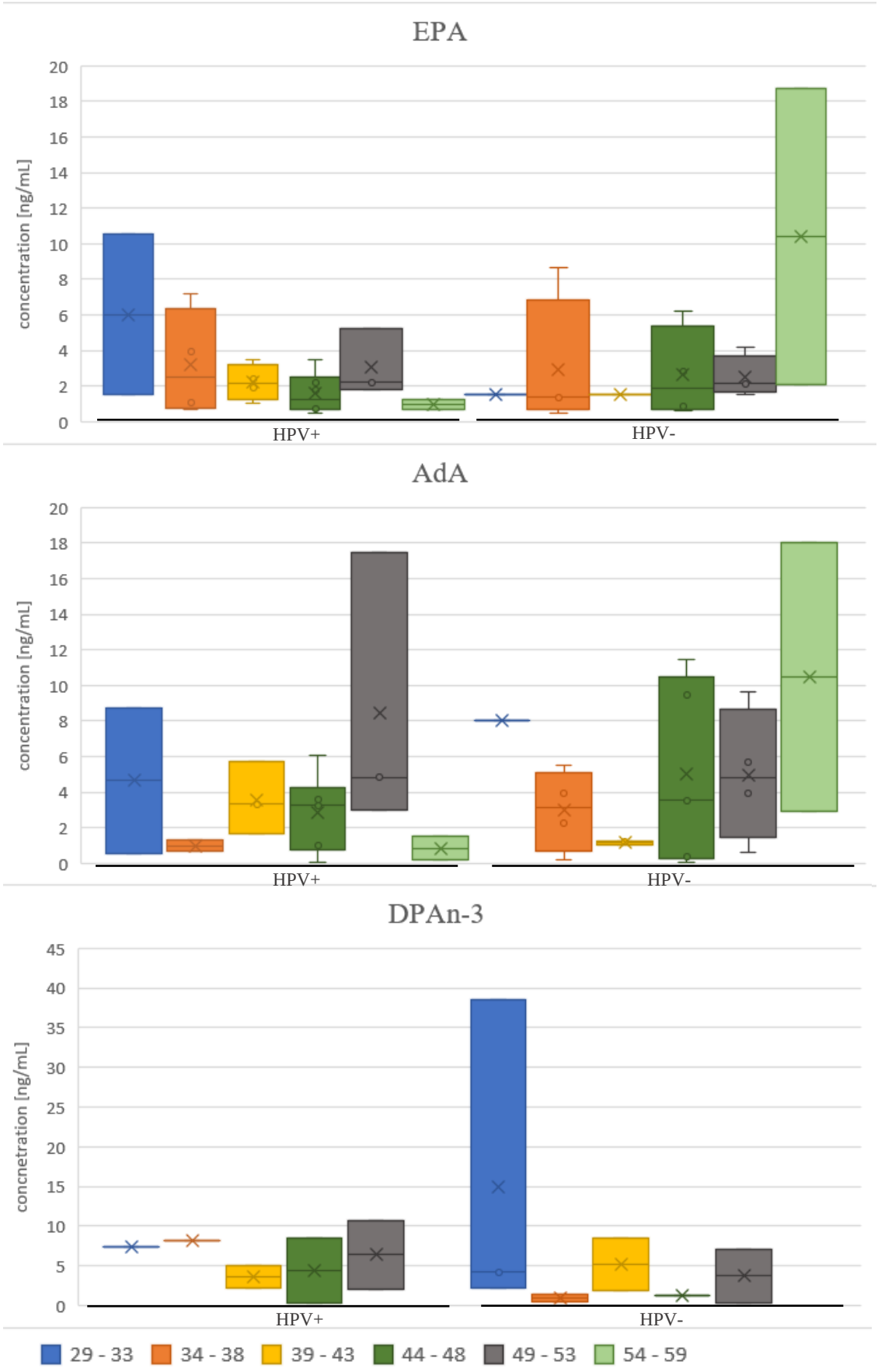

Figure 14: continued.

