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ANALYSIS OF EXPRESSION OF GENES ENCODING ENZYMES CYP3A4, CYP2B6, AND ABCB1 TRANSPORTER IN PATIENTS WITH HIV AND HCV INFECTIONS TREATED WITH ANTIVIRAL MEDICATIONS

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ANALIZA EKSPRESIJE GENA KOJI KODIRAJU SINTEZU ENZIMA CYP3A4, CYP2B6, I ABCB1 TRANSPORTERA KOD BOLESNIKA SA HIV I HCV INFEKCIJOM LEČENIH ANTIVIRUSNIM LEKOVIMA

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ANALYSIS OF EXPRESSION OF GENES ENCODING CYP3A4, CYP2B6 ENZYMES AND ABCB1 TRANSPORTER IN PATIENTS WITH HIV AND HCV INFECTION TREATED WITH ANTIVIRAL MEDICATION

SUMMARY

Background: The superfamily of CYP P450 enzymes and the family of ABC transporters are essential in determining how antiretroviral drugs and anti-HCV drugs are processed affecting further therapeutic success of occurrence of adverse effects. Among these enzymes, CYP3A4 is the most widely distributed metabolic enzyme, predominantly found in the small intestine and liver is responsible for metabolizing various drug classes, including protease inhibitors, non-nucleoside reverse transcriptase inhibitors, and CCR5 receptor antagonists. On the other hand, CYP2B6 plays a significant role in processing nucleoside reverse transcriptase inhibitors, such as efavirenz. ABC transporters are also involved in all stages of drug pharmacokinetics and can be found in different tissues and organs such as the brain, liver, and small intestine. ABCB1, in particular, acts as a transporter for a wide range of substances, including several antiretroviral drugs and integrase inhibitors. However, studies have revealed notable variations in the activity of CYP P450 enzymes and the ABC transporter system due to liver tissue damage or applied therapeutics. These variations often manifest as changes in mRNA levels and protein expression in different pathological conditions. Since HCV co-infection is common among people living with HIV due to similar transmission routes, there are approximately 5 million registered cases of HIV/HCV co-infection worldwide. Research has demonstrated that HIV infection accelerates the progression of liver damage in co-infected patients with HCV. Recent studies have suggested that the extent of liver fibrosis can also impact the activity of the CYP enzyme system, in addition to genetic factors. The level of inflammation in liver tissue serves as an indicator of the acute intensity of infection, liver damage, and response to treatment protocols. *In vitro* and animal studies have shown that inflammation affects the activity of CYP P450 enzymes and the ABC transporter family through complex transcriptional and post-transcriptional mechanisms. The activity of metabolic enzymes and transporters is believed to be dependent on the nature and duration of inflammatory mediators. Three proposed mechanisms explain how inflammation alters the activity of metabolic enzymes and transporters: activation of the nuclear factor-kB (NF-kB) pathway by pro-inflammatory cytokines, leading to transcriptional inhibition; post-transcriptional mechanisms involving enzyme degradation through nitric oxide synthesis; and epigenetic modifications, such as changes in methylation processes in the promoter regions of enzymes and transporters.

Considering that many drugs used for HIV and HCV treatment are primarily metabolized by these enzymes and transporters, it is crucial to investigate the impact of chronic inflammation, liver damage, and long-term therapy on their activity levels. Any changes in the activity of metabolic enzymes and transporters pose risks for adverse or toxic effects of antiretroviral and anti-HCV medications, as well as the potential failure to achieve therapeutic drug concentrations. This risk is particularly significant in the treatment of chronic infections characterized by rapid viral replication and immune evasion.

Objectives: The objectives of this research were to examine the gene expression levels of metabolic enzymes CYP3A4 and CYP2B6, as well as the ABCB1 transporter, in a population of

patients with HIV infection, HCV infection, and HIV/HCV co-infection. Gene expression was compared to constitutive (housekeeping) genes and among patient groups. Additionally, the study investigated whether factors such as the degree of liver damage (fibrosis) and the level of liver inflammation (grade), treatment administration, and biochemical parameters such as liver enzymes (ALT, AST), CRP, as well as alcohol or cigarette use, influenced the gene expression of these enzymes and transporters.

Methods: The study was conducted as a cross-sectional study. The patient selection was performed at the Clinic for Infectious and Tropical Diseases "Dr Kosta Todorović" of the University Clinical Center of Serbia, in a cohort of patients who already underwent screening and met the clinical criteria for liver biopsy. From this patient group, based on the inclusion and exclusion criteria for the study, the following were included: all adult patients with serologically confirmed HIV infection and adult patients with serologically confirmed HCV infection as well as HIV/HCV co-infection. The first arm of research consisted of group of patients infected with hepatitis C virus, and this group was further divided into two subgroups: HCV mono-infected patients who did not receive treatment and HCV mono-infected patients who received treatment (PEG IFN and DAA). The second arm of research consisted of three groups of patients: HCV mono-infected patients who did not receive treatment, HIV mono-infected patients, and HIV/HCV co-infected patients. Exclusion criteria included hepatitis of unexplained etiology, autoimmune hepatitis, associated liver diseases, use of medications or substances that are inhibitors or inducers of CYP3A4 and CYP2B6 enzymes and ABCB1 transporter.

Each liver sample was divided into two parts, one snap frozen in liquid nitrogen for gene expression analysis, and the other fixed in paraffin and analyzed for the degree of liver damage and inflammation grade. Gene expression was determined through tissue homogenization, followed by conversion to cDNA, and then qRT-PCR was performed as well as data analysis using the Pfaffl method. Expression levels above two-fold were considered an increased expression, while levels below 0.5 were considered decreased expression of test genes. The degree of fibrosis and the level of liver inflammation were determined using histopathological methods.

Results: In all, 54 patients from three group—those with HIV, HCV, and HIV/HCV co-infection—were enrolled in this cross-sectional research. The sample contained 30 (55.6%) patients with HCV infection, 9 (16.7%) patients with HIV infection, and 15 (27.8%) patients who were co-infected with HIV and HCV. 14 (46.7%) of the HCV patients in the group were receiving treatment, whereas 16 (53.3%) were not receiving treatment at this point. Seven (50.0%) of the HCV patients that were treated received PEG IFN, while seven (50.0%) received DAA. All HIV patients got antiretroviral medication (ART), including those who were co-infected.

In comparison to both the HIV and HCV mono-infected groups, the co-infected patient group showed significantly increased expression of the CYP2B6 gene. Comparing the HCV mono-infected group receiving PEG INF therapy to the HCV mono-infected group getting DAA medicine or receiving no treatment, statistically significant overexpression of the CYP2B6 gene was also observed. Alterations in CYP2B6 expression were not associated with the degree of liver fibrosis or hepatic inflammation in a statistically significant way. No significant associations with the biochemical and environmental factors were also discovered.

In both mono- and co-infected individuals from the HIV-infected group, there had been a higher expression of the *CYP3A4*. Additionally, a link was discovered between increasing platelet counts and increased *CYP3A4* expression, as well as between the degree of hepatic

inflammation and high *CYP3A4* expression. The degree of liver fibrosis, however, was not correlated with the expression of these genes. When comparing between the HIV mono-infected, HCV mono-infected, and HIV/HCV co-infected groups, the expression of the ABCB1 transporter gene did not demonstrate statistical significance. Additionally, it was not related to the severity of liver fibrosis or liver inflammation. However, a relationship between lamivudine usage and decreased ABCB1 expression was found in our study.
Conclusions: The results of this study demonstrate a significant increase in CYP2B6 gene expression in individuals with HIV/HCV co-infection. Furthermore, elevated levels of CYP3A4 mRNA are strongly correlated with the presence of HIV infection. Additionally, individuals on lamivudine showed a tendency for ABCB1 to be downregulated. Surprisingly, no links regarding gene expression and liver damage, inflammation, or drug usage were discovered. These findings highlight the need for more research to fully comprehend the complex relationships among gene expression, viral infection or co-infection, liver disease, and treatment responses in this particular complex patient group.

Keywords: metabolic enzymes, metabolic transporters, HIV, HCV, gene expression, chronic inflammation, liver fibrosis, CYP3A4, CYP2B6, ABCB1

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ANALIZA EKSPRESIJE GENA KOJI KODIRAJU SINTEZU ENZIMA CYP3A4, CYP2B6, I ABCB1 TRANSPORTERA KOD BOLESNIKA SA HIV I HCV INFEKCIJOM LEČENIH ANTIVIRUSNIM LEKOVIMA

Sažetak

Uvod: Superfamilija enzima CYP P450 i familija ABC transportera su ključne u farmakokinetskim procesima kojima podležu antiretrovirusni lekovi i lekovi protiv hepatitis C virusne infekcije, što ima značajni efekat na uspeh terapije i pojavu neželjenih efekata. Među tim enzimima, CYP3A4 je najrasprostranjeniji metabolički enzim, koji se dominanto nalazi u jetri i tankom crevu, a odgovoran je za metabolizam različitih klasa lekova, uključujući inhibitore proteaze, nenukleozidne inhibitori reverzne transkriptaze i antagoniste CCR5 receptora. Sa druge strane, CYP2B6 ima značajnu ulogu u metabolisanju nukleozidnih inhibitora reverzne transkriptaze, poput efavirenza. ABC transporteri takođe su uključeni u sve faze farmakokinetike lekova i mogu se naći na različitim tkivima i organima kao što su mozak, jetra i tanko crevo. ABCB1, posebno, funkcioniše kao transporter za širok spektar supstanci, uključujući nekoliko antiretrovirusnih grupa lekova, poput inhibitora integraze. Međutim, istraživanja su pokazala značajne varijacije u aktivnosti enzima CYP P450 i ABC transportera usled oštećenja tkiva jetre ili hronične primene terapije. Ove varijacije se često manifestuju kao promene nivoa iRNK i ekspresije proteina u različitim patološkim stanjima kao što su hronične virusne infekcije. Budući da je ko-infekcija HCV-om česta kod osoba koje žive sa HIV-om zbog sličnih puteva prenosa, širom sveta zabeleženo je oko 5 miliona registrovanih slučajeva ove ko-infekcije. Podaci iz dostupne literature su pokazali da HIV infekcija ubrzava progresiju oštećenja jetre kod ko-inficiranih pacijenata sa HCV-om. Nedavne studije su ukazale da stepen fibroze jetre takođe može uticati na aktivnost metaboličkih enzima, pored genetičkih faktora. Nivo inflamacije u jetri takodje služi kao pokazatelj akutnog intenziteta infekcije, oštećenja jetre i odgovora na terapijske protokole. *In vitro* i animalne studije su pokazale da inflamacija utiče na aktivnost enzima CYP P450 i familije ABC transportera putem složenih transkripcionih i posttranskripcionih mehanizama. Veruje se da je aktivnost metaboličkih enzima i transportera zavisna od prirode i trajanja inflamatornih medijatora. Predložena su tri mehanizma kojima inflamacija utiče na aktivnost metaboličkih enzima i transportera: aktivacija nuklearnog faktora-kB (NF-kB) putem proinflamatornih citokina, što dovodi do transkripcionog inhibiranja; posttranskripcioni mehanizmi koji uključuju degradaciju enzima putem sinteze azot monoksida; i epigenetičke modifikacije, kao što su promene u metilaciji u promotorskim regionima enzima i transportera.

Kako se mnogi antiretrovirusni i lekovi koji se koriste u terapiji HCV infekcije metabolišu putem ovih enzima i transportera, od suštinske je važnosti istražiti uticaj hronične inflamacije, oštećenja jetre i dugotrajne terapije na njihovu aktivnost. Svaka promena u aktivnosti metaboličkih enzima i transportera predstavlja rizik od neželjenih ili toksičnih efekata kao i potencijalnog neuspeha u postizanju terapijskih koncentracija leka. Ovaj rizik je posebno značajan u lečenju hroničnih infekcija koje karakteriše brza replikacija i visoka mutagenost virusa.

Ciljevi: Ciljevi ovog istraživanja su bili da se ispita nivo ekspresije gena koji kodiraju metaboličke enzime CYP3A4 i CYP2B6 kao i transporter ABCB1 u populaciji pacijenata obolelih od HIV infekcije, HCV infekcije i HIV/HCV koinfeckije. Ekspresija gena je poređena sa konstitutivnim (*house keeping*) genima kao i među grupama pacijenata. Pored toga, ispitivano

je da li na ekspresiju gena ovih enzima i transportera utiču faktori kao što su stepen oštećenja (fibroza) i nivo zapaljenja jetre (gradus), primena terapije kao i biohemijski paramentri kao što su enzimi jetre (ALT, AST), CRP, kao i upotreba alkohola ili cigareta.

Metode: Studija je rađena kao studija preseka. Selekcija pacijenata izvršena je na Klinici za infektivne i tropske bolesti „Dr Kosta Todorović“ Univerzitetskog kliničkog centra Srbije u kohorti pacijenata koji su već ispunili kliničke kriterijuma za biopsiju jetre. Iz ove grupe pacijenata u odnosu na kriterijuma za uključivanje i isključivanje u istraživanje su uključeni: svi punoletni pacijenti sa serološki potvrđenom dijagnozom HIV infekcije i punoletni pacijenti sa serološki potvrđenom dijagnozom HCV infekcije. Pacijenti su dalje podeljeni u podgrupe u odnosu na terapijski protokol kojim su lečeni. Prvu grupu činili su pacijenti inficiranih hepatitis C virusom, a dalje je ova grupa bila podeljena na dve podgrupe: HCV mono-inficirane pacijente koji nisu primal terapiju i HCV mono-inficirane pacijente koji su primal terapiju (PEG IFN i DAA). Drugu grupu pacijenata činile su tri podgrupe.HCV mono-inficirani pacijetni koji nisu primal terapiju; HIV mono-inficirani pacijenti i HIV/HCV ko-inficirani pacijenti. Kriterijumi za isključivanje bili su hepatitisi neobjašnjene etiologije, autoimuni hepatitis, udružena oboljenja jetre, upotreba lekova ili supstanci koje su inhibitori ili induktori enzima CYP3A4 i CYP2B6 i transportera ABCB1.

Svaki uzorak jetre je bio podeljen na dva dela od kojih je jedan zamrznut u tečnom azotu i dalje ispitivan za ekspresiju gena a drugi fiksiran parafinom i analiziran za stepen oštećenja jetre i gradus inflamacije jetre. Ekspresija gena odredjivana je kroz postupak homogenizacije tkiva jetre, zatim prevodjenja u CDNK a zatim je rađen *qRT-PCR* i analiza podataka metodom po Pfaffl-u. Nivo ekspresije iznad dva stepena je smatran povišenom ekspresijom a ispod 0.5 je smatran sniženom ekspresijom. Histopatološkim metodama je određivan stepen fibrize i nivo zapaljenja jetre.

Rezultati: Ova studija preseka uključila je ukupno 54 pacijenta iz tri grupe - pacijenti sa HIV infekcijom, pacijenti sa HCV infekcijom i pacijenti sa HIV/HCV ko-infekcijom. Uzorak je obuhvatio 30 (55,6%) pacijenata sa HCV infekcijom, 9 (16,7%) pacijenata sa HIV infekcijom i 15 (27,8%) pacijenata koji su bili ko-inficirani sa HIV-om i HCV-om. Od pacijenata sa HCV infekcijom, njih 14 (46,7%) je primalo antivirusnu terapiju, dok njih 16 (53,3%) nije primalo terapiju. Sedam (50,0%) pacijenata koji su primali anti-HCV terapiju koristilo je PEG IFN, dok je sedam (50,0%) koristilo lekove iz grupe DAA. Svi pacijenti sa HIV-om su primali antiretrovirusnu terapiju (ART), uključujući obe grupe pacijenata, HIV monoinficirane i HIV/HCV koinficirane.

Povišena ekspresija gena koji kodiraju enzim CYP2B6 primećena je u grupi ko-inficiranih pacijenta, kada je ova grupa poredjena sa obe grupe monoinficiranih, HIV i HCV. Pored toga, statistički značajno veća eskresija gena koji kodiraju CYP2B6 vidjena je i u grupi HCV monoinficiranih pacijenata koji su koristili PEG INF terapijski režim. Ova grupa poredjena je sa grupom HCV monoinficiranih koji su koristili DAA lekove kao i sa HCV monoinficiranim pacijentima koji nisu bili na anti-HCV terapiji. Nije primećena statistički značajna povezanost izmedju promenjene ekspresije CYP2B6 i stepena fibroze jetre ili gradusa zapaljenja jetre. Takodje, nije pronadjena značajna povezanost sa biohemijskim i faktorima sredine koji su praćeni.

Veća ekspresija gena koji kodiraju enzim CYP3A4 primećana je kod svih pacijenata koji su pripadali grupi HIV inficiranih, kod monoinficiranih kao i koinficiranih. Takodje, korelacija je postojala izmedju gradusa zapaljenja jetre i visoke ekspresije *CYP3A4* kao i izmedju visokih vrednosti trombocita i povećane eksresije *CYP3A4.* S druge strane, nije postojala značajna

povezanost *CYP3A4 sa* sa stepenom fibroze jetre. Ekspresija gena transportera ABCB1 nije pokazala statističku značajnost u poredjenju izmedju grupa HIV monoinficiranih, HCV monoinficiranih i HIV/HCV koinficiranih. Takodje nije pokazala korelaciju sa stepenom fibroze jetre i gradusa zapaljenja, medjutim u našem istraživanju zabeležena je korelacija između smanjene ekspresije ABCB1 i upotrebe lamivudina.
Zaključak: Rezultati ovog istraživanja pokazuju značajno povećanje ekspresije gena CYP2B6 kod osoba sa HIV/HCV koinfekcijom. Osim toga, u našem istraživanju pokazano je das u visoke vrednosti *CYP3A4* iRNK su snažno povezane sa prisustvom HIV infekcije. Takođe, kod osoba koje su koristile lamivudin primećena je tendencija smanjene ekspresije gena ABCB1. Nasuprot ovim rezultatima, nisu pronađene veze između ekspresije gena i stepena oštećenja i zapaljenja jetre ili upotrebe specifičnih lekova. Ovi rezultati ukazuju na potrebu za daljim istraživanjem kako bismo potpuno razumeli kompleksne veze između ekspresije gena, virusne infekcije ili koinfekcije, bolesti jetre i odgovora na terapiju kod ove specifične kohorte pacijenata.

Ključne reči: metabolički enzimi, metabolički transporteri, HIV, HCV, ekspresija gena, hronična inflamacija, fibroza jetre, CYP3A4, CYP2B6, ABCB1

Naučna oblast: Medicinske nauke

Uža naučna oblast: Medicinska farmakologija

UDK broj:

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1. INTRODUCTION

1.1 HIV infection: Overview and definition of human immunodeficiency virus infection

Human immunodeficiency virus (HIV) is a retrovirus that primarily targets the human immune system, specifically CD4+ T-cells, leading to a progressive immunodeficiency syndrome known as AIDS (Acquired Immunodeficiency Syndrome). HIV is transmitted through specific body fluids, including blood, semen, vaginal fluids, breast milk, and rectal fluids. The most common modes of transmission are unprotected sex, sharing contaminated needles, and mother-to-child transmission during pregnancy, childbirth, or breastfeeding. HIV infection is one of the leading causes of morbidity and mortality worldwide, with around 39 million people being infected at the end of 2021. and 650 000 people died from HIV related conditions (Summary of the HIV epidemic, 2021.WHO).