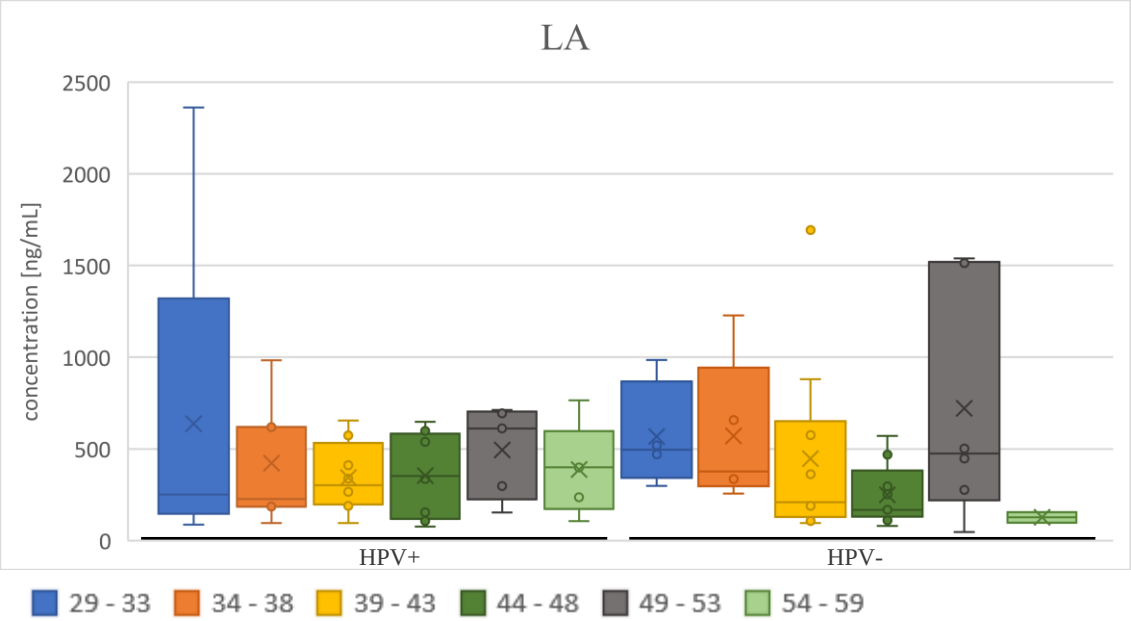

Figure 14: continued.

HPV+ samples were then divided into 3 groups based on the severity of the patient´s condition. Box plots were plotted (Figure 15). They showed the concentration of metabolite when comparing patients with no neoplasia or malignancy in their case history, atypical squamous cells of undetermined significance (ASC-US) and low-grade squamous intraepithelial lesion (LSIL). LSIL represent the more severe condition in comparison with ASC-US.

HPV- samples were excluded from this analysis, since only a few ASC-US (3 out of 37 samples) and patients with no neoplasia or malignancy could be compared. LSIL does not occur in HPV- patients

There was no significant value for any of the detected lipid metabolite. Although, 8-HETE, anti-inflammatory metabolite, was detected only in patients with no neoplasia or malignancy. TxB2, pro-inflammatory metabolite, reached the higher concentration in LSIL – the more severe health condition. As for DHA, EPA, AdA and DPAn-3 lipid metabolites, they were only measured in patients with no neoplasia or malignancy. Therefore, the values within groups could not be compared due to one value only in ASC-US and LSIL.