HIV is a member of the Retroviridae family. Its genome is made up of two strands of RNA and the enzyme reverse transcriptase (RT), and it is encased in a p24 protein-based capsid. This virus also has a capsid, but it also has an outer membrane made up of the transmembrane protein gp41 and the surface glycoprotein gp120. HIV utilizes receptors on the surface of CD4+ T lymphocytes to enter host cells. The viral gp120 glycoprotein binds to the CD4+ T cell receptor, causing conformational changes in gp120 that make it possible for it to attach to the CCR5 receptor. This is how the virus enters the CD4+ T cell. The gp41 glycoprotein is simultaneously activated by the complex that is produced, mediating the fusion of the viral envelope with the host cell membrane and the virus' entry into the cell. The capacity of the HIV virus to convert viral RNA into deoxyribonucleic acid (DNA), which is made possible by the enzyme viral RNA-dependent DNA polymerase, also known as reverse transcriptase, is one of its distinguishing characteristics. The RNA genome is converted into double-stranded DNA by reverse transcriptase, which then moves into the nucleus and combines with the DNA molecules of the host cell. The resultant DNA is integrated into the genome of the infected cell by the integrase enzyme, creating a provirus that is dormant until the infected cell is activated. When a provirus is activated, it behaves like any other gene in the cell and starts to produce new viral RNA molecules based on proviral DNA that is subsequently carried into the cytoplasm. New viral particles are created from precursor viral proteins in the cytoplasm under the control of HIV protease. An immature viral particle is made up of the RNA viral genome and viral proteins; it develops during the process of budding through the cell membrane. As a result of this process, mature virus particles are produced, starting an effective HIV infection. HIV stays permanently incorporated in host cells as a provirus. During the S-phase of the cell cycle, the virus replicates alongside the cellular DNA. As a result, once an infection has become entrenched, it usually lasts a lifetime (Naif M. 2013.).

1.2. HIV pathogenesis: mechanisms and processes involved in HIV pathogenesis

The pathogenesis of HIV infection involves a series of complex interactions between the virus and the immune system. During the course of infection, HIV replicates continuously, leading to a high viral load in the bloodstream. The virus also exhibits a high mutation rate, resulting in genetic diversity and the emergence of drug-resistant strains. HIV's ability to evade immune surveillance is facilitated by several mechanisms, including antigenic variation, which allows the virus to change its surface proteins and avoid recognition by the immune system (Madham S. et al., 2023).

The progressive depletion of CD4+ T-cells is a hallmark of HIV infection. This depletion occurs due to multiple factors, including direct viral killing of infected cells, chronic immune activation and inflammation, and cell-mediated immune responses targeting HIV-infected cells. The loss of CD4+

T cells weakens the immune system, making individuals more susceptible to opportunistic infections and certain malignancies).

In the acute phase of HIV infection, during the first weeks, there is intense viral replication in CD4+ T-cells in secondary lymphoid organs, accompanied by massive viremia. Viral replication stimulates HIV-specific cytotoxic lymphocytes that eliminate infected lymphocytes and/or suppress viral replication through antiviral factors (chemokines), resulting in a decline in viremia, which coincides with seroconversion, the appearance of anti-HIV antibodies (Levy et al., 1993).

Chronic HIV infection is characterized by intense viral replication, which constantly mutates, leading to the continuous activation of new clones of HIV-specific CD4+ and CD8+ lymphocytes, while evading immune surveillance. This state of hyper activation eventually leads to immune system exhaustion. It has been shown that the level of viral replication in the body and the degree of lymphocyte activation are directly proportional, and both of these indicators are associated with rapid progression to clinically manifest immunodeficiency (Blanco et al., 2001).

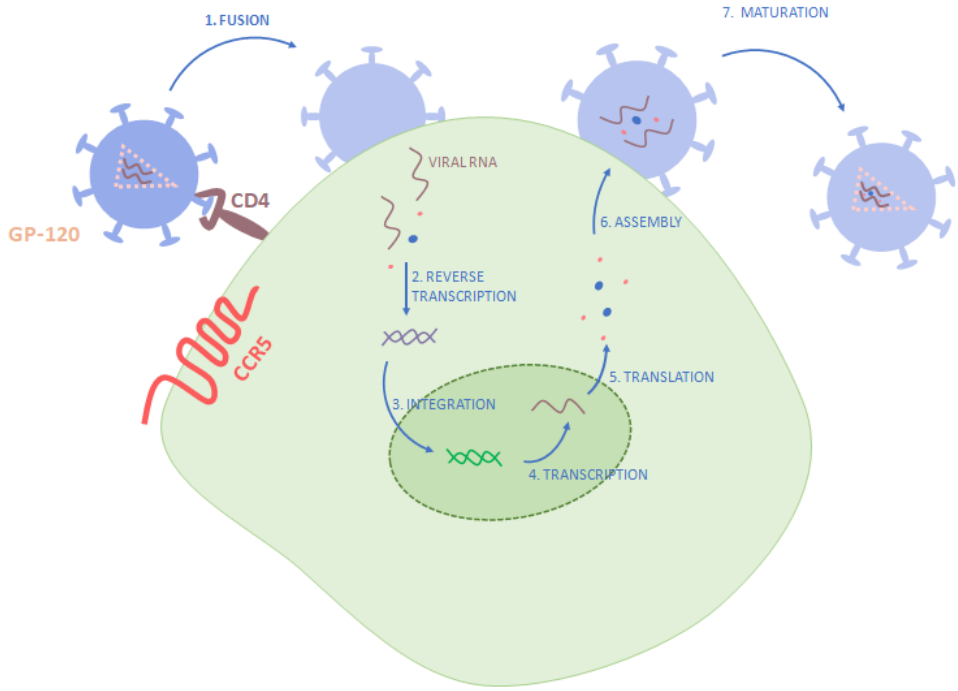

Figure 1. HIV pathogenesis process

HIV primarily infects CD4+ T cells and through various mechanisms leads to the gradual elimination of entire clones of CD4+ T cell lymphocytes. Over time, there is a reduced ability of the immune response to effectively combat the HIV virus itself, as well as other infections for which preserved cellular immune function is crucial for prevention and control. On the other hand, due to impaired hematopoiesis and lymphocyte differentiation, the production of naive lymphocytes is decreased, so the immune system cannot effectively respond to infectious agents it encounters for the first time. Therefore, infections that occur within the context of weakened immune responses during

HIV infection are referred to as opportunistic infections. These include infections caused by pathogens such as parasites (*Pneumocystis carinii*, *Toxoplasma gondii*, *Cryptosporidium*, *Isospora belli*, *microsporidia*), fungi (*Candida spp*., *Cryptococcus neoformans*, *Aspergillus spp*.), certain bacteria (*Salmonella spp*., *Mycobacterium tuberculosis*, *Mycobacterium avium complex*), certain DNA viruses, mainly from the herpes virus family (herpes simplex virus, cytomegalovirus, varicella-zoster virus, Epstein-Barr virus, human herpesvirus type 6 and human herpesvirus type 8), as well as human papillomaviruses, parvovirus B19, and others (Florence et al., 2003). Their reactivation occurs in cases of weakened cellular immunity, which is a characteristic feature of HIV-induced immune dysfunction.

HIV infection progresses through different stages and clinical manifestations, culminating in the development of full clinical AIDS. This usually occurs several years after the entry of the HIV virus into the body. The length of the incubation period varies individually, ranging from several months to more than ten years, and is influenced by individual differences in the immune response to HIV and the specific cytopathic characteristics of the virus itself. HIV infection goes through an acute and a chronic phase. The acute phase is characterized by intense and uncontrolled viral replication in CD4+ T-cell lymphocytes in secondary lymphoid organs such as the spleen and lymph nodes. This is accompanied by massive viremia, with viral RNA copies in the plasma ranging from 10^9 to 10^{10} copies/ml. Viral replication stimulates HIV-specific cytotoxic lymphocytes, which eliminate infected lymphocytes and/or suppress viral replication with antiviral factors (chemokines), leading to a decline in viremia, which coincides with seroconversion. Chronic HIV infection is characterized by ongoing and intense viral replication. The virus continuously mutates, leading to the activation of new clones of CD4+ and CD8+ T lymphocytes. This constant evasion of immune surveillance eventually results in immune system exhaustion (Madham S. et al., 2023).

1.3. Clinical manifestations and symptoms of HIV infection

HIV infection can be either asymptomatic or symptomatic. During the asymptomatic phase, there are no clinical signs of infection, but there are clear laboratory parameters indicating immune system dysfunction. As the immune system weakens further, the first symptoms and signs of the disease appear, characterized by the development of opportunistic infections and/or opportunistic tumors.

The progression of HIV infection is monitored by determining the absolute and relative numbers of CD4+ T cell lymphocytes in peripheral blood and by measuring the viral load in the plasma (plasma viral load, pVL). The classification of HIV infection is based on the clinical presentation and the CD4+ T - cell count, which is a necessary laboratory parameter for the definitive diagnosis of the stage of HIV infection. According to the classification by the U.S. Centers for Disease Control and Prevention (CDC), three clinical categories of HIV infection are distinguished based on the clinical presentation. These categories are A, B, and C, further divided into three subgroups based on the CD4+ T - cell count and labeled as 1, 2, and 3. Categories A1, A2, and A3 represent clinically asymptomatic HIV infection. Categories B1, B2, and B3 include individuals with neurological manifestations of HIV infection and opportunistic infections that do not meet the criteria for AIDS (peripheral neuropathy, minor opportunistic infections, and cervical intraepithelial neoplasia). Categories C1, C2, and C3 encompass all AIDS-defining illnesses, indicating the presence of AIDS. According to the CDC

classification of HIV disease, a diagnosis of AIDS can be made if the absolute CD4+ T - cell count is below 200 cells / mm3, regardless of clinical signs. All individuals included in the A3, B3, and C1 - C3 categories of the CDC classification are considered to have AIDS (CDC, https://www.cdc.gov/nchs/hus/sources-definitions/hiv-disease.htm).

1.4. HIV diagnostics: Diagnostic methods for HIV detection and assessment

Early detection of HIV infection is crucial for the timely initiation of treatment and preventing disease progression. Over the years, significant advancements have been made in HIV diagnostics, allowing for accurate and efficient detection of the virus.

Enzyme-Linked Immunosorbent Assay (ELISA) is an antibody-based laboratory test widely used for HIV diagnostics. It detects HIV-specific antibodies or antigens in a patient's blood sample. ELISA is the primary screening test for HIV, often followed by confirmatory tests such as Western blot or immunofluorescence assays. It is highly reliable and widely available, making it an essential tool in HIV testing.

Polymerase Chain Reaction (PCR) is commonly used for early detection of HIV infection, especially during the window period when antibody tests may not yet detect the virus. It is also used for monitoring treatment response, detecting drug resistance mutations, and diagnosing HIV in infants born to HIV-positive mothers. PCR has greatly improved the accuracy and efficiency of HIV diagnostics and plays a crucial role in disease management. PCR is highly sensitive and can detect very low levels of HIV genetic material in a sample, even during the early stages of infection. It also, shows great specificity as it targets specific regions of the viral genome, allowing for accurate detection of HIV without cross-reactivity with other viruses or substances. Another strength of this method is quantification as PCR can provide information about the viral load, which is important for monitoring disease progression and treatment response.

In summary, both PCR and ELISA are important tools in HIV diagnostics, each with its strengths and limitations. PCR offers high sensitivity and is particularly useful for early detection and monitoring viral load, while ELISA provides wide availability, cost-effectiveness, and established methodology for screening a large population. The choice of test depends on various factors such as the purpose of testing, available resources, and the stage of infection (Hollingsworth TD et al., 2008.).

1.5. HIV treatment options

Currently, a total of 29 drugs are registered, classified into 6 groups based on their mechanism of action: nucleoside/tide reverse transcriptase inhibitors (NRTIs), non-nucleoside/tide reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), CCR5 receptor antagonists, and fusion inhibitors (FIs). Initiation of combination antiretroviral therapy (cART) is recommended for all individuals with serologically confirmed HIV infection, regardless of CD4+ T-cell count, in order to reduce morbidity and mortality associated with

HIV infection and to prevent viral transmission (2). According to the recommendations of the European AIDS Clinical Society (EACS), cART commonly consists of combinations of two drugs from the group of nucleoside reverse transcriptase inhibitors (NRTIs) with one drug from the group of integrase inhibitors (INSTIs) (2 NRTIs + 1 INSTI). Another option is a combination of two drugs from the group of NRTIs with one non-nucleoside reverse transcriptase inhibitor (2 NRTIs + 1 NNRTI), and a dosing regimen that involves simultaneous use of one NRTI drug with one INSTI drug is also recommended (NRTI+INSTI) (EACS Guidelines, v11.1 2022). Table 1. presents a comprehensive list of presently available antiretroviral drugs categorized based on their respective mechanisms of action.

Table 1. Classification of antiretroviral drugs based on their mechanism of action.

NRTIs	**NNRTIs**	**PIs**	**FI**	**CCR5 receptor antagonists**	**INSTI**
zidovudine (AZT), didanosine (ddI), stavudine (d4T), lamivudine (3TC), abacavir (ABC), emtricitabine (FTC), tenofovir disoproxil fumarate/tenofovir alafenamide (TDF/TF).	nevirapine (NVP), efavirenz (EFV), etravirine (ETV), rilpivirine (RPV), doravirine (DOR).	saquinavir (SQV), ritonavir (RTV), lopinavir (LPV), fosamprenavir (FOS), atazanavir (ATV), tipranavir (TPV), and darunavir (DRV).	enfuvirtide (T20), fostemsavir (FTR).	maraviroc (MVC).	raltegravir (RAL), dolutegravir (DTG), elvitegravir (EVG), bictegravir (BIC), cabotegravir (CAB).

2. HCV infection: Overview and definition of hepatitis C viral infection

Hepatitis is an inflammatory disease of the liver of various etiologies. The clinical presentation, prognosis, and treatment depend on the underlying cause of the disease. Abdominal pain, jaundice, elevated temperature, and enlarged liver are the most common clinical manifestations of acute hepatitis (Kasting ML et al., 2018). Chronic forms of the disease typically present with nonspecific symptoms such as fatigue, weakness, loss of appetite, and dull pain beneath the right rib cage. Some chronic hepatitis forms may exhibit few symptoms and signs, only becoming apparent when there has been prolonged inflammation leading to the replacement of hepatocytes with fibrous tissue, or when liver cirrhosis has developed (Thrift AP et al., 2017). The most common causes of acute hepatitis are hepatotropic viruses such as hepatitis C virus (HCV).

The global prevalence of hepatitis C virus (HCV) infection is estimated to be 1.6%, ranging from 1.3% to 2.1%, which corresponds to approximately 115 million individuals (range: 92-149 million) (Kasting ML et al., 2018.). Hepatitis C virus infection leads to acute hepatitis, and 40 - 80% of affected individuals will develop chronic infection. About 20 - 30% of chronic hepatitis cases progress with severe clinical consequences. Chronic HCV infection increases the risk of developing liver cirrhosis and hepatocellular carcinoma (HCC). The annual risk of hepatic decompensation (jaundice, ascites, variceal bleeding, encephalopathy) is 3 - 4%, while the risk of HCC development is 3 - 7% (Kasting ML et al., 2018.). After the onset of these complications, the five-year survival rate is approximately 50%. Chronic C viral infection is the primary indication for liver transplantation in the developed world.

The prevalence of hepatitis C viral infection varies by geographical region. It is lowest in the United Kingdom and Scandinavian countries, ranging from 0.01% to 0.1%, while slightly higher rates are observed in the United States and Western Europe (around 0.5%). Mediterranean countries such as Italy (0.7%) and Spain (0.9%) have higher prevalence rates in Europe. High prevalence rates are also reported in Asian countries, such as Japan (1.5%) and China (2.5%) (Martinello M et al., 2016; Sarin SK et al., 2020). In our country, the prevalence ranges from 0.3% to 0.7%. Geographic variations in HCV prevalence are thought to be related to the presence or absence of different risk factors. For example, HCV infection in the United States and Australia is predominantly observed in younger individuals (aged 30-49), males who are intravenous drug users, while infection in individuals over 50 years of age is almost always associated with transfusion of contaminated blood in their youth (Martinello M et al., 2016; Kasting ML et al., 2018; Sarin SK et al., 2020.).

The source of infection is always a human with acute or chronic HCV infection. Considering that HCV can be found in all body fluids, it is not surprising that the prevalence of HCV infection is higher in "high-risk groups" than in the general population. Definition of high-risk groups include intravenous drug users, patients who have received blood transfusions or blood products, patients on hemodialysis, individuals engaged in high-risk sexual behaviors, and healthcare workers. Although rare, vertical transmission from mother to fetus is also possible.

2.2. HCV Pathogenesis: Mechanisms and processes involved in HCV pathogenesis

The genus Hepacivirus of the Flaviviridae family contains the hepatitis C virus. It is a small particle that ranges in size from 45 to 65 nm and is made up of a capsid and a lipid sheath (Figure 2). A double-layer lipoprotein envelope made up of host-derived lipids and the E1 and E2 glycoproteins expressed by the virus surrounds the single-stranded (+) RNA found in the capsid. Both, chronic and acute versions of the illness are possible. Acute hepatitis C frequently has no symptoms and remains undiagnosed. However, between 40 and 80 percent of those who have chronic hepatitis C go on to develop liver cirrhosis, the last stage of the chronic liver disease, which is characterized by vague or nonexistent symptoms (Lin W et al., 2013.).

Due to transcription mistakes and a lack of repair mechanisms, the HCV genome is extremely diverse and prone to frequent mutations. The structural (S) region and non-structural (NS) region are two coding regions in the genome, together with two non-coding sections (5' and 3'). Although they are not involved in the creation of viral structural proteins, the non-coding areas are crucial for viral RNA replication. The structural components of the virus are the core, E1, and E2 proteins, whereas the non-structural protein group consists of NS2, NS3, NS4, NS4A, NS4B, NS5, and NS5B. The C protein is thought to be involved in hepatocellular oncogenesis, whereas the E1 and E2 glycoproteins help the virus enter hepatocytes, even if the mechanism of oncogenesis in individuals with chronic HCV infection is still not entirely known. The E2 region of the viral genome is the most varied. The chronic nature of the sickness and viral persistence in the body are both explained by the genome's high degree of diversity, which enables the virus to elude antibodies. Viral replication is aided by non-structural proteins (Gupta et al., 2013). Peripheral blood mononuclear cells and hepatocytes are where replication predominantly takes place. Cryoglobulinemia, B-cell lymphoma, and monoclonal gammopathies are a few immunological diseases that can arise in people with chronic HCV infection as a result of viral replication in peripheral blood mononuclear cells (Everhart et al., 2009).

Hepatitis C virus (HCV) may be divided into six genotypes (1–6), each of which has a number of subgroups (identified by lowercase letters). HCV demonstrates tremendous genetic variability. Quasispecies, which refers to genetic variation within a single subtype, exemplifies the genetic heterogeneity of the virus within an infected person. (Messina JP et al., 2015; Shah R. et al., 2021.)

Numerous studies have examined the connection between genotypes and disease pathogenicity and the rate at which diseases advance. According to certain research, subtype 1b of genotype 1, in particular, is more harmful than other subtypes, and individuals infected with it are more likely to develop liver cirrhosis and hepatocellular carcinoma (Russo FP et al., 2022.). However, other scientists contend that the viral genotype has little bearing on the rate at which the illness progresses (Luna-Cuadros MA et al., 2022.).

It is well established that a number of factors affect how the HCV infection progresses and develops. The course and result of HCV infection are greatly influenced by the host's cellular and humoral immune response as well as genetic predisposition. Patients with "self-limiting acute hepatitis" have specific CD4+ T cells that detect the NS3 protein in the presence of HLA class I antigens, triggering an intense immunological proliferative response and the generation of IFN-. Chronically infected people's CD4+ T cell response declines and disappears throughout the chronic stage of the illness. The strongest antigens that CD4+ T cells can identify include NS4 and Protein C. Different

cytokines that are produced by CD4+ T-cells, such as IL-2 and IFN, also have immunomodulatory effects on B and CD8+ T-cells. Additionally proven is the cytotoxic activity of CD8+ T cells that detect antigens in the context of HLA class II antigens. Hepatocytes' surfaces have viral peptides that CD8+ lymphocytes can identify and eliminate. However, individuals with chronic C viral infection as well as those with acute hepatitis have specialized cytotoxic T cells, demonstrating that this cytotoxic response is inadequate to eradicate the virus. The presence of CD8+ T cells is associated with hepatocellular injury in chronic HCV infection. The establishment of CD4+ and CD8+ mediated immune responses takes several days or weeks. Natural killer cells (NK cells) are the first line of defense in the interim. Although, they don't have antigen-specific receptors, yet they nonetheless help virus-infected cells to lyse. NK cells start to function when a virus first infects a person. Type 1 interferon (IFN-/-) and IL-12 are the factors that trigger their activation. IFN, TNF, chemokines, and interferon-gamma-inducible protein-10 (IP-10) are all secreted by activated NK cells.