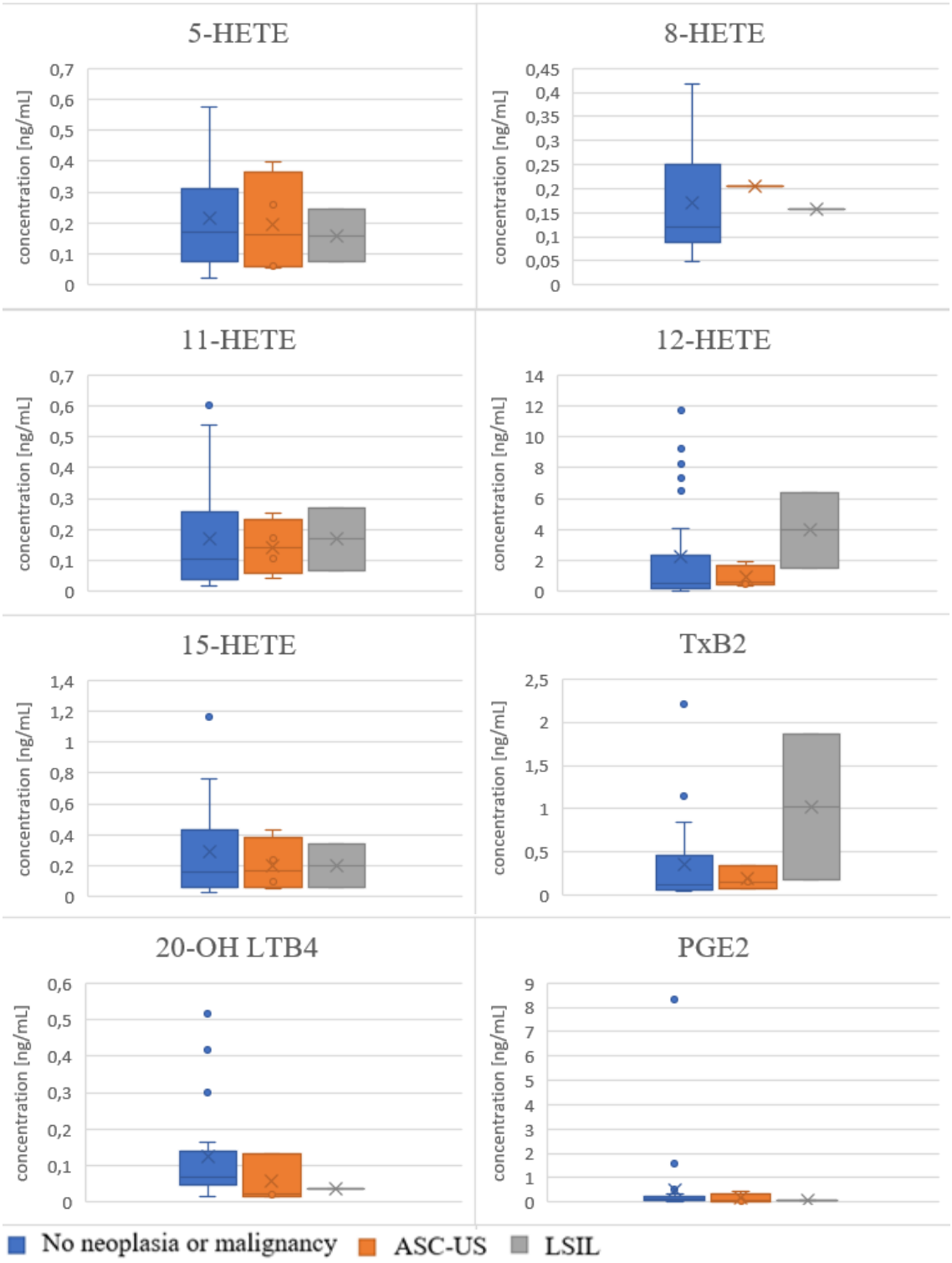

Figure 15: The box plotted comparing HPV+ samples based on severity of the condition of the patients. Healthy controls (blue), ASC-US (orange) and LSIL (grey) were represented there.

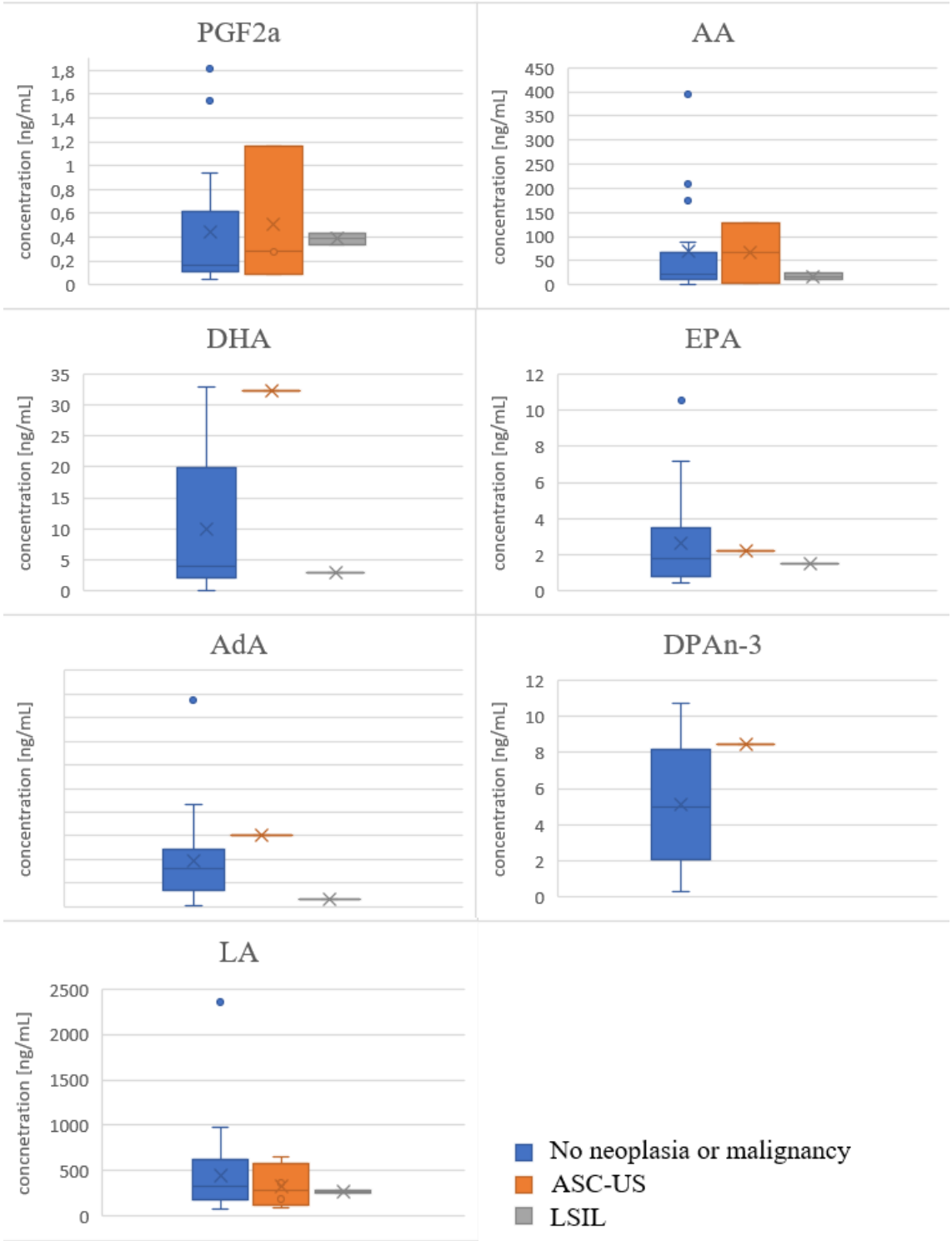

Figure 15: continued.

5 DISCUSSION

Herein, the lipidomic profile was identified. It consisted of 15 fatty acid metabolites which, to the different extent, could influence the induction and resolution of the inflammatory process.

Any significant differences were reported between HPV-positive and HPV-negative in cervicovaginal smear samples using targeted lipidomic approach. This finding rejected the alternative hypothesis that there was a corelation between HPV+ and HPV- samples.

Nevertheless, differences in fatty acid metabolites regarding the age group in HPV-positive and HPV- negative individuals were recognized. This included 5-HETE, 11-HETE, 15-HETE, PGF2a and especially TXB2 as lipid metabolites that discriminated positive from negative individuals. However, the results in identified lipids did not show any trend that could provide clear evidence of the presence of pro-inflammatory lipid metabolites in HPV+ and anti-inflammatory metabolites in both HPV+ and HPV- samples.

Recently, fatty acid metabolites have been studied to determine their function in various biological environments. The effect of the lipid metabolites seems to be tissue specific due to isoforms of their receptors (Harizi et al., 2008).

5-HETE operates as eicosanoid produced by diverse cell types in the human body. It has a capacity to contribute to the up-regulation of acute inflammatory and thus, is considered to have a pro-inflammatory activity (Tam, 2013).

11-HETE, anti-inflammatory metabolite, was reported to be significantly higher in those with hyperplastic polyps and adenomas of colon. Furthermore, since oxylipids such as 5- and 15-HETE have a role as signaling molecules involved in inflammation regulation, these oxylipids may have important functions in inflammation-associated polyp presence (Austin Pickens et al., 2019)

15-HETE was one of the pro-resolving lipid metabolites which was found at a lower abundance in influenza infected animals when comparing to healthy controls (Tam, 2013). 15-HETE have anti-inflammatory activity as it seems to block IL-6 secretion from macrophages (Krönke et al., 2009, p. 15).