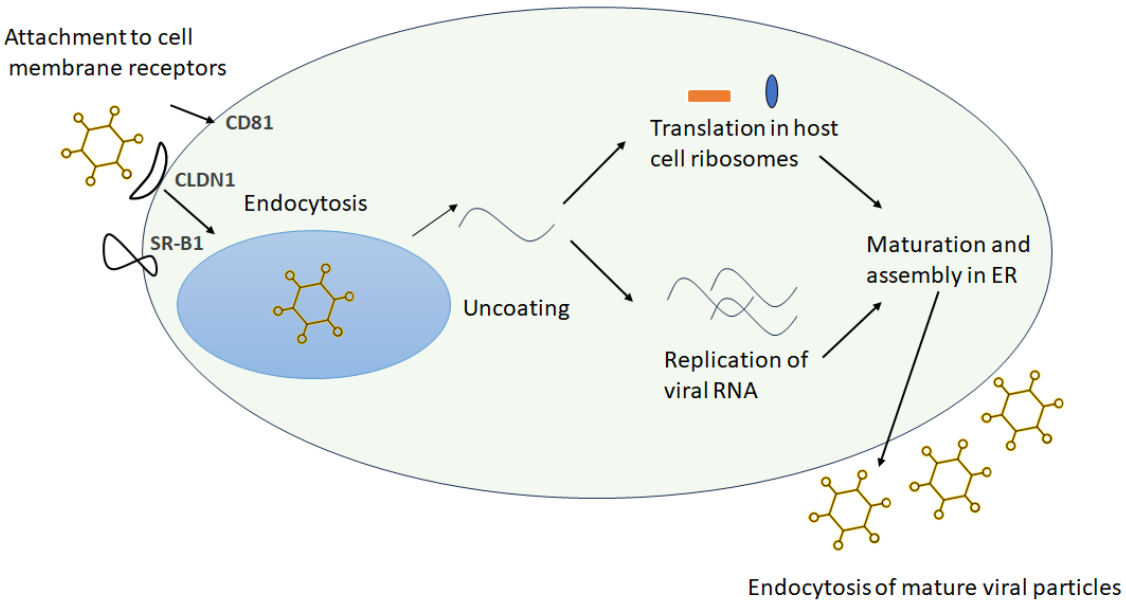

Figure 2. HCV pathogenesis process

Hundreds of interferon-stimulated genes (ISGs) are activated by IFN - through the interferon alpha receptor (IFN-R) and the JAK-STAT signaling pathway (JAK stands for Janus kinase, STAT for signal transducer and activator of transcription), which is how IFN - exerts its antiviral effect. In particular, JAK1 and TYK2 (tyrosine kinase) activation results in the phosphorylation of STAT1 and STAT2, which bind to the interferon-stimulated response element (ISRE), one of the multiple ISG promoters. Antiviral action is brought on by its activation within the infected cell. On the other hand, activation of suppressor ISGs (SOCS1, SOCS3) prevents JAK1 and TYK2 from blocking the phosphorylation of STAT1 and STAT2, hence preventing the antiviral impact. Similar to IFN, IFN inhibits viral replication by using a distinct receptor on the surface of the infected cell to activate the JAK-STAT signaling pathway, which is how it exerts its antiviral effect (Chen Y et al., 2017.).

Usually 6 to 12 weeks after an acute infection, the body also produces antibodies in response to the presence of HCV. Anti-HCV antibodies are produced against protein C, NS3, NS4, and the hypervariable region E2 proteins, and they are neutralizing.

Only 10 – 20% of infected persons manage to remove the virus from the body during the acute phase of the disease, while the remaining acquire chronic infection, showing that the immune system is unable to clear the virus from the body despite a robust cellular and humoral immune response. There is still some confusion over the precise causes of this. By failing to produce enough IFN -, CD8+ T cells are thought to play a significant part in the emergence of chronic infection (Hofmann M. et al., 2018.).

2.3. HCV clinical presentation: Clinical manifestations and symptoms of HCV infection

An asymptomatic acute phase of the illness is thought to occur in 50 – 90% of people with HCV infection (Lin M., 2013.). The development of a chronic form of the illness is one of the primary hallmarks of acute HCV infection. Only 10% to 20 % of infected people recover from the virus during the first three months, whereas 40% to 80 % experience persistent illness. Chronic infections can take decades to develop. The development of liver cirrhosis is accelerated by the presence of diabetes mellitus, alcohol usage, HIV co-infection, or other hepatotropic viruses. 10% to 40% of those with chronic hepatitis C will develop liver cirrhosis, depending on the cofactors present. The most significant contributing factor in the development of chronic infection into liver cirrhosis is thought to be alcohol use. It can increase up to three times the chance of getting cirrhosis, according to studies. Previous studies have also shown that while illness development is slower in younger people and women, it is quicker when infection occurs at an older age, notably in those over 50. HIV co-infection also hastens disease development, most likely by lowering CD4+ T cell counts.

An inadvertent finding of increased liver enzymes (alanine aminotransferase, or ALT, and aspartate aminotransferase, or AST) during regular biochemical testing occurs in around 70% of individuals with chronic hepatitis C. Unspecific symptoms including exhaustion, appetite loss, easy fatigability, and dull ache beneath the right ribcage may be experienced by a small percentage of individuals. There may also be extrahepatic symptoms of the illness, such as membranous glomerulonephritis, lichen planus, lymphocytic sialoadenitis, and cryoglobulinemia. According to several scientists, non-Hodgkin lymphoma and persistent C infections are related. Occasionally, the

disease's ultimate stage is identified by severe cirrhosis, liver failure, and hepatocellular cancer. Hepatocellular carcinoma is thought to be most often caused in Europe by HCV infection. These patients' death risk is 33% within one year (Russo FP. et al., 2022.)

The liver damage brought on by other hepatotropic viruses, primarily HBV and HDV, autoimmune liver diseases, non-alcoholic steatohepatitis (NASH), liver damage brought on by alcohol and drugs, and metabolic diseases (Wilson's disease, hemochromatosis, -1 antitrypsin deficiency) must all be taken into account in the differential diagnosis of chronic hepatitis C.

2.4. HCV diagnostics: Diagnostic methods for HCV detection and assessment

The clinical presentation, biochemical, and virological studies all contribute to the diagnosis. HCV RNA in serum is found using molecular methods like PCR, both qualitatively and quantitatively, whereas anti -HCV antibodies are detected using serological methods like ELISA testing. The most recent assays have exceptional sensitivity and can detect viral replication at levels as low as 10 IU/ml and as high as 10^7 IU/ml. This new generation of assays' great sensitivity is crucial for "response-guided therapy" (RGT).

For determining the extent of liver damage, including fibrosis staging and activity grading, liver biopsy continues to be the gold standard. METAVIR, Ishak, and Knodell are the three most widely utilized scoring systems in Europe and the Republic of Serbia. Prior to starting medication, it is crucial to identify individuals who already have liver cirrhosis by histopathological analysis of liver biopsy specimens since these patients have a decreased likelihood of obtaining sustained virological response (SVR). There are additional non-invasive procedures available in addition to the invasive biopsy to assess the degree of liver fibrosis. The fibrosis stage may be evaluated by transient elastography (TE), however the necroinflammatory activity and disease etiology are not disclosed. Although they are appropriate, serum biochemical markers of fibrosis are also often employed.but they are applicable only in patients with significant fibrosis (METAVIR score F2-F4) (European Association for the Study of the Liver, 2019).

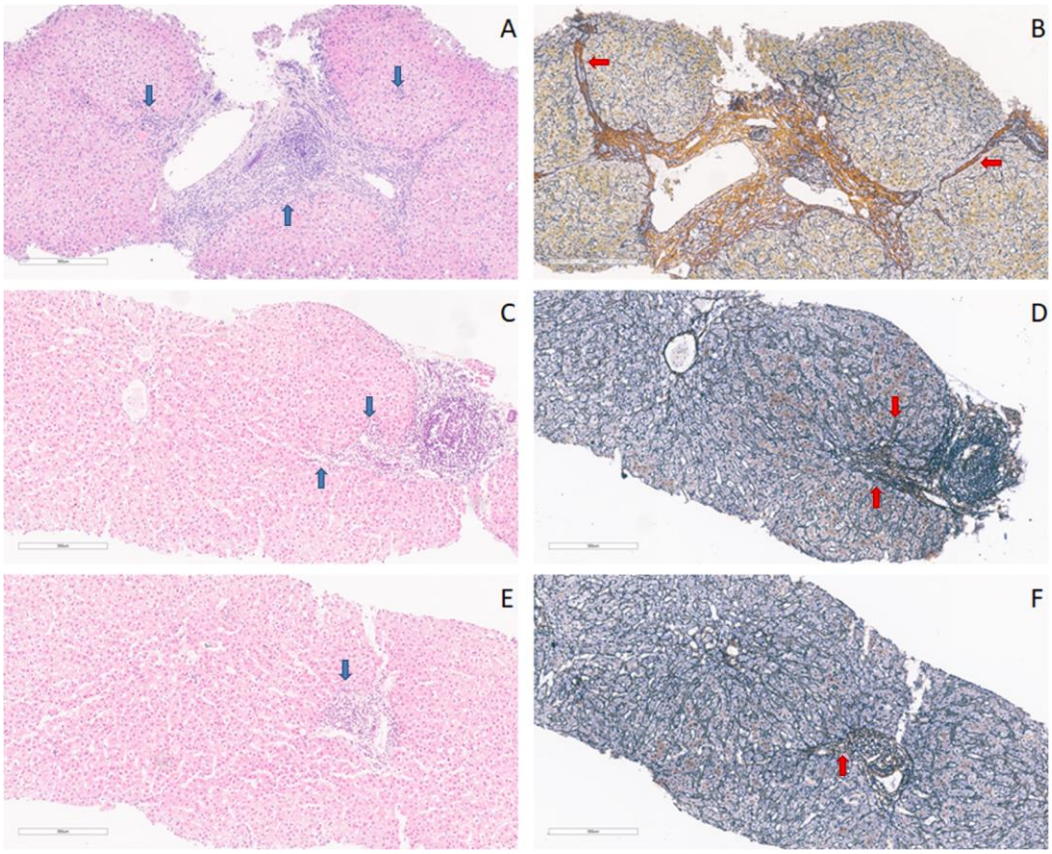

Figure 1

Figure 3. Liver tissue needle biopsy samples, heterogeneous findings, with blue arrows marking necro-inflammatory activity, and red arrows marking reticulin fibers A) showing moderate necro-inflammatory activity- A2, METAVIR score, from HIV/HCV co-infected patient,, hematoxylin-eosin stain, magnification 10X; B) cirrhosis -F4, METAVIR score, from HIV/HCV co-infected patient, Reticuline-silver stain, magnification 10X; C) mild necro-inflammatory activity - A1, METAVIR score, from HCV infected patient,, hematoxylin-eosin stain, magnification 10X; D) moderate fibrosis, periportal with few septa, -F1, METAVIR score, from HCV infected patient; Reticuline-silver stain, magnification 10X; E) minimal necroinflammatory activity, hematoxylin-eosin stain - A1, METAVIR score, from HIV infected patient,, magnification 10X; F) no fibrosis -F0, METAVIR score, from HIV infected patient, Reticuline-silver stain, magnification 10X.

2.5. HCV treatment options

The treatment recommendations for HCV infection have evolved significantly over the years, and the primary goal is to achieve a sustained virologic response (SVR), which is defined as the absence of detectable HCV RNA in the blood 12 weeks after completing treatment. Until recently, the standard therapy involved the combined use of pegylated interferon α (PEG-IFN) and ribavirin (RBV). With this treatment, SVR was achieved in only 40% of patients with genotype 1, and it was associated with frequent and numerous adverse effects, highlighting the need for clear definition of predictive factors for a favorable clinical and virological response to therapy (Sung PS. et al., 2012.). The relatively low therapeutic efficacy and numerous adverse effects have prompted the initiation of new research aimed at the synthesis and development of drugs that are potent enough to eliminate the virus without causing unwanted side effects, allowing patients to lead a normal life. Thus, in 2011, direct-acting antiviral drugs (DAAs) were introduced into clinical practice for the first time. The advent of direct-acting antiviral drugs ushered in a new era in the treatment of HCV-infected individuals. There was a rapid increase in the number of patients who responded successfully to this form of therapy. Additionally, significantly better results were observed in patients with advanced-stage disease, those who had been re-infected, and those who had previously shown no response to treatment (Sagnelli E. et al., 2021.).

There are three classes of direct-acting antiviral drugs:

1. NS3/4A protease inhibitors: boceprevir, telaprevir, simeprevir, asunaprevir, paritaprevir.

2. Polymerase inhibitors: sofosbuvir, dasabuvir.

3. NS5A replication complex inhibitors: daklatasvir, ledipasvir, ombitasvir.

Current treatment recommendations for HCV infection include usage of DAA therapy while the choice of DAA regimen depends on several factors, including HCV genotype, prior treatment history, presence of liver cirrhosis, and potential drug-drug interactions.

For treatment of genotype 1 there are several highly effective DAA regimens available, including combination therapies of glecaprevir/pibrentasvir, sofosbuvir/velpatasvir, and elbasvir/grazoprevir. For patients living with genotype 2 there are available combinations of sofosbuvir/velpatasvir or glecaprevir/pibrentasvir recommended as first-line therapies. For. genotype 3 HCV infection sofosbuvir/velpatasvir or glecaprevir/pibrentasvir are preferred treatment options, with extended treatment duration in some cases and for patients living with genotype 4, 5, or 6 glecaprevir/pibrentasvir or sofosbuvir/velpatasvir are recommended as first-line treatments.

There are special considerations for patients with compensated cirrhosis although DAA regimens are generally effective and well-tolerated in this patient population. Treatment duration may be extended based on the specific regimen and clinical considerations. For patients with decompensated cirrhosis selection of DAA regimens and treatment duration is individualized, considering liver function, comorbidities, and potential drug interactions (Sagnelli E. et al., 2021.).

3. HIV/HCV co-infection: Definition and overview

HIV and HCV co-infections, due to the high incidence and the accompanying illness and mortality, which affects around 5 million people globally, is a serious public health problem (Gobran ST. et al., 2021.). HIV and HCV co-infection has complicated effects on how this co-infection develops, on the effectiveness of the treatments, and management of the patients. The global frequency varies by area, with greater rates seen in those who engage in high-risk activities including drug injection and unguarded sex. The chance of co-infection is enhanced in these groups because of common transmission channels such as sharing needles and sexual contact. The pathogenesis of HIV/HCV co-infection is complex and multifaceted. HIV-induced immunosuppression plays a crucial role in HCV replication and disease progression. There are several different processes included in this complex co-infection (Merwat SN. et al., 2011.; Gobran ST. et al., 2021.; Bar N. et al., 2022.):

1. Accelerated Liver Disease Progression: HIV infection can accelerate the progression of liver disease in individuals with HCV. Co-infected individuals tend to experience more rapid liver fibrosis, leading to an increased risk of developing cirrhosis and other complications such as hepatocellular carcinoma. The exact mechanisms behind this acceleration are not fully understood but may involve interactions between the viruses and the immune system.

2. Higher HCV viral load: HIV co-infection is associated with higher HCV viral loads, meaning that co-infected individuals tend to have higher levels of HCV in their blood compared to those with HCV alone. This elevated viral load can contribute to more severe liver damage and faster disease progression.

3. Impaired immune response: HIV weakens the immune system by specifically targeting CD4+ T cells, which play a crucial role in coordinating the immune response. While HIV primarily infects CD4+ T cells, which play a crucial role in coordinating the immune response, HCV is a hepatotropic virus that primarily targets hepatocytes. In individuals co-infected with HIV and HCV, the presence of HIV has several implications for the course of HCV infection. The depletion of CD4+ T-cells by HIV affects the balance between viral replication and immune control of HCV. It results in higher HCV viral loads and a reduced ability to control HCV infection, leading to a more rapid progression of liver disease. Co-infected individuals often experience chronic immune activation and inflammation due to HIV infection. Persistent immune activation contributes to the impairment of the immune response against HCV and promotes liver damage. The immune dysregulation further accelerates the progression of liver fibrosis and increases the risk of developing cirrhosis and hepatocellular carcinoma.

4. Increased risk of liver-related complications: Co-infection with HIV and HCV can lead to a higher risk of liver-related complications. These may include decompensated cirrhosis leading to liver failure, hepatocellular carcinoma and hepatic encephalopathy.

5. Drug-drug interactions: HIV and HCV treatments can interact with each other, which necessitates careful management.

6. Impact on HIV treatment: Liver disease resulting from HCV co-infection can complicate the management of HIV. Liver damage may affect the choice and dosage of antiretroviral drugs used so close monitoring is necessary to minimize potential adverse effects.

Early diagnosis has an immense positive effect on treatment and disease prognosis. The use of diagnostic testing, such as molecular and serological assays, is used to confirm HIV and HCV infections. Assessing liver fibrosis and cirrhosis is also crucial for figuring out the severity of liver disease and directing therapy choices (Gobran ST. et al., 2021.; Bar N. et al., 2022.)
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The treatment of HIV-HCV co-infection requires a comprehensive approach that addresses the management of both viral infections simultaneously. The goals of treatment are to achieve virologic suppression of HIV and to achieve sustained virologic response (SVR) for HCV. Early initiation of ART is recommended for all individuals with HIV/HCV co-infection, regardless of CD4+ T cell count. This helps to reduce HIV-related morbidity, mortality, and the risk of HIV transmission.
When selecting of ART regimens considerations should be made for potential drug-drug interactions with HCV direct-acting antivirals (DAAs). Certain antiretroviral drugs, such as PI and NNRTI, may interact with HCV DAAs and require dose adjustments or alternative ART choices. Treatment for HCV in individuals with HIV/HCV co-infection typically involves interferon-free regimens consisting of oral DAAs. The choice of DAA regimen depends on HCV genotype, prior treatment history, and the presence of liver cirrhosis. The duration of HCV treatment varies depending on HCV genotype, baseline characteristics, and treatment response. In general, treatment durations of 8 to 12 weeks are recommended for most individuals with HIV/HCV co-infection. Regular monitoring of HIV viral load and HCV RNA levels is essential to assess treatment response and adjust therapy if needed (Gobran ST. et al., 2021.; Bar N. et al., 2022.).

An additional very important consideration for these patients includes liver function monitoring: liver function tests, including liver enzyme levels and markers of liver fibrosis, should be monitored regularly to assess liver health and guide treatment decisions.

The pathogenesis of HIV-HCV co-infection involves a complex interplay between the two viruses, immune dysregulation, and liver damage. HIV-induced immune dysfunction and chronic immune activation contribute to increased HCV replication, accelerated liver fibrosis, and a heightened risk of liver-related complications. Managing co-infection requires comprehensive care strategies that include both antiretroviral therapy for HIV and direct-acting antiviral therapy for HCV, along with addressing immune dysregulation and liver health. Understanding the pathogenesis of coinfection is crucial for developing effective prevention strategies and optimizing treatment approaches.

4. Metabolic enzymes and transporters

The Human Genome Project (HGP) identified 57 different cytochrome P450 (CYP) enzymes in human organisms. They are classified into 18 families and 43 subfamilies based on similarities in gene sequences. Among the phase I biotransformation enzymes, the cytochrome P450 family stands out for its catalytic versatility. This superfamily of enzymes represents the most important enzymatic composition involved in the biotransformation of many drugs. Physiological substrates of these enzymes include steroids, fatty acids, prostaglandins, leukotrienes, and biogenic amines, while xenobiotics include drugs, plant toxins, and toxic environmental substances. Cytochromes predominantly catalyze oxidation reactions, acting as monooxygenases, oxidases, and peroxidases, but they can also participate in reduction reactions. Functional molecules that serve as conjugation sites in phase II reactions are formed during phase I metabolism reactions. The highest concentrations of P450 enzymes are found in the membranes of the endoplasmic reticulum, primarily in the liver, although these enzymes can be found in almost all tissues and organs (intestines, lungs, kidneys, brain, lymphocytes, placenta). Therefore, P450 enzymes play an important role in regulating the intensity and duration of drug activity, detoxification of xenobiotics, and activation of xenobiotics into toxic, mutagenic, and carcinogenic metabolites (Knights KM . et al., 2016; Manikandan P. et al., 2018.)

Liver enzymes from the CYP P450 family involved in biotransformation are classified into three main genetic subfamilies: CYP1, CYP2, and CYP3. The liver also contains the CYP4 subfamily, whose substrates include certain fatty acids and eicosanoids, as well as a smaller number of xenobiotics (Taninger et al., 1999). On the other hand, not all CYP enzymes are involved in drug metabolism. Twelve CYP enzymes are responsible for metabolizing up to 93.0% of drugs. CYP 1A2, 2B6, 2D6, 2C9, and 2C19 are responsible for the metabolism of approximately 40.0% of drugs, and in combination with CYP 3A4, this number increases to as much as 60.0% (Knights KM . et al., 2016; Manikandan P. et al., 2018.).

The quantity and activity of P450 enzymes can vary among individuals due to genetic and external factors. Decreased enzyme activity may result from genetic mutations that inhibit enzyme synthesis or lead to the production of inactive forms of metabolic enzymes. It can also be caused by external factors, such as exposure to different infectious diseases or xenobiotics, which can suppress or induce the expression of P450 enzymes or inhibit/inactivate existing enzymes. Inhibition of cytochrome P450 by one drug can disrupt the biotransformation of another drug, potentially leading to increased pharmacological or toxic effects of the latter. In this regard, the inhibition of CYP P450 enzymes mimics genetically caused insufficient expression of these enzymes. Increased enzyme activity can be the result of genetic duplications that result in enhanced P450 enzyme expression or can be induced by external factors, such as xenobiotics that induce the synthesis of cytochrome P450 or stimulate existing enzymes. Induction of cytochrome P450 by one drug can enhance the metabolism of another drug, thereby reducing or increasing its therapeutic effect. Allelic variants resulting from mutations in the wild-type gene are another source of individual variability in P450 enzyme activity. Substitutions of amino acids can increase or more commonly decrease enzyme activity. External factors that can influence the quantity and activity of cytochrome P450 enzymes include medications (e.g., barbiturates, rifampin, isoniazid), food (e.g., cruciferous vegetables such as kale, turnips, cabbage; grapefruit), lifestyle habits (alcohol consumption, smoking), and diseases (diabetes, infections, hyper/hypothyroidism) as well as liver damage and reparation process (Zhou J et al., 2020; Drozdzik M. et al., 2021.).

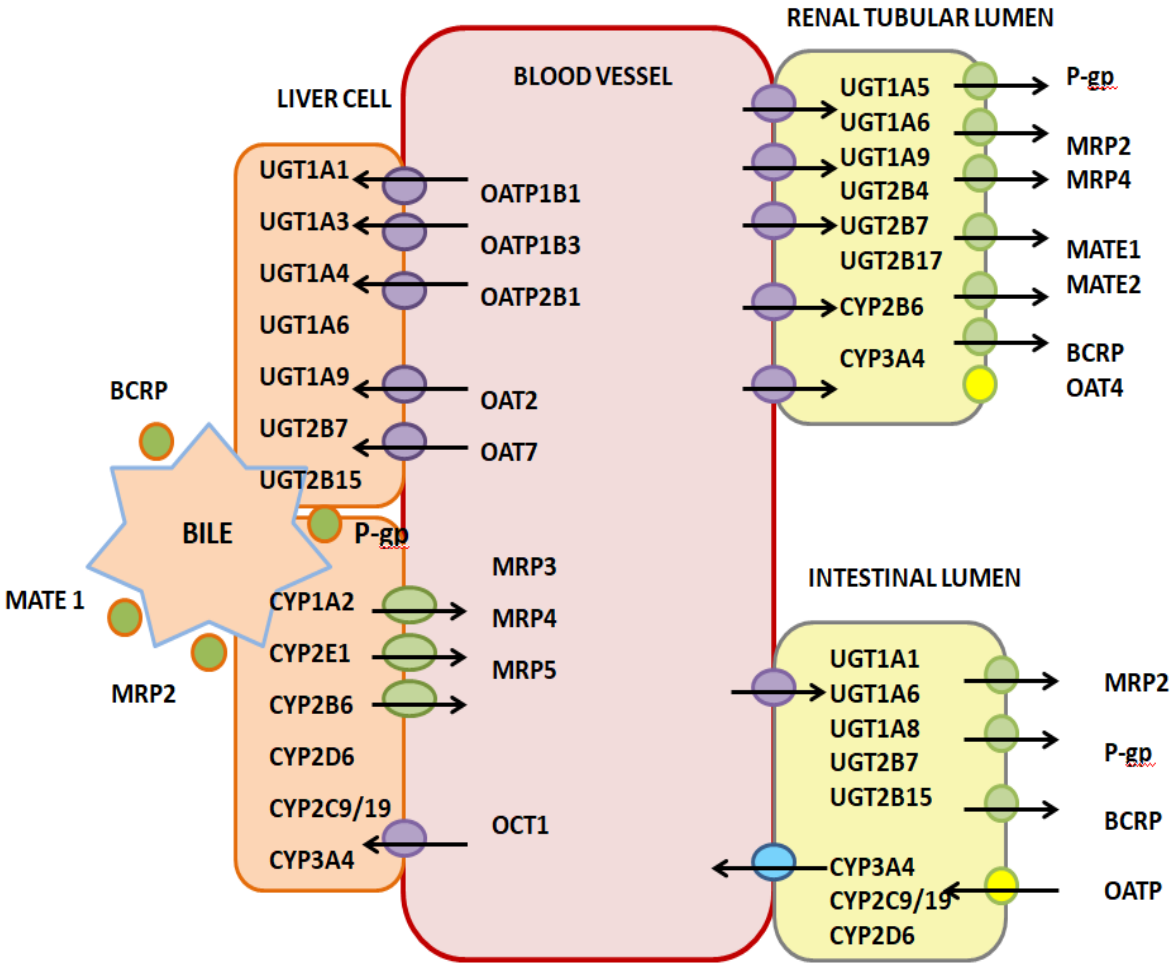

Figure 4. Common localization of clinically most important metabolic enzymes and transporters.

4.1. CYP2B6 - Localization, physiological effects, clinical significance, and therapeutic implications

CYP2B6 is a member of the cytochrome P450 superfamily of enzymes, which play a crucial role in the metabolism of endogenous and exogenous compounds. CYP2B6 is predominantly localized in the endoplasmic reticulum of hepatocytes, where it participates in the biotransformation of various xenobiotics, including drugs, toxins, and environmental pollutants. Although its expression is highest in the liver, CYP2B6 is also found in extrahepatic tissues such as the lungs, intestines, brain, and kidneys, albeit at lower levels. CYP2B6 plays a vital role in the metabolism of numerous clinically relevant drugs. It exhibits broad substrate specificity and contributes to the biotransformation of compounds such as antiretroviral drugs such as efavirenz and nevirapine, anticancer drugs like cyclophosphamide, antidepressants such as bupropion and sertraline, analgesics like methadone and fentanyl. CYP2B6 is involved in the metabolism of endogenous compounds, including steroids, fatty acids, and hormones. For instance, it contributes to the metabolism of testosterone, estradiol, progesterone, and retinoic acid. Most of the direct-acting antiviral (DAA) drugs used in the treatment of hepatitis C virus (HCV) infection are not primarily metabolized by CYP2B6. However, there are some exceptions where CYP2B6 may play a minor role in the metabolism of certain DAA drugs: daclatasvir is an NS5A inhibitor used in combination with other DAAs for the treatment of HCV. While daclatasvir is primarily metabolized by cytochrome P450 enzymes CYP3A4 and CYP3A5, studies have suggested that CYP2B6 can also contribute to its metabolism to a lesser extent. Similarly, although the primary metabolic pathway for elbasvir is through CYP3A4, CYP2B6 has been identified as a minor contributor to its metabolism (Desta Z et al., 2021.)

Interindividual variability in CYP2B6 expression and activity can significantly impact drug pharmacokinetics. Genetic polymorphisms in the CYP2B6 gene have been identified, leading to altered enzyme function and variable drug response. These variations contribute to differences in drug efficacy, toxicity, and adverse effects among individuals. Genetic variations in the CYP2B6 gene can lead to significant interindividual differences in drug response and pharmacokinetics (Ahmed S. et al., 2016; Neary M. et al., 2017.). Several genetic variants of CYP2B6 have been identified, including single nucleotide polymorphisms (SNPs) that can affect the enzyme's activity and expression. One of the most well-studied genetic variants is CYP2B6*6, which is characterized by a G-to-T change at position 516 in the gene (Ward BA. et al., 2003.). This variant is associated with decreased enzyme activity, resulting in slower metabolism of drugs metabolized by CYP2B6. The influence of CYP2B6 genetic variations on drug pharmacokinetics is evident in the metabolism of various medications. For example, the antiretroviral drug efavirenz is metabolized primarily by CYP2B6. Individuals carrying the CYP2B6*6 variant may have higher drug exposure and slower clearance of efavirenz, leading to an increased risk of adverse effects (Wang P. et al., 2019.) Similarly, bupropion, a medication used for smoking cessation and depression, is primarily metabolized by CYP2B6. Genetic variations in CYP2B6 can influence bupropion's clearance and efficacy (Mohamed MF. et al., 2021.). The impact of CYP2B6 genetic variations extends beyond individual drugs. Since CYP2B6 plays a role in the metabolism of numerous medications, including anticancer agents, antimalarials, anesthetics, and opioids, genetic variations can affect the effectiveness, toxicity, and dosage requirements of these drugs. Pharmacogenetic studies have shown that individuals with specific CYP2B6 variants may require adjusted dosing regimens to optimize therapeutic outcomes and minimize the risk of adverse reactions.

The influence of CYP2B6 genetic variations on drug pharmacokinetics is evident in the metabolism of various medications. For example, the antiretroviral drug efavirenz is metabolized primarily by CYP2B6. Individuals carrying the CYP2B6*6 variant may have higher drug exposure and slower clearance of efavirenz, leading to an increased risk of adverse effects. Similarly, bupropion, a medication used for smoking cessation and depression, is primarily metabolized by CYP2B6. Genetic variations in CYP2B6 can influence bupropion's clearance and efficacy (Pereira SA et al., 2008; Apostolova N. et al., 2015; Calza L. et al., 2015.).

Several transcription factors have been identified to play a role in regulating CYP2B6 expression. For instance, the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) are nuclear receptors that can activate CYP2B6 gene transcription (Chen Q. et al., 2015.). Their binding to specific response elements within the gene's regulatory regions can enhance gene expression. Epigenetic modifications, such as DNA methylation and histone modifications, can also influence CYP2B6 gene expression. DNA methylation involves the addition of a methyl group to the DNA molecule, often resulting in gene silencing. Studies have shown that DNA methylation patterns within the CYP2B6 promoter region can affect its expression levels. Similarly, histone modifications, which alter the structure of chromatin, can impact gene accessibility and transcriptional activity (Ngayo MO. et al., 2021.). Certain drugs can modulate CYP2B6 gene expression through drug-drug interactions. For example, some antiepileptic drugs, such as carbamazepine and phenytoin, have been shown to induce CYP2B6 expression. Induction occurs when drugs activate specific nuclear receptors, such as CAR and PXR, resulting in increased CYP2B6 transcription and subsequent enzyme activity.

Understanding the differences in CYP2B6 gene expression is important in personalized medicine and optimizing drug therapy. Variations in gene expression can contribute to interindividual differences in drug metabolism, efficacy, and adverse reactions. Pharmacogenomic studies aim to identify these differences to guide individualized treatment decisions, such as selecting appropriate drug doses or alternative medications, particularly for drugs primarily metabolized by CYP2B6.

4.2. CYP3A4 Localization, physiological effects, clinical significance, and therapeutic implications

CYP3A4 is primarily localized in the liver, specifically within the endoplasmic reticulum of hepatocytes, where it plays a crucial role in drug metabolism. However, it is important to note that CYP3A4 is not limited to the liver and is also expressed in extrahepatic tissues, including the small intestine, lungs, kidney, and various other organs. The expression of CYP3A4 can be influenced by various factors, such as age, sex, hormonal status, presence of infection, type of infection, genetic polymorphisms, and exposure to certain drugs or environmental factors. It exhibits substantial interindividual variability in its expression levels, which can result in differences in drug metabolism and response among individuals (Werk AN. et al., 2014.).

As a member of the cytochrome P450 enzyme superfamily, CYP3A4 is involved in the oxidative metabolism of a wide range of endogenous substances and exogenous compounds, including drugs, toxins, and xenobiotics. It catalyzes the metabolism of these compounds by introducing functional groups (such as hydroxyl, epoxide, or desethyl groups) to make them more water-soluble for

elimination from the body. The broad substrate specificity of CYP3A4 allows it to metabolize a vast array of drugs, including anticancer agents, immunosuppressants, cardiovascular drugs, antivirals, and many others. It is estimated that CYP3A4 is responsible for metabolizing around 50% of currently prescribed drugs (Werk AN. et al., 2014.). This makes it a crucial enzyme in drug metabolism and contributes to the variability in drug responses and therapeutic outcomes observed among individuals. Moreover, CYP3A4 can be influenced by drug-drug interactions, where concomitant administration of multiple drugs can either inhibit or induce its activity. Inhibition of CYP3A4 can lead to increased drug concentrations and potential toxicity, while induction can result in decreased drug levels and reduced efficacy. Therefore, understanding the potential for drug interactions involving CYP3A4 is essential in optimizing drug therapy and preventing adverse effects.

As a key enzyme in drug metabolism, CYP3A4 is involved in the biotransformation of a wide range of medications, including antiretrovirals and drugs used in the treatment of HCV and HBV infections. Its involvement in the metabolism of these drugs can have significant clinical implications, including drug interactions and variations in therapeutic responses. Many different groups of antiretrovirals undergo metabolism through this enzyme. Many PIs are substrates of CYP3A4. Examples include: atazanavir, indinavir, lopinavir, saquinavir, tipranavir. Some NNRTIs are also metabolized by CYP3A4. These include efavirenz, nevirapine, delavirdine. Although INSTIs are primarily metabolized by glucuronidation or other pathways, there can be minor involvement of CYP3A4 for some drugs (Chan C. et al., 2018.).

Several DAAs used in the treatment of HCV infection are substrates of CYP3A4. These include: grazoprevir, paritaprevir, simeprevir, velpatasvir, voxilaprevir as well as NS5A inhibitors such as daclatasvir, ledipasvir, ombitasvir (Saldarriaga O, et al. 2021.).

Understanding the involvement of CYP3A4 in the metabolism of antiretrovirals and anti-HCV, medications is essential for optimizing drug therapy. Drug-drug interactions involving CYP3A4 substrates may necessitate dose adjustments, therapeutic drug monitoring, or the selection of alternative treatment options to ensure efficacy and minimize the risk of adverse effects. Healthcare professionals should consider these factors when prescribing or managing drug regimens for patients receiving these medications.

4.3. Metabolic transporters involved in the metabolism of antiretroviral and drugs used in HCV infection therapy

Metabolic transporters are membrane proteins whose primary function is to facilitate the movement of molecules across the cells. They play a role in transporting nutrients and endogenous substrates such as carbohydrates, amino acids, nucleotide bases, vitamins, as well as protecting the body from toxins that arise from various dietary habits or environmental factors. However, it is a fact that transporter specificity is not limited to their physiological role. As a consequence, proteins that function as transporters have an important role in the bioavailability, efficacy, and pharmacokinetics of a wide range of drugs. Moreover, since drugs can compete with endogenous substrates for the same transporters, they can have different effects on organism homeostasis (Nigam SK., et al. 2015.).

Most transporters are expressed in tissues that serve as barriers, such as the liver, kidneys, gastrointestinal tract, placenta, and brain. Cells in these tissues are typically polarized. For example, enterocytes in the intestines and proximal tubule cells in the kidneys have an apical part facing the lumen of the organ and a basolateral part in contact with circulation. Hepatocytes are polarized toward the canalicular membrane facing the bile ducts, and on the other side, they face the sinusoidal membrane in contact with circulation. In most tissues, the expression of transport proteins is concentrated on one side of the polarized cell (apical or basolateral). This orientation of transport proteins plays a significant role in drug transport to the appropriate tissues and organs (Ahmed S. et al., 2016; Neary M. et al., 2017.).

Transport proteins can be categorized based on several characteristics. They can be influx or efflux transporters, depending on the direction in which they transport substrates across the cell membrane. This type of division is often used in cellular-level studies. In this definition, transporters that pump substrates out of cells are called efflux transporters, while those that transport substrates into the cell are called influx transporters.

Another way to classify transporters is based on their role in pharmacokinetic and pharmacodynamic processes. In this classification, transporters that carry substrates into the systemic circulation are called absorptive transporters, while those that eliminate substrates from the blood into bile ducts, urine, or the gastrointestinal system are called secretory transporters.

Most transporters can also be divided into two major families, ABC and SLC transporters. ABC transporters, or ATP-binding cassette transporters, are a family of membrane transport proteins that require ATP hydrolysis to carry out their function of transporting substrates across cell membranes. Therefore, transporters from the ABC family are primarily active transporters. This family of transporters includes eight subfamilies, and the most well-studied and well-known transporters in this group are the multidrug resistance protein (MDR), multidrug resistance-associated protein (MRP), and breast cancer resistance protein (BCRP) (Ahmed S. et al., 2016; Neary M. et al., 2017; Hardwick RN et al., 2011.).

Members of the SLC family, or solute carriers, are classified as facilitated transporters because they utilize the electrochemical difference between two environments or the ionic gradient generated by the activity of primarily active transporters to transport substrates against the gradient. Unlike ATP-

dependent transporters, SLC transporters do not possess an adenosine binding site. Most drug transporters belong to the SLC family.

Considering the significant role that drug transporters play in the absorption, distribution, and elimination of endogenous toxins and drugs, impaired function of these membrane proteins plays a crucial role in intra- and inter-individual variations that affect the therapeutic efficacy and toxicity of drugs. The main factors that can regulate transporter function include hormones, protein kinases, nuclear receptors, structural proteins, and the presence of diseases. These factors can influence transporter activity at multiple levels: (i) when and how often the gene encoding the transporter will be transcribed (transcriptional control), (ii) how the primary RNA transcript is processed (RNA control), (iii) which mRNA in the cytoplasm is translated into ribosomes (translational control), and (iv) how the transporter is modified and constructed (posttranslational control). Posttranslational modification can affect the physical and chemical properties of transporters, their binding, conformation, distribution, stability, and, ultimately, their activity. Regulation of transporter activity occurring at the gene level usually requires hours or days of activity and is therefore referred to as long-term or chronic regulation. Chronic regulation typically occurs when the organism undergoes significant changes, such as development or the onset of a disease. On the other hand, posttranslational regulation usually occurs within minutes or hours and is therefore referred to as short-term or acute regulation.

4.4. ABCB1/P glycoprotein: Localization, physiological effects, clinical significance, and therapeutic implications

Among transport proteins, the best-studied transporter is P-glycoprotein (P-gp), encoded by the ABCB1 or MDR1 gene (multidrug resistance) (Benish RL., et al. 2010.). P-gp is an integral membrane protein responsible for the efflux of substances from the cell and its membrane to the circulation. Its physiological function is to protect the cell from toxic compounds. Numerous drugs are substrates of this transporter, and therefore, the activity of P-gp affects their pharmacokinetic parameters, interactions, and therapeutic efficacy. This fact was initially observed in malignant cells, where high expression and activity of MDR1 confer resistance to various drugs. P-gp is also present in various non-malignant cells and organs, including the intestines, placenta, kidneys, liver, pancreas, testes, blood-brain barrier, lymphocytes, and macrophages. The expression of P-glycoprotein in these tissues results in reduced drug absorption from the gastrointestinal tract, increased drug elimination into bile and urine, and slower penetration of certain drugs into the central nervous system. The clinical significance of P-glycoprotein's role depends on its localization in tissues, the therapeutic index of the substrate drug, and interindividual variability. Regarding variability, studies on the polymorphisms of the ABCB1 gene have shown a significant correlation between certain genotypes and haplotypes with changes in the pharmacokinetics and interactions of clinically important drugs such as digoxin, talinolol, tacrolimus, and HIV protease inhibitors (Sui H. et al., 2014.).