Interestingly, 5-, 11- and 15-HETEs together were significantly associated with polyp type of colon cancer (Austin Pickens et al., 2019)

TxB2 is associated with delayed resolution of inflammation, so it represents pro-inflammatory metabolite. The higher concentration was found in HPV+ samples

regarding age group 39 – 43 years old. On the other hand, in the age group 49 – 59 years old, higher concentration of this metabolite was detected in the HPV- samples. TXB2 imbalance relates to PGE2, precursor of PGF2 metabolite. TXB2 and PGF2a are derived from one common precursor, instable prostaglandin PGG2, which is converted to PGH2. (Holtzman, 1992).

PGF2a, a pro-inflammatory lipid mediator, was determined significantly higher in HPV+ samples in age group 39 – 43 years old individuals. PGF2a is elevated in patients with chronic inflammatory disease (such as osteoarthritis or rheumatoid arthritis) (Ricciotti & FitzGerald, 2011).

In accordance with this results, Ilhan *et al*. also reported they did not observe any significant changes in HPV samples compering patient sample and negative control. However, they also tested the relationship of vaginal microbiota and host microenvironment with its specific metabolites as this could be determining factor of a degree of inflammation. The relevation of the interaction in the complex virus-host-microbiome in the context of cervical cancer might have significant potential for future metabolome targeted diagnostics and therapies (Ilhan et al., 2019).

The cervicovaginal smear samples were originally collected and used for HPV genotyping analysis, so several steps in pre-analytical phase was not suitable for later lipid metabolite analysis. Firstly, when sample was collected, it was stored in 20 mL of 50% methanol medium. This could mean that samples were too diluted. In other words, if the dilution factor of methanol was reduced, thus concentration of samples was higher, more metabolites could be captured during the analysis. Then, collected samples were stored undefined time (from sample collection till DNA analysis for genotypization) at room temperature because lipid metabolite analysis was not considered at that moment. Room temperature may have damaged the thermolabile lipids and biased the results. Unfortunately, another exposure to room temperature occurred during transport to the LUMC laboratory. This could be an explanation why I only identified 15 probably the most abundant fatty acid metabolites out of 76 possible.

Additionally, genital inflammation was associated with various groups of lipids. It was discussed that ceramides and ergosterol have potential to reveal more about the relationship of lipids in cervicovaginal microenvironment and HPV (Ilhan et al., 2019; Singh et al., 2009; Urbanek et al., 2022).

Ceramides are formed by binding a fatty acid to the amino group of sphingosine through an amide bond. Among the most frequently represented fatty acids in ceramides

are palmitic, stearic, nervonic and lignoceric acids (Castro et al., 2014). Ceramides with different carbon chain lengths (c14; c16; c16:1; c18; c18:1; c20; c22; c24; c24:1; c26) are most often found in the cell membrane. If found in the sample freely, in an unbound form within the membrane, they can serve as a marker of cell apoptosis.

Ergosterol belongs to sterols that represent essential elements of fungal cellular membranes that maintain membrane structural integrity, fluidity, and permeability (Jordá & Puig, 2020). It has been identified as an immunoactive lipid that induces a necrotic and inflammatory programmed cell death (Rodrigues, 2018). Candida albicans is a yeast having ergosterol in its membrane. This yeast has an ability to cause genital infection. Thus, ergosterol might serve as a biomarker of genital yeast infection. Infection then causes weakened immunity and therefore a greater possibility of acquiring viral infection (Bhattacharya et al., 2020).

6 CONCLUSION

Recently, several groups have investigated how lipid mediators might influence inflammation during an infection caused by various microbial intruders (Tam, 2013). This thesis has undertaken the task of determining the lipidomic profile of cervicovaginal smear sample. However, it turned out that none of these lipid metabolites could be considered as pro-inflammatory/ pro-resolution biomarker in HPV+ samples at the moment. If there is some significant difference in HPV+ and HPV- samples, they must be hidden under the limit of detection during MS analysis. To capture these metabolites, the pre-analytical phase, specifically sample collection, must be optimized. Additional dataset and further experiments will be required to determine the biological significance of fatty acid metabolites in sample of cervicovaginal smear.