Well-known substrates of P-glycoprotein include antimicrobial agents (erythromycin, protease inhibitors, fluconazole, clarithromycin, ketoconazole, rifampin), antiepileptics (phenytoin, phenobarbital, carbamazepine, lamotrigine, felbamate), statins (atorvastatin, fluvastatin, fenofibrate, lovastatin, simvastatin), immunosuppressants (cyclosporine, tacrolimus), calcium channel blockers (amlodipine, felodipine, nifedipine, verapamil), psychotropic drugs (alprazolam, diazepam, fluoxetine,

paroxetine), as well as dexamethasone, digoxin, prednisolone, and propafenone (Elmeliegy M. et al., 2020.).

Several antiretroviral, anti-HCV drugs have been identified as substrates of ABCB1. Some examples include protease inhibitors like saquinavir, ritonavir, NNRTIs like efavirenz, nevirapine and delavirdine; NRTIs such as zidovudine, lamivudine. Also, DAA drugs such as telaprevir, boceprevir, ledipasvir, ombitasvir are substrates for P-gp as well.

Gene expression changes in ABCB1 can have significant implications for drug response and treatment outcomes. Genetic polymorphisms in the ABCB1 gene can affect the expression and function of the transporter. Single nucleotide polymorphisms such as C3435T, G2677T/A, and C1236T, have been associated with altered ABCB1 expression levels (Milojkovic M., et al. 2011.). Individuals carrying specific variants may exhibit differences in drug disposition and response. For example, some studies suggest that certain ABCB1 polymorphisms may influence the pharmacokinetics of antiretroviral drugs and affect treatment outcomes in HIV-infected patients (Ahmed S. et al., 2016; Neary M. et al., 2017;). Certain drugs or co-administered substances can influence ABCB1 expression through various mechanisms. Some drugs, referred to as ABCB1 inducers or activators, can upregulate the expression of ABCB1. On the other hand, ABCB1 inhibitors may downregulate its expression or inhibit its activity. These interactions can occur at the transcriptional or post-transcriptional level and can have implications for drug efficacy and toxicity. Understanding these interactions is crucial in avoiding suboptimal treatment outcomes and potential drug interactions. Various disease states can influence ABCB1 expression levels.

Overall, gene expression changes in ABCB1 can significantly impact drug response, drug interactions, and treatment outcomes. Understanding the factors influencing ABCB1 expression, such as genetic variations, drug interactions, and disease conditions is crucial in individualizing treatment strategies, predicting drug efficacy, and minimizing potential adverse effects. Further research and comprehensive studies are needed to unravel the complex mechanisms underlying ABCB1 expression changes and their clinical implications.

5. Inflammation influence on metabolic enzymes and transporter activity

The gene expression and function of metabolic enzymes and transporters responsible for drug metabolism and disposition can be significantly impacted by inflammation. This can lead to changes in medication pharmacokinetics, drug-drug interactions, and therapeutic outcomes. Tumor necrosis factor-alpha (TNF-α) and other cytokines are examples of inflammatory mediators that can modify the expression of genes encoding metabolic enzymes and transporters. The transcription of these genes can be increased or decreased by various inflammatory mediators via activating signaling pathways. For example, pro-inflammatory cytokines have been demonstrated to suppress the expression of cytochrome P450 enzymes, including CYP1A1, CYP1A2, CYP2C9, and CYP3A4 (Drozdzik M. et al. 2021.).

Three mechanisms have been proposed to explain how inflammation leads to altered activity of metabolic enzymes and transporters: 1) activation of the nuclear factor-kappa B (NF-kB) pathway by pro-inflammatory cytokines, contributing to the inhibition of transcription of metabolic enzymes and transporters; 2) post-transcriptional mechanisms involving the degradation of metabolic enzymes and transporters through the synthesis of nitric oxide; and 3) epigenetic changes in the methylation process in the promoter regions of metabolic enzymes and transporters (Klomp F. et al., 2020.; Lenoir C et al., 2021.; Gwak K et al., 2020.)

Inflammation can directly impact the functioning of metabolic enzymes and transporters in addition to altering gene expression. Through post-translational alterations like phosphorylation or nitrosylation, cytokines and other inflammatory mediators can control the activity of enzymes (Lenoir C et al., 2021.; Gwak K et al., 2020.). These alterations may lead to altered drug metabolism by increasing or decreasing enzymatic activity. As an illustration, it has been demonstrated that several cytochrome P450 enzymes, such as CYP2C9 and CYP2D6, are less active when pro-inflammatory cytokines are present (Lenoir C et al., 2021.; Gwak K et al., 2020.).

Another way of inflammation influencing function of metabolic enzymes and transporters is by affecting integrity and functioning of numerous biological barriers or organs such as liver, which are involved in medication absorption, metabolism, and elimination. For instance, intestinal epithelial drug transporters like P-gp might be affected in terms of expression and function by gastrointestinal tract inflammation. Additionally, inflammatory stimuli can activate nuclear factor-kappa B (NF-B) and other transcription factors, causing them to express and activate a number of metabolic enzymes. This induction is thought to be a protective mechanism designed to speed up elimination of inflammatory substances. It may, however, also result in increased drug metabolism and decreased drug exposure, which may jeopardize the therapeutic effectiveness of some medications (Lenoir C et al., 2021.).

On the other hand, since the liver plays a significant role in drug metabolism, inflammation can as well have a negative effect on liver function. Hepatic fibrosis, liver damage, and decreased drugs clearance can all be caused by hepatic inflammation. Affected drug pharmacokinetics, elevated drug toxicity, and decreased medication effectiveness may result from this (Hanada K., 2012.). Using biochemical measures like ALT and AST as well as integrated biochemical indexes like the AST to platelet ratio index, the degree of liver damage is clinically measured. For forecasting fibrosis levels, techniques based on biochemical and demographic factors, such as the FIB-4 system and Forns index, as well as specific diagnostic techniques, are also utilized. Another way of measuring liver fibrosis is

elastography, a non-invasive technique based on specialist ultrasound examination. However, liver biopsy analysis, with fibrosis staging and inflammation grading being the primary histological markers, continues to be the gold standard for determining liver damage (Asrani SK et al., 2019.).

The METAVIR score is often used in clinical practice today to categorize the extent of liver fibrosis, which ranges from the lack of fibrosis to the presence of mild, moderate, and severe fibrosis as well as cirrhosis (stages F0-F4). To increase the accuracy and repeatability of outcomes, many changes of existing scoring systems have been suggested. According to recent research, evaluating the level of liver fibrosis is crucial since it is a non-genetic component that may affect the CYP enzyme system's function (Asrani SK et al., 2019.).

Available *in vitro* and animal studies has demonstrated the impact of inflammation on the activity of CYP P450 enzymes and the ABC transporter family through multiple and complex transcriptional and post-transcriptional mechanisms. The literature primarily suggests an inhibitory effect of inflammation on enzyme and transporter activity, although some studies have shown an induction of liver enzyme activity in the presence of higher levels of inflammation. The activity of metabolic enzymes and transporters is also believed to depend on specific isoforms of CYP P450 enzymes and the nature of inflammatory mediators, as well as the origin and duration of inflammation (Lenoir C et al., 2021.; Gwak K et al., 2020.).

Both HIV and HCV infections may regulate the expression levels of *CYP2B6, CYP3A4* and *ABCB1* genes through complex mechanisms involving viral proteins, inflammatory mediators and cytokines. As a result, alterations in CYP2B6, CYP3A4, and ABCB1 expression and activity in HIV- and HCV-infected individuals can have profound effects on pharmacokinetics and therapeutic outcomes. Literature data mainly show an inhibitory effect of inflammation on enzyme activity (Wolffenbüttel L et al., 2004; Hardwick RN et al., 2011.), although some studies have shown induction of liver enzyme activity in the presence of higher levels of inflammation (Hanada K., 2012.). The nature of inflammatory mediators, i.e., origin and duration of inflammation, may contribute to changes in the activities of metabolic enzymes and transporters Wolffenbüttel L et al., 2004; Hardwick RN et al., 2011.). Studies assessing gene expression of metabolic enzymes and transporters in HIV, HCV, and HCV/HIV co-infected patients are limited, and to our knowledge, few studies have been performed in human liver tissue. Previous studies have shown less variation in gene expression levels as an indicator of enzyme activity compared to protein abundance, enzyme catalytic activity, or the presence of polymorphisms (Hardwick RN et al., 2011; Talal A. et al., 2020; Stanke-Labesque F et al. 2020). Comparing the expression of genes encoding metabolic enzymes and transporters with constitutive genes (housekeeping genes) provides insight into their potential expression patterns in this particular population.

2. RESEARCH OBJECTIVES:

1. To conduct an analysis of the gene expression of *CYP2B6, CYP3A4,* and *ABCB1* transporter in a cohort of patients infected with HIV, patients infected with HCV, and patients co-infected with HIV and HCV.

2. To investigate the association between the degree of fibrosis, level of inflammation, and the expression levels of genes encoding *CYP2B6, CYP3A4*, and *ABCB1* transporter in patients infected with HIV, patients infected with HCV, and patients co-infected with HIV and HCV.

3. To examine the potential impact of different therapeutic regimens on the expression of genes encoding *CYP2B6, CYP3A4*, and *ABCB1* transporter in patients infected with HIV, patients infected with HCV, and patients co-infected with HIV and HCV.

4. To examine the potential impact of different biochemical and inflammatory factors on the expression of genes encoding *CYP2B6, CYP3A4*, and *ABCB1* transporter in patients infected with HIV, patients infected with HCV, and patients co-infected with HIV and HCV.

3. MATERIALS AND METHODS

3.1. Inclusion and exclusion criteria

Inclusion criteria were age older than 18, both, male and female and serologically confirmed diagnosis of HIV and HCV infection. Exclusion criteria were hepatitis of unexplained etiology, autoimmune hepatitis, associated liver diseases, use of all known drugs or substances that are inhibitors or inducers of liver enzymes (*CYP2B6* and *CYP3A4*) and transporter (*ABCB1*). All patients signed informed consent for participation in the study in accordance with the guidelines included in the Declaration of Helsinki and with approval from Ethics Committee of Faculty of Medicine, University of Belgrade (protocol code: 17/I-8 12.1.2023).

3.2. Liver biopsy

A liver biopsy was performed as part of diagnostic and therapeutic protocols in patients for whom it was indicated. Those who had already completed screening and qualified for a liver biopsy were chosen for the study's patient selection process from a pre-established group. Biopsy was performed using Menghini 14 gauge needle. Each tissue cylinder obtained by biopsy was at least 2 mm wide and at least 20 mm in length. After sampling, the liver tissue was divided into two parts. One part was fixed in formalin and used to determine the stage of liver fibrosis and grade of liver inflammation. The remaining liver tissue was snap-frozen over liquid nitrogen and stored at a temperature of -80°C until further analyses were performed (qRT-PCR). All samples were collected at VI and XI department at the Clinic for Infectious and Tropical Disease “Dr Kosta Todorovic”, UKCS, Belgrade, Serbia and stored at the Department of Pharmacology, Clinical Pharmacology and Toxicology, Medical Faculty, University of Belgrade, Belgrade, Serbia.

3.3. Tissue homogenization and RNK extraction from liver tissue

All procedures were carried out in an RNase-free environment and all solutions were made up using RNase-free water/reagents. CK 14 microtubes were used for homogenization. Each sample was placed on ice. With a metal spatula, each sample was removed from collection tubes and placed into CK14 microtubes. 500 µl of QIAzol Lysis Reagent were added to each tube. CK14 microtubes were then placed in the Minilys® personal homogenizer and homogenized for 20 seconds at 5000 rpm. Homogenate was then centrifuged at 12,000 x g for 10 min at 4°C to remove insoluble material. The supernatant was carefully transferred to a new 2 ml collection tube. Tubes containing the homogenates were placed at room temperature (15–25°C) for 5 min. This step was done to promote the dissociation of nucleoprotein complexes. The next step included adding 100 µl of chloroform per 500 µl QIAzol Lysis Reagent. Microtubes were placed in the vortex for 15 seconds. After resting at room temperature for 2–3 min each microtube was centrifuged at 12,000 x g for 15 min at 4°C. After centrifugation, the sample separates into 3 phases: an upper, clear aqueous phase containing RNA; milky white interphase; and a lower, red, organic phase. The upper aqueous phase was then transferred to clean 2 ml collection

tubes. In each microtube 250 µl isopropanol was added and then mixed by vertexing. All the samples were then placed at room temperature for 10 minutes and then placed into a centrifuge at 12,000 x g for 10 min at 4°C. A small white pellet was formed after centrifugation containing the RNA at the bottom of the tube. The liquid was carefully aspirated and the supernatant was discarded. In each sample at least 500 µl of 75% ethanol per 500 µl QIAzol Lysis Reagent were added. Each sample was then placed in a centrifuge at 12,000 x g for 5 minutes at 4°C. Ethanol supernatant was carefully removed and each sample air dried until the pellet become translucent (roughly 30 min). The RNA pellet was redissolved in the appropriate volume of RNase-free water (20-50 µl, depending on pellet size). For each sample RNA Clean-Up Protocol was performed according to manufacturer instructions.

3.4. cDNA synthesis

All procedures were carried out in an RNase-free environment and all solutions were made up using RNase-free water/reagents and RNaseZAP™ (Sigma-Aldrich) was used for surfaces. For cDNA synthesis TaqMan® Reverse Transcription Reagents (Thermofisher, Cat No. N8080234) were used. Mastermix included RT Buffer, MgCl2, dNTP mix, RNase Inhibitor, MultiScribe (RT enzyme), and random hexamers. 400ng of RNA was reverse transcribed to produce cDNA, which was then diluted to a concentration of 5ng/µl for use in RT-qPCR. RNA quality and quantity were assessed by spectrophotometry using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

3.5. Real-Time qRT-PCR and data analysis

All reagents were thawed on ice and kept on ice until required. TaqMan Gene Expression Master Mix was twirled gently to mix the components. For each condition to be tested, cDNA + H2O in combination were pipetted into two separate sterile, nuclease-free 0.2 ml PCR tubes (one was used for the test gene and one was used for the housekeeping gene) and mixed well by vortexing gently for 5 seconds. qPCR Mastermix was prepared for each gene to be studied e.g. GAPDH (housekeeping gene) and test gene (CYP3A4, CYP2B6 andABCB1) and added to each sample tube.

Expression via RT-qPCR was analyzed for all genes on 96-well white Hard-Shell™ PCR plates (Bio-Rad, Hemel Hempstead, UK, Cat. No. HSP-9601) using Mastermix (TaqMan® Gene Expression Master Mix; Cat. No. 4369016, Applied Biosystems, Waltham, MA, USA), with each well containing 100ng of cDNA (20µl per well). All samples were run in triplicate. Following sample Mastermix addition, the plate was sealed using optically clear adhesive seal sheets (Thermo-Scientific, Cat. No.: AB-1170), and centrifuged for 1 minute at 1300xg, 4° C to ensure proper mixing of reagents and to remove air bubbles. For the blank wells, Sigma H2O was used together with TaqMan® Gene Expression Assay/2X TaqMan® Gene Expression Master Mix.

The following TaqMan® Gene Expression Assay (Life Technologies) probes were used: CYP2B6: Hs04183483_g1; CYP3A4: Hs00604506_m1; ABCB1: Hs00184500_m1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): Hs02758991_g1. Plates quantification was performed using Bio-Rad Opticon Monitor™ Analysis software (version 3.1.32)

Fold changes in gene expression were determined as described by Pfaffl (Pfaffl M., 2001). Change above 2-fold and below 0.5-fold was considered increased or decreased gene expression.

Tissue homogenization and RNK extraction from liver tissue, cDNA synthesis and real-time qRT-PCR were done at the Department of Translational Medicine at the University of Liverpool.

3.6. Histopathological and histochemical processing of samples

After fixation in formalin through standard histopathological tissue processing, each tissue sample was embedded in paraffin blocks, stained with the standard hematoxylin-eosin method, and histopathologically analyzed. Data on the stage of fibrosis and the level of inflammatory activity was obtained from the patient's medical records.

In order to quantify the stage of fibrosis silver impregnation staining of reticular fibers was performed on sections from each block, followed by contrast staining with hematoxylin. The level of liver fibrosis was classified based on the METAVIR score, as the absence of fibrosis, the presence of mild, moderate, severe fibrosis, and cirrhosis (stages F0-F4). The degree of inflammation was classified based on the METAVIR score; such as the absence of inflammation, the presence of minimal changes, and light, moderate, and high-intensity inflammation.

3.7. Immunohistochemical staining

In order to quantify the intensity of the inflammatory process in the liver, immunohistochemical staining was performed on 4mm sections from each tissue block. After deparaffinization and antigen retrieval, each sample was labeled with a primary antibody against CD8 (Novocastra). Immunohistochemical staining was conducted manually according to the manufacturer's instructions. Visualization of positive staining was performed using the standard streptavidin-biotin technique (DAKO LSAB+ kit).

3.8. Histopathological and morphometric analysis

The analysis of immunohistochemically stained tissue sections was carried out using a light microscope (Olympus D52), after which four representative fields will be analyzed from each sample at 200x magnification. Each field of view (FOV) will be captured as an image, and the micrographs will then be analyzed using FIJI software (https://imagej.net/software/fiji/downloads).

The degree of fibrosis will be quantified on reticulin-stained preparations and presented as the percentage of representative fields of view covered by connective tissue.

The degree of inflammatory activity in liver tissue will be quantified by identifying CD8+ cytotoxic lymphocytes and presented in two ways: the absolute number of lymphocytes in representative fields of view and the percentage of the area covered by lymphocytes in representative fields of view (Stankovic M. et al., 2016.).

Table 2. METAVIR scoring system.

METAVIR scoring system					
Severity of chronic hepatitis	Piecemeal Necrosis (PMN)	+	Lobular Necrosis (LN)	=	Histological Activity Score
0 = none	PMN 0	+	LN 0	→	A0
1 = mild	PMN 0 PMN 1	+ +	LN 1 LN 0 or 1	→	A1
2 = moderate	PMN 0 or 1 PMN 2	+ +	LN 2 LN 0 or 1	→	A2
3 = severe	PMN 2 or 3 PMN 3	+ +	LN 2 LN 0 or 1 or 2	→	A3

Table 3. Description of METAVIR scoring system

METAVIR scoring system		
Description	Fibrosis level (Stage)	Severity of fibrosis
No fibrosis / No scarring	F0	
Portal tract fibrosis without septa formation / Minimal scarring	F1	Mild to moderate fibrosis
Portal tract fibrosis with infrequent/rare septa formation (Scarring around vessels within the liver)	F2	Significent fibrosis
Numerous septa, but no cirrhosis (Bridging fibrosis)	F3	Severe / advanced fibrosis
Cirrhosis / advanced scarring	F4	

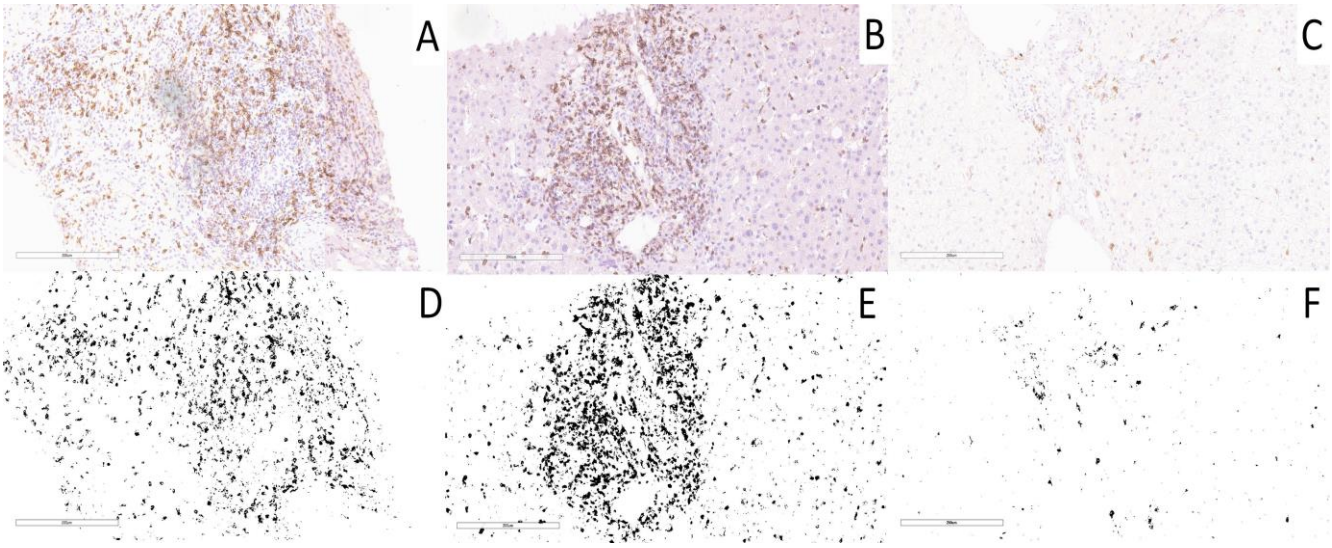

Figure 5. .Immunochemically stained liver tissue for CD8 positive lymphocytes showing severe, A3 METAVIR score A), moderate, A2 METAVIR score B), and minimal to no necroinflamмatory activity, A0-A1 METAVIR score C), magnification 200x; 16bit formatted micrographs, thresholder to emphasize brown color of 3,3′-Diaminobenzidine chromogen used for immunohistochemical staining D), E), and F) for assessment of number of CD8 positive lymphocytes 200x.

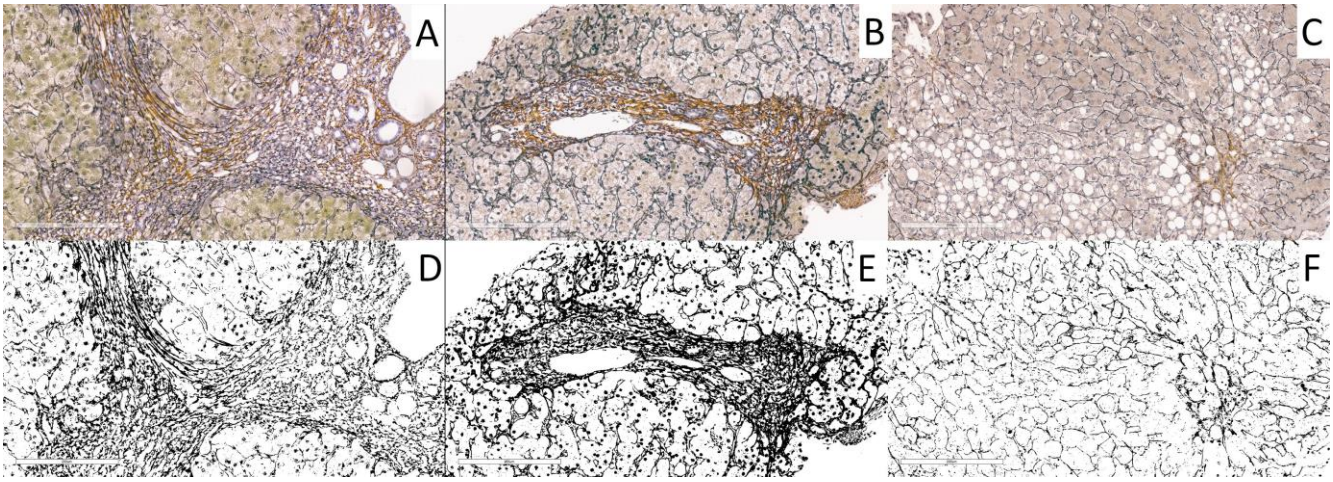

Figure.6. Slides showing cirrhosis, F4 METAVIR score A), severe fibrosis, F3 METAVIR score B), and minimal to no fibrosis, F0-F1 METAVIR score C), Reticuline silver stained, magnification 200x; 8bit formatted micrographs, tthresholdto emphasize fibrous tissue D), E), and F) for assessment of area percentage in field of view 200x.

3.9. Statistical analysis

Categorical variables are shown as percentages and absolute numbers. Depending on the distribution of the data, numerical variables were displayed as means with standard deviations or medians with the 25th–75th percentile. ANOVA with LSD was employed as a posthoc test to evaluate differences between the three patient groups, while Student's t-test or a Mann-Whitney test was utilized to evaluate differences in numerical variables between the two groups. Both the Chi-square test and the Fisher exact test were used to assess categorical data. Depending on the data scale used in the analyses, correlations were looked at using correlation coefficients (Pearson or Spearman correlation coefficient). The significance threshold was set at 0.05. The IBM SPSS 21 (Chicago, IL, USA, 2012) software program was used for the statistical analysis. The data analysis was conducted at the Department of statistic at the Faculty of Medicine, University of Belgrade.

4. RESULTS

Study population

In this cross-sectional study, a total of 54 patients were divided into three groups: patients infected only with HCV, patients infected with only HIV, and those co-infected with both HIV and HCV. The sample consisted of 30 (55.6%) patients with HCV infection, 9 (16.7%) patients with HIV infection, and 15 (27.8%) patients with co-infection of HIV/HCV (as shown in Table 4, Figure 7.).

Table 4. Infection frequency in the study population

Group	HCV without therapy	HIV	HCV/HIV	HCV receiving therapy		
				Total	PEG-IFN	DAA
Frequency (N)	16 (53.3%)	9 (16.7%)	15 (27.8%)	14 (46.7%)	7 (50.0%)	7 (50.0%)

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct acting antivirals;

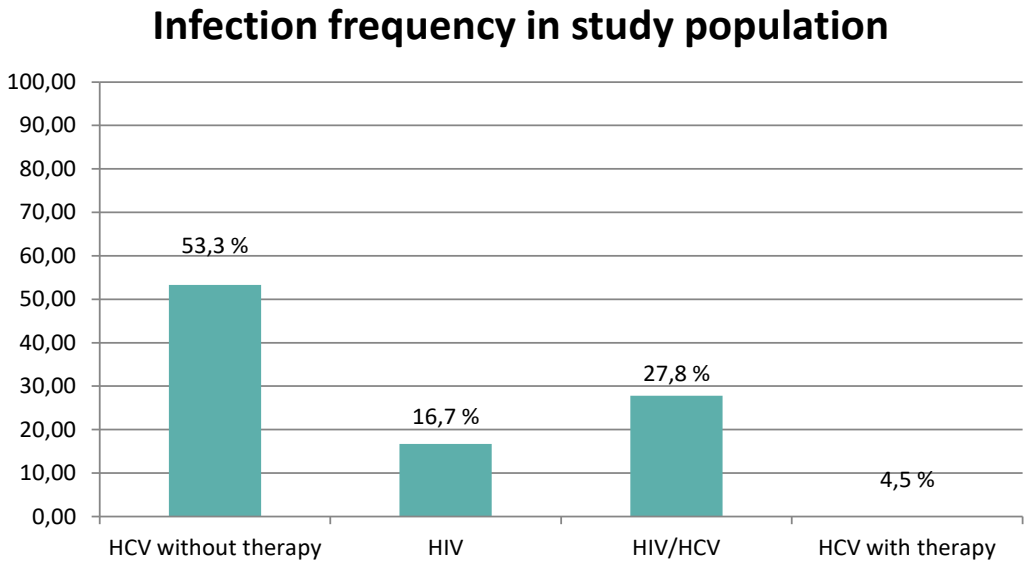

Figure 7. Infection frequency in study population

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus;

14 (46.7%) of the HCV patients were receiving treatment, whereas 16 (53.3%) were not. Seven (50.0%) of the HCV patients were treated with PEG-IFN, while seven (50.0%) received DAA. All HIV patients got antiretroviral medication (ART), including those who were co-infected with HCV and

HIV. Lamivudine (50%) was given to 12 patients, dolutegravir (45.8%) to 11, and efavirenz (58.3%) was given to 14 patients as part of cART. None of the co-infected individuals (Table 5) had HCV therapy.

Table 5. Use of all medications in the study population.

	Infection	ART	HCV therapy		Other medications
			PEG-IFN	DAA	
1.	HIV	EFV+ABC/LAM			ASP 100mg daily BIS 1.25mg daily
2.	HIV	DTG+EMT/TDF			
3.	HIV	DTG+EMT/TDF			
4.	HIV	DTG+EMT/TDF			
5.	HIV	EFV+ABC/LAM			
6.	HIV	DTG+EMT/TDF			
7.	HIV	DTG+EMT/TDF			
8.	HIV	DTG+EMT/TDF			
9.	HIV	DTG+EMT/TDF			
10.	HIV/HCV	DTG+EMT/TDF	no	no	
11.	HIV/HCV	DTG+EMT/TDF	no	no	SIL 420mg daily
12.	HIV/HCV	EFV+ABC/LAM	no	no	
13.	HIV/HCV	EFV+ABC/LAM	no	no	
14.	HIV/HCV	DTG+EMT/TDF	no	no	
15.	HIV/HCV	EFV+ABC/LAM	no	no	
16.	HIV/HCV	DTG+EMT/TDF	no	no	
17.	HIV/HCV	DTG+EMT/TDF	no	no	
18.	HIV/HCV	EFV+ABC/LAM	no	no	
19.	HIV/HCV	EFV+ABC/LAM	no	no	
20.	HIV/HCV	DTG+EMT/TDF	no	no	
21.	HIV/HCV	DTG+EMT/TDF	no	no	
22.	HIV/HCV	DTG+EMT/TDF	no	no	
23.	HIV/HCV	EFV+ABC/LAM	no	no	
24.	HIV/HCV	DTG+EMT/TDF	no	no	LOR 1mg daily
25.	HCV		no	no	SIL 420mg daily
26.	HCV		no	no	
27.	HCV		no	no	
28.	HCV		no	no	
29.	HCV		no	no	
30.	HCV		no	no	
31.	HCV		no	no	
32.	HCV		no	no	
33.	HCV		no	no	
34.	HCV		no	no	
35.	HCV		no	no	
36.	HCV		no	no	
37.	HCV		no	no	
38.	HCV		no	no	
39.	HCV		no	no	
40.	HCV		no	no	
41.	HCV			SOF/VEL	
42.	HCV			SOF/VEL	
43.	HCV		PEG-IFN		
44.	HCV		PEG-IFN		
45.	HCV		PEG-IFN		
46.	HCV			SOF	LOR 1mg daily
47.	HCV			SOF/VEL	
48.	HCV		PEG-IFN		
49.	HCV			SOF	
50.	HCV		PEG-IFN		ASP 100mg daily
51.	HCV		PEG-IFN		
52.	HCV			SOF/VEL	
53.	HCV		PEG-IFN		ASP 100mg daily
54.	HCV			SOF	

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; ART-antiretroviral therapy; DTG-dolutegravir; EMT-emitricabine; TDF-tenofovir disoproxil fumarate; EFV-efavirenz; ABC-abacavir; LAM-lamivudine; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals; SOF-sofobuvir; VEL- velpatasvir; SIL-silymarin; LOR-lorazepam; ASP- acetylsalicylic acid; BIS-bisoprolol;

The average patient age was 50±13, 20 (37.0%) patients were female and 34 (63.0%) were male. All of the patients were white, and their median BMI ranged from 23.6 kg/m2 (25^{th} - 75^{th} percentile: 20.4 - 25.8). The median CD4+ T - cell count was 656 cells/ml (25^{th}-75^{th} percentile: 459 - 928), the median ALT was 96 U/L (25^{th}-75^{th} percentile: 69 - 120), and the median AST was 60.5 U/L (25^{th}-75^{th} percentile: 38 - 89). Twenty-two (40.7%) patients were consuming tobacco, and twelve (22.2%) reported alcohol consumption. Table 6 provides an overview of the key clinical data for the study population.

Table 6. The summary of the main clinical data of the study population

Clinical data	**HCV untreated**	**HIV**	**HCV/HIV**	**HCV receiving therapy**		
				Total	**PEG-IFN**	**DAA**
Age*	47.81 (±12.82)	57.22 (±18.46)	49.20 (±10.78)	48.5 (±8.81)	51.29 (±10.44)	45.71 (±6.42)
BMI*	23.18 (±4.09)	23.06 (±2.12)	22.61 (±3.94)	25.16 (±2.80)	24.46 (±2.77)	25.98 (±2.84)
Gender female**	43.8% (n=7)	11.1% (n=1)	26.7% (n=4)	42.9% (n=6)	42.9% (n=3)	42.9% (n=3)
Narcotics**	6.3% (n=1)	11.1% (n=1)	13.3% (n=2)	7.1% (n=1)	0% (n=0)	14.3% (n=1)
Alcohol**	25% (n=4)	11.1% (n=1)	13.3% (n=2)	35.7% (n=5)	42.9% (n=3)	28.6% (n=2)
Tobacco**	43.8% (n=7)	33.3% (n=3)	40% (n=6)	42.9% (n=6)	42.9% (n=3)	42.9% (n=3)
AST***	53 (33 - 62)	78 (56-87)	59 (37-78.5)	86 (61-118.5)	87 (63-107)	85 (41-127.5)
ALT***	83 (40-100.75)	98 (85-115)	89 (69-113)	111 (98-179.5)	115 (98-150)	106 (93-183.5)
CRP***	2.5 (1.3-5.25)	2 (2-3)	3 (1-5.5)	2.7 (2-4)	2 (2-3)	4 (2.1-4.5)

Mean (±Standard deviation); **Percentage within the group (N); ***Median (25^{th}-75^{th} percentile);

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; PEG IFN - pegylated interferon; DAA- direct-acting antivirals

Table 7. Comparison of clinical data between the groups of HIV, HCV, and HIV/HCV co-infected patients

Clinical data	HCV untreated	HIV	HCV/HIV	*p* value
Age*	47.81 (±12.82)	57.22 (±18.46)	49.20(±10.78)	*p*=0.240
BMI*	23.18 (±4.09)	23.06 (±2.12)	22.61(±3.94)	*p*=0.908
Gender female**	43.8% (n=7)	11.1% (n=1)	26.7%(n=4)	*p*=0.218
Narcotics**	6.3% (n=1)	11.1% (n=1)	13.3%(n=2)	*p*=0.800
Alcohol**	25% (n=4)	11.1% (n=1)	13.3%(n=2)	*p*=0.589
Smoking**	43.8% (n=7)	33.3% (n=3)	40%(n=6)	*p*=0.878
AST***	53 (33-62)	78 (56-87)	59 (37-78.5)	*p*=0.198
ALT***	83 (40-100.75)	98 (85-115)	89 (69-113)	*p*=0.295
CRP***	2.5 (1.3-5.25)	2 (2-3)	3 (1-5.5)	*p*=0.689

Mean (±Standard deviation); **Percentage within the group (N); ***Median (25^{th}-75^{th} percentile);

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; BMI-body mass index; AST-aspartate aminotransferase; ALT-alanine transaminase; CRP-C-reactive protein.

All patients, HIV mono-infected, HCV mono-infected, HIV/HCV co-infected as well as groups of HCV mono-infected treated and untreated patients were compared between groups for basic patient characteristics. Variables such as age, BMI, gender, narcotics, alcohol and tobacco consumption were analyzed and compared. Also, clinical parameters like AST, ALT and CRP levels were described and compared between these groups of patients. This comparison is for three groups of patients (HIV, HCV mono-infected and HIV/HCV co-infected) presented in Table 4 while for HCV mono-infected treated and untreated patients presented in Table 7.

In Table 8. and Table 9. are presented basic patients characteristics in the group of treated or untreated mono-infected HCV patients. .

Table 8. Comparison of clinical data between the groups of HCV-infected treated and untreated patients

Clinical data	HCV untreated	HCV receiving therapy	*P*
Age*	47.81(±12.82)	48.5 (±8.81)	*p*=0.867
BMI*	23.18 (±4.09)	25.16 (±2.80)	*p*=0.150
Gender female**	43.8% (n=7)	42.9% (n=6)	*p*=0.961
Narcotics**	6.3% (n=1)	7.1% (n=1)	*p* = 1.00
Alcohol**	25% (n=4)	35.7% (n=5)	*p* = 0.694
Smoking**	43.8% (n=7)	42.9% (n=6)	*p* = 0.969
AST***	53 (33-62)	86 (61-118.5)	*p* = 0.005
ALT***	83 (40-100.75)	111(98-179.5)	*p* = 0.012
CRP***	2.5 (1.3-5.25)	2.7 (2-4)	*p* = 0.728

Mean (±Standard deviation); **Percentage within the group (N); ***Median (25^{th}-75^{th} percentile); HCV-hepatitis C virus; BMI-body mass index; AST-aspartate aminotransferase; ALT-alanine transaminase; CRP-C-reactive protein.

Table 9. Comparison of clinical data between the groups of HCV infected treated patients

Clinical data	HCV receiving therapy		*P*
	PEG-IFN	DAA	
Age*	51.29 (±10.44)	45.71(±6.42)	*p* = 0.252
BMI*	24.46 (±2.77)	25.98(±2.84)	*p* = 0.349
Gender female**	42.9% (n=3)	42.9% (n=3)	*p*=1.000
Narcotics**	0% (n=0)	14.3% (n=1)	*p*=1.000
Alcohol**	42.9% (n=3)	28.6% (n=2)	*p*=1.00
Tobacco**	42.9% (n=3)	42.9% (n=3)	*p*=1.00
AST***	87 (63-107)	85 (41 - 127.5)	*p* = 0.805
ALT***	115 (98-150)	106 (93 - 183.5)	*p* = 0.902
CRP***	2 (2-3)	4 (2.1-4.5)	*p* = 0.165

Mean (±Standard deviation); **Percentage within the group (N); ***Median (25^{th}-75^{th} percentile); HCV-hepatitis C virus; PEG IFN - pegylated interferon; DAA- direct-acting antivirals

Parameters used for measuring liver damage, liver fibrosis stadium and necro-inflammatory activity levels were compared in between these groups as well. This data is presented in Table 10. – 13 for necro-inflammatory activity.

Table 10. Necro-inflammatory activity values in liver biopsy samples measured with METAVIR score in groups of HIV, HCV and HIV/HCV co-infected patients

Groups	**Liver activity**		
	Minimal	**Moderate**	**Severe**
HCV without therapy	81.3% (n=13)	18.8% (n=3)	0% (n=0)
HIV	66.7% (n=6)	33.3% (n=3)	0% (n=0)
HIV/HCV	46.7% (n=7)	40% (n=6)	13.3% (n=2)

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; Minimal-A0 and A1 METAVIR score; Moderate - A2 METAVIR score; Severe - A3 METAVIR score;

Table 11. Comparison of correlation of necro-inflammatory activity values in liver biopsy samples measured with METAVIR score in groups of HIV, HCV and HIV/HCV co-infected patients

Groups	**Liver activity**		***p* value**
	Minimal	**Moderate/Severe**	
HCV without therapy	81.3% (n=13)	18.8% (n=3)	1 vs 2 p=0.630
HIV	66.7% (n=6)	33.3% (n=3)	1 vs 3 p=0.066
HIV/HCV	46.7% (n=7)	53.3% (n=8)	2 vs 3 p=0.423

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; Minimal-A0 and A1 METAVIR score; Moderate-A2 METAVIR score; Severe-A3 METAVIR score; 1*, HCV+ without HCV therapy; 2*, HIV+; 3*, HIV+/ HCV+ without HCV therapy;

Table 12. Correlation of necro-inflammatory activity values in liver biopsy samples measured with METAVIR score in groups of HCV-infected treated and untreated patients

Groups	Liver activity		*p* value
	Minimal	**Moderate/Severe**	
HCV without therapy	81.3% (n=13)	18.8% (n=3)	$p = 0.236$
HCV with therapy	57.1% (n=8)	42.9% (n=6)	

HCV-hepatitis C virus; Minimal-A0 and A1 METAVIR score; Moderate-A2 METAVIR score; Severe-A3 METAVIR score;

Table 13. Correlation of necro-inflammatory activity values in liver biopsy samples measured with METAVIR score in groups of HCV infected treated

Groups		Liver activity		*p* value
		Minimal	**Moderate /Severe**	
HCV with therapy	PEG-IFN	57.1% (n=4)	42.9% (n=6)	$p = 1.000$
	DAA	57.1% (n=4)	42.9% (n=3)	

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals; Minimal-A0 and A1 METAVIR score; Moderate-A2 METAVIR score; Severe-A3 METAVIR score;

Additional parameters measured and compared between groups were the level of liver damage or liver fibrosis stage. For this measurement, the METAVIR score was used. Patients were compared between groups, HIV and HCV mono-infected with co-infected patients. In another research arm, a comparison was made between HCV mono-infected patients, both, treated or untreated. This data is presented in Table 14 – 17.