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8 ABBREVIATIONS

5-HETE	5-hydroxyeicosatetraenoic acid
5-LOX	5-lipoxygenase
8-HETE	8-hydroxyeicosatetraenoic acid
8-iso-PGF2α-d4	8-iso-prostaglandin-2α-d4
9-HODE	9-hydroxyoctadecadienoic acid
9-HOTrE	9-hydroxyoctadecatrienoic acid
11-HETE	11-hydroxyeicosatetraenoic acid
12-HETE	12-hydroxyeicosatetraenoic acid
12-LOX	12-lipoxygenase
13-HDoHE	13-hydroxydocosahexaenoic acid
13-HODE	13-hydroxyoctadecadienoic acid
13-HOTrE	13-hydroxyoctadecatrienoic acid
15-HETE	15-hydroxyeicosatetraenoic acid
15-HETE-d8	15-hydroxyeicosatetraenoic acid-d_8
15-LOX	15-lipoxygenase
14(15)EET-d11	14(15)-epoxyeicosatrienoic acid
14-HDoHE	14-hydroxydocosahexaenoic acid
17-HDoHE	17-hydroxydocosahexaenoic acid
18-HEPE	18-hydroxyeicosapentaenoic acid
20-OH LTB4	20-hydroxy-leukotriene B4
AA	Arachidonic acid
AdA	Adrenic acid
AIN	Anal intraepithelial neoplasia
ALA	Alpha-linolenic acid
ASC-US	Atypical cells of undermined significance
CADM1	Cell Adhesion Molecule 1
cAMP	Cyclic adenosine monophosphate
CIN	Cervical intraepithelial neoplasia
CLP	Calibration line for quantification of polyunsaturated fatty acids
CLR	Calibration line for quantification of non-polyunsaturated fatty acids

COX	Cyclooxygenase pathway
CpG site	5'—Cytosine—phosphate—Guanine—3 site
DB	Dot blot hybridization
DHA	Docosahexaenoic acid
diHETrE	Dihydroxyeicosatrienoic acid
DNA	Deoxyribonucleic acid
DPAn-3	Docosapentaenoic acid
E1, E2, E3, E4, E5, E6, E7, E8	Early region HPV genes
EET	Epoxyeicosatrienoic acids
EGFR	Epidermal growth factor receptor
EM	Extension mix
EPA	Eicosapentaenoic acid
ESI	Electrospray ionization
HETE	Hydroxy fatty acid
HPETE	hydroxyperoxyl
HPLC	High performance liquid chromatography
HPV	Human Papillomavirus
EQC	External quality controls
HR-HPV	High risk-HPV
HSIL	High-grade squamous intraepithelial neoplasia
HSPG	Heparin sulphate proteoglycan
IQC	Internal quality controls
IMTM	Institute of Molecular and Translational Medicine
IS	Internal standard
ISH	*In situ* hybridization
krpm	kilo revolutions per minute
L1, L2	Late region HPV genes
LA	Linoleic acid
LC-MS	Liquid chromatography – mass spectrometry
LCR	Long coding region
LOX	Lipoxygenase pathway
LR-HPV	Low risk-HPV
LSIL	Low-grade squamous intraepithelial lesion

LTB_4	Leukotriene B4
LTB4-d4	Leukotriene B4-d4
LUMC	Leiden University Medical Center
MAL	T-lymphocyte Maturation Associated Protein
MeOH	Methanol
miR-124-2	microRNA-124-2
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
ng/mL	Nanogram/mililiter
NASBA	Nucleic acid sequence-based amplification
ORF	Open reading frame
p16/Ki67	Proteins P16 and Ki67
P_{97}, P_{670}, P_{E8}	HPV promotors
p53	Tumour protein p53
Pap test	Papanikolaou test
PC	Principal component
PC5/6	Proprotein convertase 5/6
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E2
PGE2-d4	Prostaglandin E2-d4
PGF_{2a}	Prostaglandin 2 alpha
PPAR	Peroxisome proliferator-activated receptor
PLS-DA	Partial Least-Squares Discriminant Analysis
pRB	Retinoblastoma protein
PUFA	Polyunsaturated fatty acid
QC	Quality control
QTrap	Quadropule Ion Trap
RNA	Ribonucleic acid
ROC	Receiver operating characteristics
RRT	Relative retention time
RT	Room temperature

SIL	Squamous intraepithelial lesion
SPE	Solid phase extraction
SPM	Specialized pro-resolving mediator
SST	System suitability test
STH	Southern transfer hybridization
TOF	Time of flight mas analyser
TXA_2	Thromboxane A2
TXB_2	Thromboxane B2
VIN	Vulvar intraepithelial neoplasia
VLP	Virus-like particle

9 ADDITIONAL FILES

Additional files 1, 2 and 3 can be found in attached Excel file “Kroupova_DP_240423”.