Table 14. Fibrosis stage values in liver biopsy samples measured with METAVIR score in groups of HIV, HCV and HIV/HCV co-infected patients

Groups	Liver fibrosis			
	No	Mild/Moderate	Significant/Severe	Cirrhosis
HCV without therapy	43.8 % (n=7)	6.3% (n=1)	25% (n=4)	25% (n=4)
HIV	33.3 % (n=3)	44.4% (n=4)	0% (n=0)	22.2% (n=2)
HIV/HCV	33.3 % (n=5)	20% (n=8)	10% (n=4)	32.5% (n=13)

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; No-F0 METAVIR score; Mild/Moderate-F1 METAVIR score; Significant/Severe-F2 and F3METAVIR score; Cirrhosis- F4 METAVIR score;

Table 15. Correlation of fibrosis stage values in liver biopsy samples measured with METAVIR score in groups of HIV, HCV and HIV/HCV co-infected patients

Groups	Liver fibrosis		*p* value
	No-Severe	**Cirrhosis**	
HCV without therapy	75% (n=12)	25% (n=4)	1 vs 2
HIV	77.8% (n=7)	22.2% (n=2)	*p* = 1.000
HIV/HCV	53.3% (n=8)	46.7% (n=7)	1 vs 3
			p = 0.273
			2 vs 3
			p = 0.389

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; No-Severe-F0-F3 METAVIR score; Cirrhosis- F4 METAVIR score; 1*, HCV+ without HCV therapy; 2*, HIV+; 3*, HIV+/ HCV+ without HCV therapy;

Table 16. Correlation of fibrosis stage values in liver biopsy samples measured with METAVIR score in groups of HCV infected treated and untreated patients

Groups	**Liver fibrosis**		***p* value**
	No-Severe	**Cirrhosis**	
HCV without therapy	75% (n=12)	25% (n=4)	$p = 0.657$
HCV with therapy	85.7% (n=12)	14.3% (n=2)	

HCV-hepatitis C virus; No-Severe-F0-F3 METAVIR score; Cirrhosis- F4 METAVIR score;

Table 17. Correlation of fibrosis stage values in liver biopsy samples measured with METAVIR score in groups of HCV infected treated patients

Groups		**Liver fibrosis**		***p-value***
		No-Severe	**Cirrhosis**	
HCV with therapy	PEG-IFN	85.7% (n=6)	14.3% (n=1)	$p = 1.000$
	DAA	85.7% (n=6)	14.3% (n=1)	

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals; No-Severe-F0-F3 METAVIR score; Cirrhosis- F4 METAVIR score;

In order to assess precise data on liver damage, aside METAVIR score, another methodology was utilized. In all tissue samples two additional parameters were assessed, the number of CD8 + and area percentage of fibrous tissue per field of view. Data on the correlation of the number of CD8+ lymphocytes per field of the view in between groups is presented in tables 18 – 20.

Table 18. Correlation of number of CD8+ lymphocytes per field of view *x200* in liver biopsy samples in groups of HIV, HCV and HIV/HCV co-infected patients

Groups	CD8 count		*p* value
	MEAN	SD	
HCV without therapy	97.11	89.37	$p = 0.616$
HIV	154.64	157.16	
HIV/HCV	127.79	105.64	

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; CD8 count-number of CD8+ lymphocytes per field of view x200

Table 19. Correlation of number of CD8+ lymphocytes per field of view *x200* in liver biopsy samples in groups of HCV infected treated and untreated patients

Groups	CD8 count		*p* value
	MEAN	SD	
HCV without therapy	97.11	89.37	$p = 0.840$
HCV with therapy	105.75	61.12	

HCV-hepatitis C virus; CD8 count-number of CD8+ lymphocytes per field of view x200

Table 20. Correlation of number of CD8+ lymphocytes per field of view *x200* in liver biopsy samples in groups of HCV infected treated patients

Groups		CD8 count		*p* value
		MEAN	SD	
HCV with therapy	**PEG-IFN**	133.5	101.12	$p = 0.494$
	DAA	91.88	45.26	

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals; CD8 count-number of CD8+ lymphocytes per field of view x200

In both research arms, in HIV and HCV mono-infected and co-infected patients as well as in HCV mono-infected, treated and untreated patients the, area of the percentage of fibrous tissue per field of view was determined. That data is presented in Tables 21 – 23.

Table 21. Correlation of area percentage of fibrous tissue per field of view *x200* in liver biopsy samples in groups of HIV, HCV and HIV/HCV co-infected patients

Groups	**Fibrosis area percentage**		***p* values**	**Posthoc**
	MEAN	**SD**		
HCV without therapy	28.01	11.46	p=0.060	1 vs 2 *p* = 0.034
HIV	17.76	8.17		1 vs 3 *p*=0.049
HIV/HCV	19.95	5.81		2 vs 3 *p*=0.622

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; Fibrosis area percentage- area percentage of fibrous tissue per field of view x200; 1*, HCV+ without HCV therapy; 2*, HIV+; 3*, HIV+/ HCV+ without HCV therapy;

Table 22. Correlation of area percentage of fibrous tissue per field of view x200 in liver biopsy samples in groups of HCV infected treated and untreated patients

Groups	**Fibrosis area percentage**		**Posthoc**
	MEAN	SD	
HCV without therapy	28.01	11.46	*p* = 0.243
HCV with therapy	22.45	6.36	

HCV-hepatitis C virus; Fibrosis area percentage- area percentage of fibrous tissue per field of view x200;

Table 23. Correlation of area percentage of fibrous tissue per field of view x200 in liver biopsy samples in groups of HCV infected treated patients

Groups		Fibrosis area percentage		Posthoc
		MEAN	SD	
HCV with therapy	PEG-IFN	18.65	3.65	$p = 0.088$
	DAA	26.26	6.51	

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals; Fibrosis area percentage- area percentage of fibrous tissue per field of view x200;

Table 24. Correlation of necro-inflammatory activity values in liver biopsy samples measured with METAVIR score and number of CD8+ lymphocytes per field of view *x200* in groups of HIV, HCV and HIV/HCV co-infected patients

Parameters	CD8+ count	*p value*
Liver activity	Rho=0.130; N=34	$p = 0.464$

Liver activity- necro-inflammatory measured with METAVIR score; CD8 count-number of CD8+ lymphocytes per field of view x200

Table 25. Correlation of fibrosis stage values in liver biopsy samples measured with METAVIR score and area percentage of fibrous tissue per field of view *x200* in groups of HIV, HCV and HIV/HCV co-infected patients

Parameters	Fibrosis area percentage	*p value*
Liver fibrosis	Rho=0.130; N=34	$p = 0.464$

Liver fibrosis- fibrosis stage measured with METAVIR score; Fibrosis area percentage- area percentage of fibrous tissue per field of view x200;

Table 26. Correlation of alcohol consumption and area percentage of fibrous tissue per field of view x200 in groups of HIV, HCV and HIV/HCV co-infected patients

Parameters	**Fibrosis area percentage**	***p* value**
Alcohol	Rho=0.370; N=34	$p = 0.031$

Fibrosis area percentage- area percentage of fibrous tissue per field of view x200;

Table 27. Correlation of use of tobacco and area percentage of fibrous tissue per field of view x200 in groups of HIV, HCV and HIV/HCV co-infected patients

Parameters	**Fibrosis area percentage**	***p* value**
Use of tobacco	Rho=0.342; N=34	$p = 0.047$

Fibrosis area percentage- area percentage of fibrous tissue per field of view x200;

Table 28. Correlation of levels of ALT and area percentage of fibrous tissue per field of view x200 in groups of HIV, HCV and HIV/HCV co-infected patients

Parameters	**Fibrosis area percentage**	***p* value**
ALT	Rho= - 0.342; N=34	$p = 0.037$

ALT-alanine transaminase; Fibrosis area percentage- area percentage of fibrous tissue per field of view x200;

***CYP2B6* expression in the study population**

Expression of *CYP2B6* in the groups of HIV, HCV, and HIV/HCV co-infected patients

In groups of patients with HIV, HCV and HIV/HCV co-infection, over 2-fold increases in *CYP2B6* expression were seen in 35.7% of untreated HCV patients, 48.3% of HIV patients, and 91.8% of these patients (Table 29, Figure 8.).

Table 29. Frequency of increased *CYP2B6* expression in the groups of HIV, HCV, and HIV/HCV co-infected patients

Groups	HCV without therapy	HIV	HIV/HCV
CYP2B6	35.7%	48.3%	81.8%

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus;

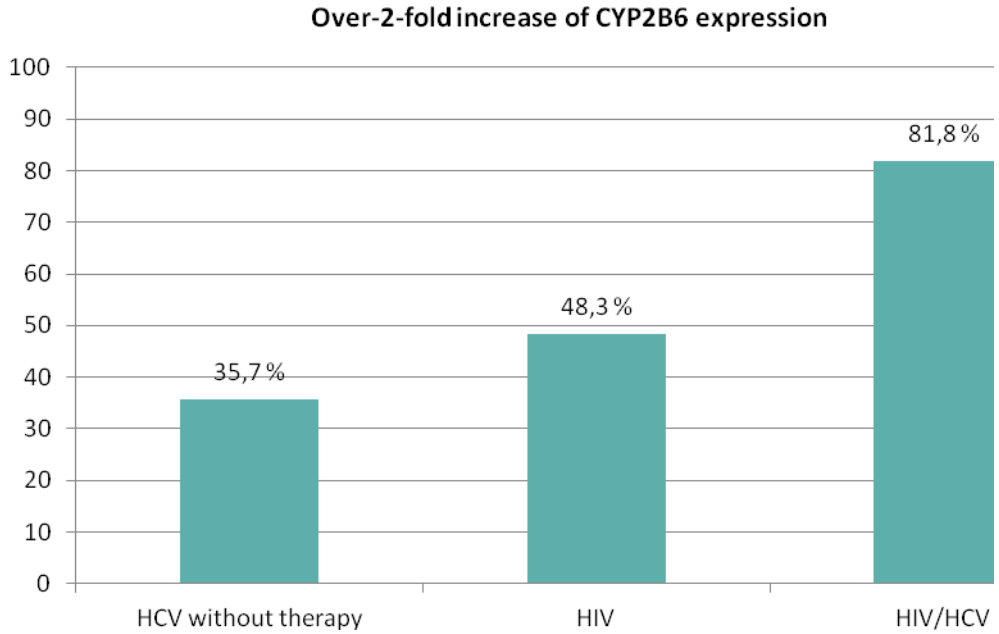

Figure 8. Frequency of increased *CYP2B6* expression in the groups of HIV, HCV, and HIV/HCV co-infected patients.

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus;

Initial variance analysis revealed a significant difference in *CYP2B6* gene expression levels across the three patient groups — those with HIV, HCV, and HIV/HCV co-infection ($p = 0.017$). A more thorough research found a significant ($p = 0.007$) difference in the expression of *CYP2B6* between groups of untreated HCV mono-infected patients and HIV/HCV co-infected patients, indicating higher expression in the co-infected patients. Similar to this, our findings revealed a difference in CYP2B6 expression between groups of HIV/HCV co-infected patients and HIV mono-infected patients that was significantly different ($p = 0.030$), indicating greater expression in a group of co-infected patients (Table 30.).

Table 30. *CYP2B6* gene expression in groups of HIV, HCV and HIV/HCV co-infected patients

Groups	***CYP2B6* expression (mean±SD)**	***p* value**	**Posthoc**
HCV without HCV therapy	1.711 ± 0.635	0.017	1* vs 3* *p* = 0.007
HIV+	1.813 ± 0.866		2* vs 3* *p* = 0.030
HIV/HCV without HCV therapy	3.673 ± 2.704		

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; 1*, HCV+ without HCV therapy; 2*, HIV+; 3*, HIV+/ HCV+ without HCV therapy;

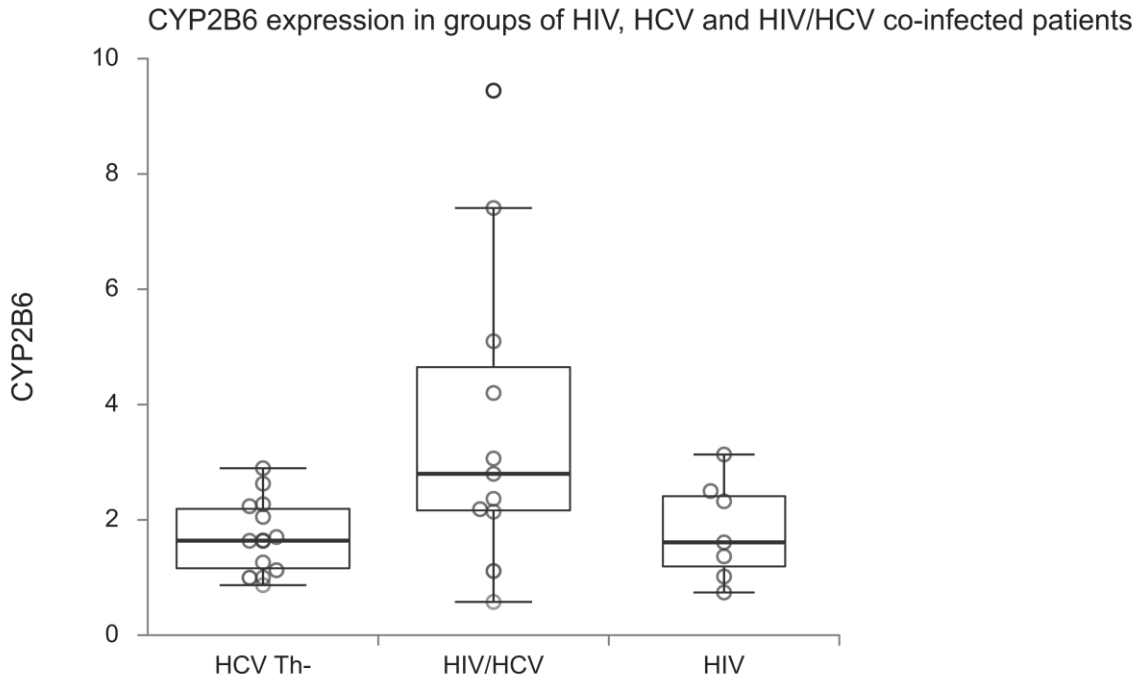

Figure 9. *CYP2B6* gene expression in groups of HIV, HCV and HIV/HCV co-infected patients.

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus;

In the group of HCV mono-infected patients, 35.7% of untreated HCV patients had *CYP2B6* expression that was over 2-fold higher, whereas 42% of patients in HCV mono-infected group who received treatment had *CYP2B6* expression that was over 2-fold higher. 16.7% of HCV mono-infected patients receiving DAA and 80% of patients receiving PEG-IFN both showed an over 2-fold rise for *CYP2B6* (Table 31, Figure 10, Figure 11.).

Table 31. Frequency of increased *CYP2B6* expression in the groups of HCV-infected treated and untreated patients

Groups	**HCV without therapy**	**HCV with therapy**		
		Total	**PEG-IFN**	**DAA**
CYP2B6	35.7%	42%	80.0%	16.7%

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals;

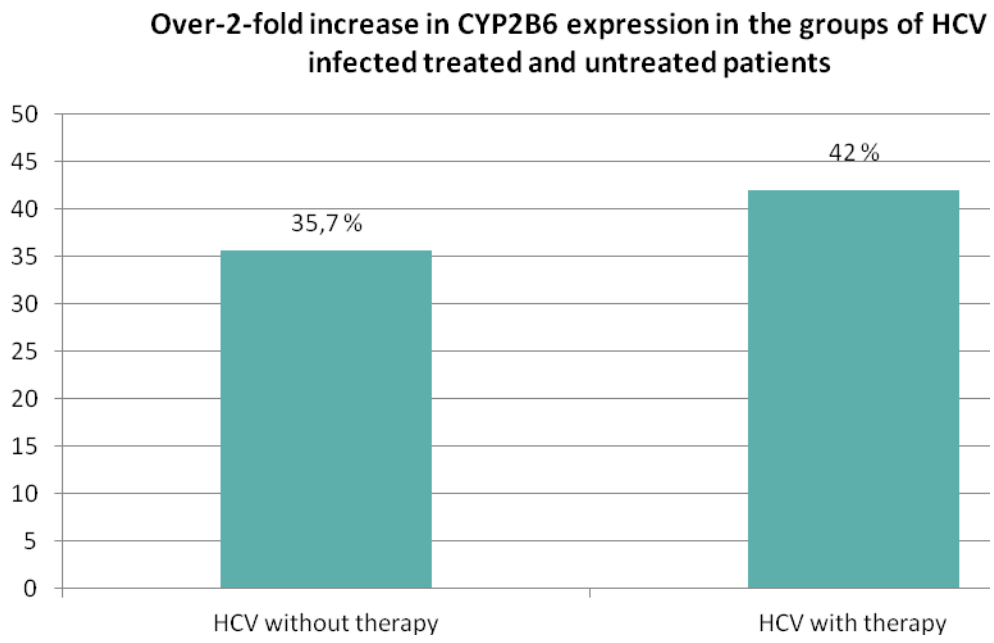

Figure 10. Frequency of increased *CYP2B6* expression in the groups of HCV-infected treated and untreated patients

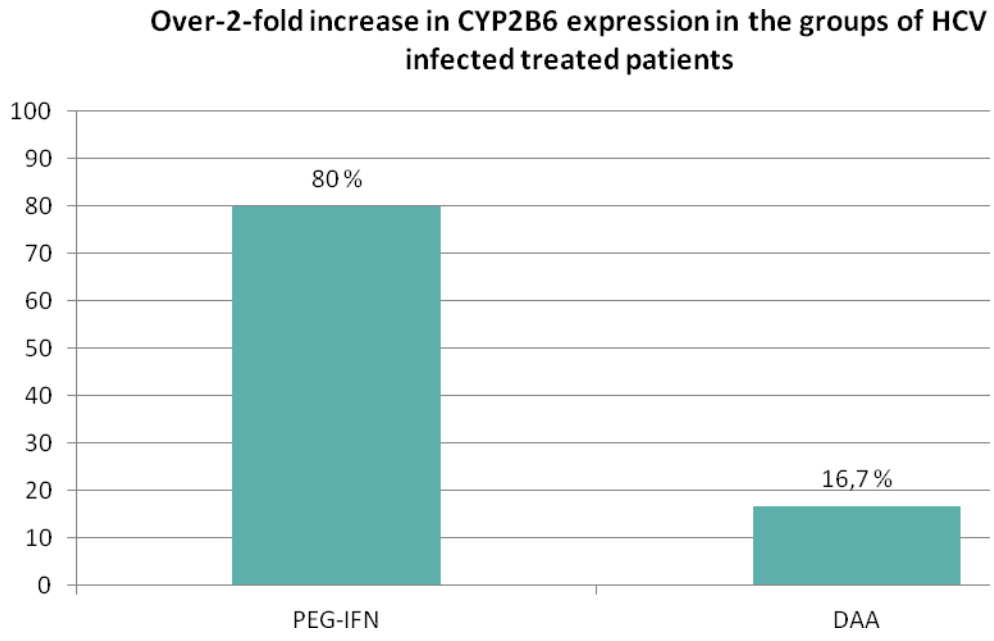

Figure 11. Frequency of increased *CYP2B6* expression in the groups of HCV infected treated patients HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals;

The results of this study showed no significant difference ($p = 0.252$) between groups of treated and untreated HCV mono-infected patients in the expression of genes encoding *CYP2B6*. Univariate analysis among the treated HCV mono-infected individuals revealed that the choice of treatment was independently linked with *CYP2B6* levels. Specifically, greater expression of *CYP2B6* genes was seen in a group of patients treated with PEG IFN compared to a group of HCV mono-infected patients who were not treated ($p = 0.021$) and compared to a group of HCV-infected patients who were treated with DAA ($p = 0.028$) (Table 32, Figure 12, Figure 13).

Table 32. *CYP2B6* gene expression in the groups of HCV infected treated and untreated patients

Groups		***CYP2B6* expression (mean±SD)**	***p* value**	**Posthoc**
HCV without HCV therapy		1.711 ± 0.635	0.252	1* vs 4* PEG IFN $p = 0.021$
HCV with HCV therapy	**Total**	2.117 ± 1.081		
	PEG IFN	2.727 ± 1.144	0.028	1* vs 4* DAA $p > 0.05$
	DAA	1.601 ± 0.781		

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals; 1*, HCV without HCV therapy; 2*, HIV; 3*, HIV/ HCV without HCV therapy; 4* . HCV with HCV therapy (Total);

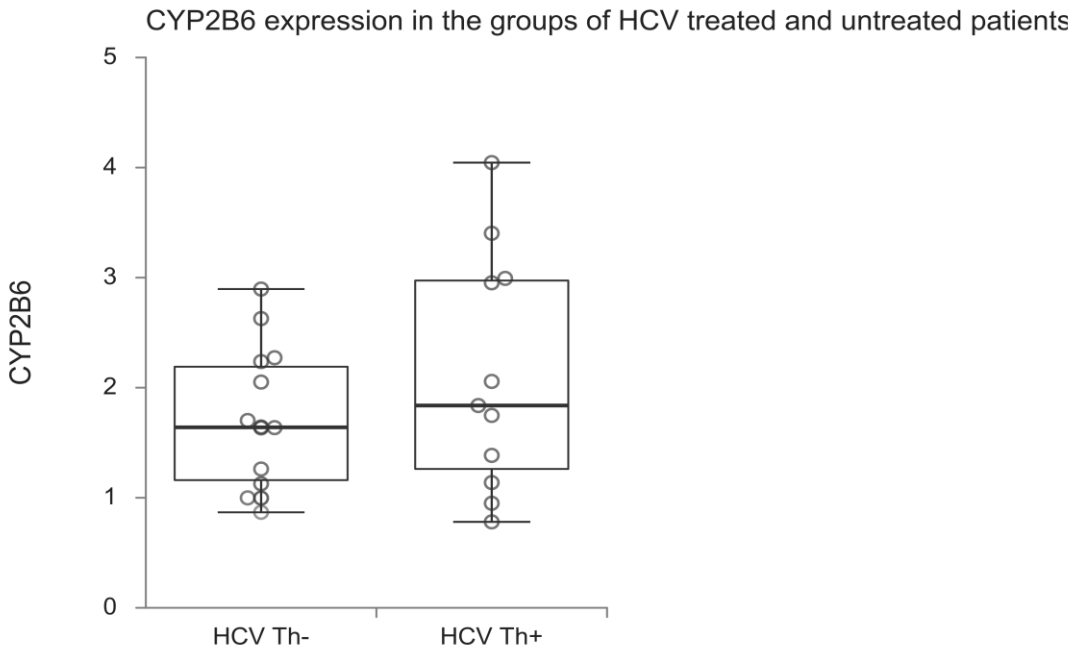

Figure 12. *CYP2B6* gene expression in the groups of HCV mono-infected treated and untreated patients

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals;

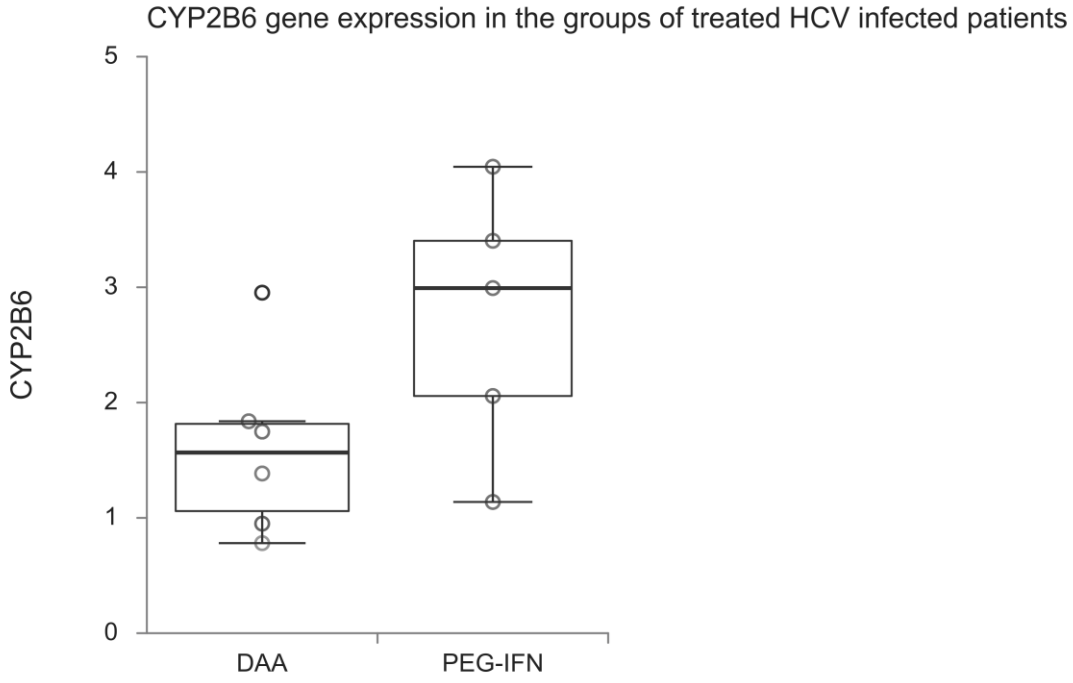

Figure 13. *CYP2B6* gene expression in the groups of HCV mono-infected treated and untreated patients
HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals;

Additionally, there was no significant difference ($p > 0.05$) in the liver fibrosis stage and grade as determined by the METAVIR scoring method.

There was also no statistically significant correlation between followed clinical and biochemical parameters with gene expression of *CYP2B6* in any of the followed groups of patients.

***CYP3A4* gene expression in the study population**

Expression of *CYP3A4* in the groups of HIV, HCV, and HIV/HCV co-infected patients

Less than a 2-fold increase of expression for *CYP3A4* was observed in 38.5% of HCV patients without HCV therapy, while over 2-fold expression increase was found in 71.4% of HIV infected and 81.8% of HIV/HCV co-infected patients (Table 33).

Table 33. Frequency of increased *CYP3A4* expression in the groups of HIV, HCV, and HIV/HCV co-infected patients

Groups	HCV without therapy	HIV	HIV/HCV
CYP3A4	61.5%	71.4%	81.8%

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus;

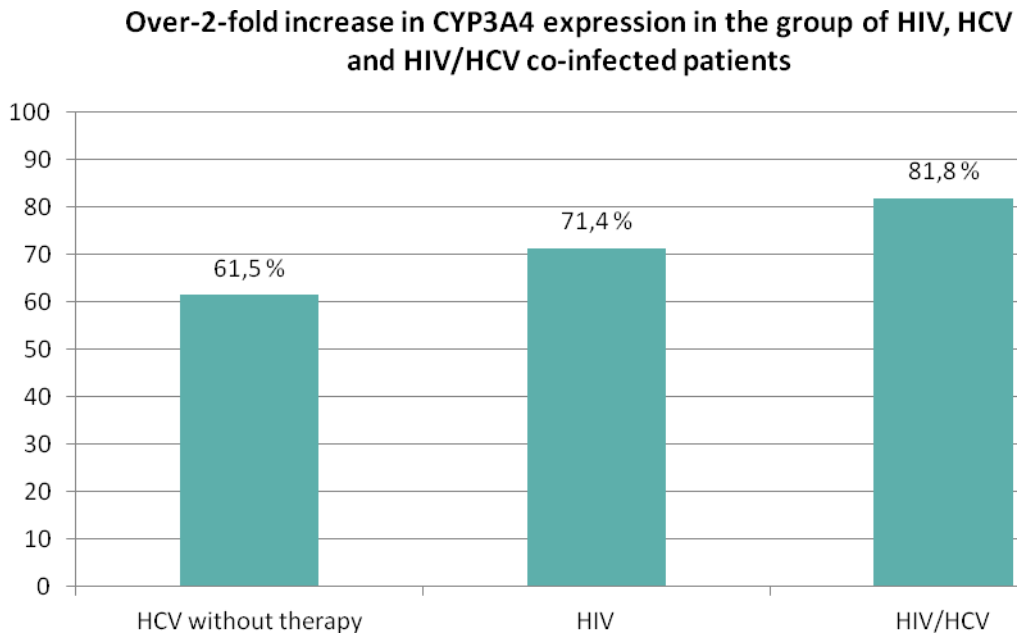

Figure 14. Frequency of increased *CYP3A4* expression in the groups of HIV, HCV, and HIV/HCV co-infected patients

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus;

Analysis has not shown a significant difference (p = 0.359) in *CYP3A4* expression between groups of HCV, HIV, and HCV/HCV+ co-infected patients (Table 34, Figure 14.).

Table 34. CYP3A4 gene expression in groups of HIV, HCV and HIV/HCV co-infected patients

Groups	*CYP3A4* expression (mean±SD)	*p* value	Posthoc
HCV without HCV therapy	2.377 ± 1.227	0.359	1* vs 3* *p* > 0.05
HIV	3.730 ± 2.696		1* vs 2* *p* > 0.05
HIV/HCV without HCV therapy	4.245 ± 4.799		2* vs 3* *p* > 0.05

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; 1*, HCV+ without HCV therapy; 2*, HIV+; 3*, HIV+/ HCV+ without HCV therapy;

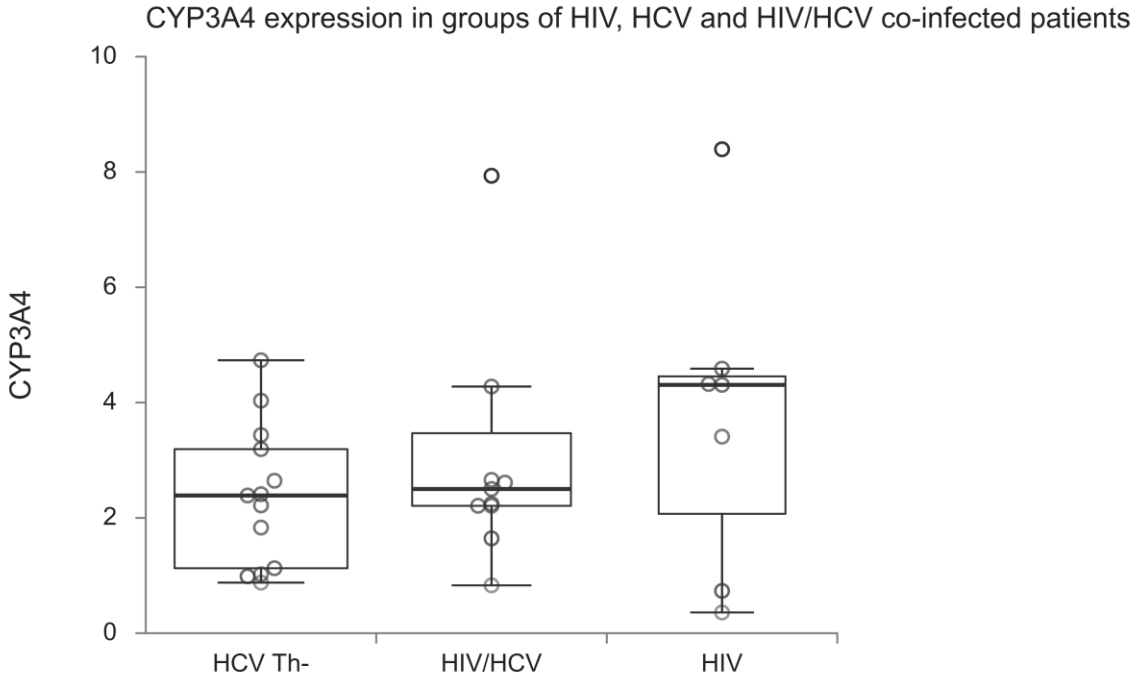

Figure 15. *CYP3A4* gene expression in groups of HIV, HCV and HIV/HCV co-infected patients

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; 1*, HCV+ without HCV therapy; 2*, HIV+; 3*, HIV+/ HCV+ without HCV therapy;

In the HCV group, an over 2-fold increase of expression for *CYP3A4* was observed in 61.5% of untreated HCV patients, and in 33.3% of treated HCV patients (Table 35, Figure 16.). In Figure 17. data is presented on the frequency of *CYP3A4* expression in the groups of HCV infected treated patients with DAA and PEG IFN.

Table 35. Frequency of increased *CYP3A4* expression in the groups of HCV-infected treated and untreated patients

Groups	HCV without therapy	HCV with therapy		
		Total	PEG-INF	DAA
CYP3A4	61.5%	33.3%	20.0%	42.9%

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals;

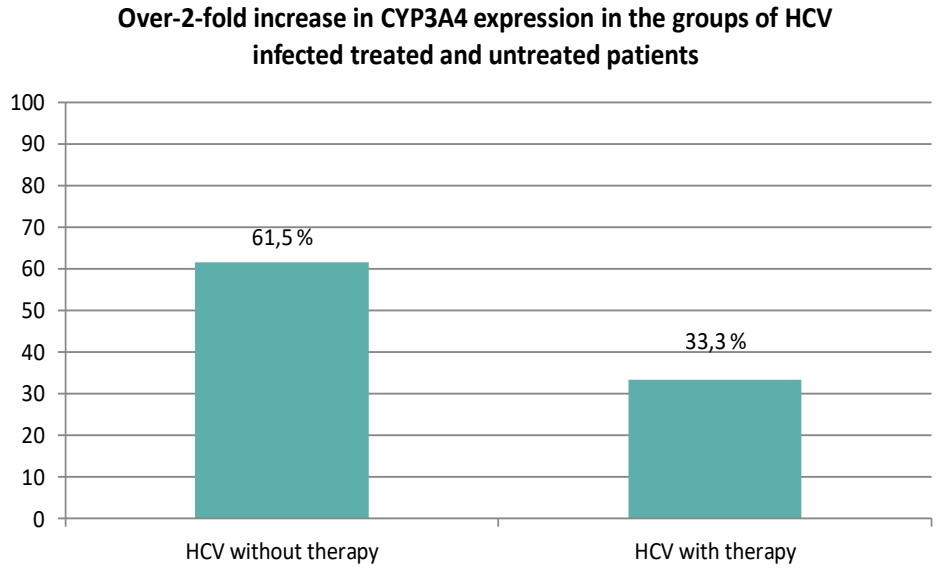

Figure 16. Frequency of increased *CYP3A4* expression in the groups of HCV-infected treated and untreated patients

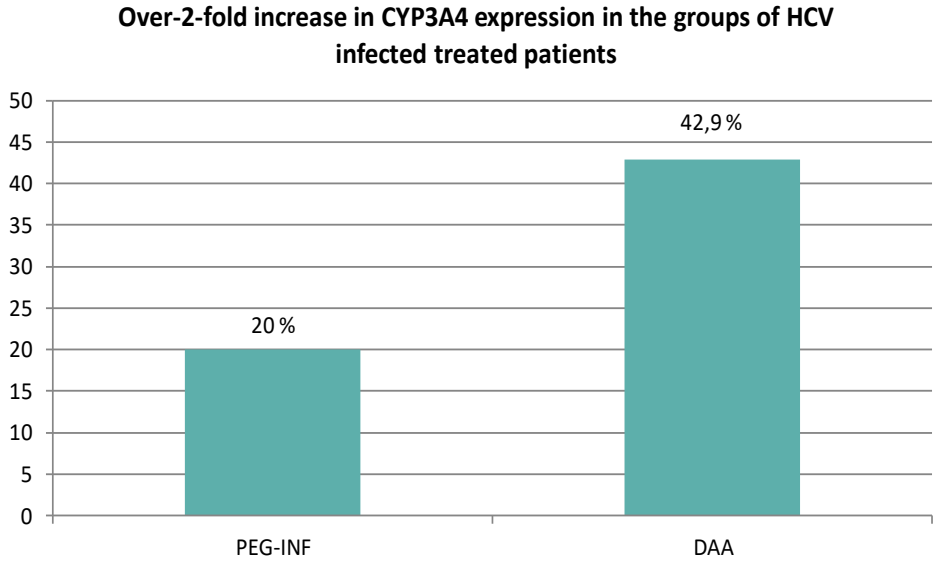

Figure 17. Frequency of increased *CYP3A4* expression in the groups of HCV-infected treated and untreated patients
HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals;

Results of this study have shown no significant difference (p = 0.149) in the expression of genes encoding *CYP3A4* in a comparison of groups of treated and untreated HCV patients (Table 36. Figure , Figure).

Table 36. *CYP3A4* gene expression in the groups of HCV infected treated and untreated patients

Groups		*CYP3A4* expression (mean±SD)	*p* value	Posthoc
HCV without HCV therapy		2.377 ± 1.227	0.149	1* vs 4* PEG IFN *p* > 0.05
HCV with HCV therapy	Total	1.637 ± 1.252		
	PEG IFN	1.174 ± 0.613	0.300	1* vs 4* DAA *p* > 0.05
	DAA	1.968 ± 1.521		

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals; 1*, HCV without HCV therapy; 2*, HIV; 3*, HIV/ HCV without HCV therapy; 4* . HCV with HCV therapy (Total);

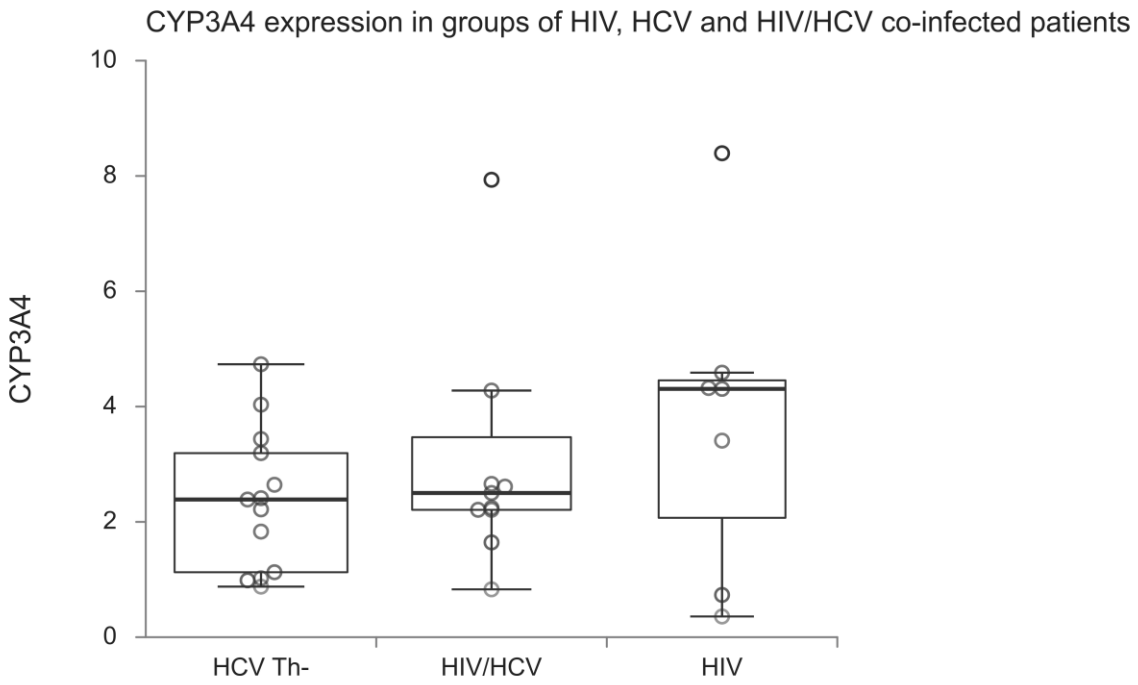

Figure 18. *CYP3A4* gene expression in the groups of HCV infected treated and untreated patients
HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals;

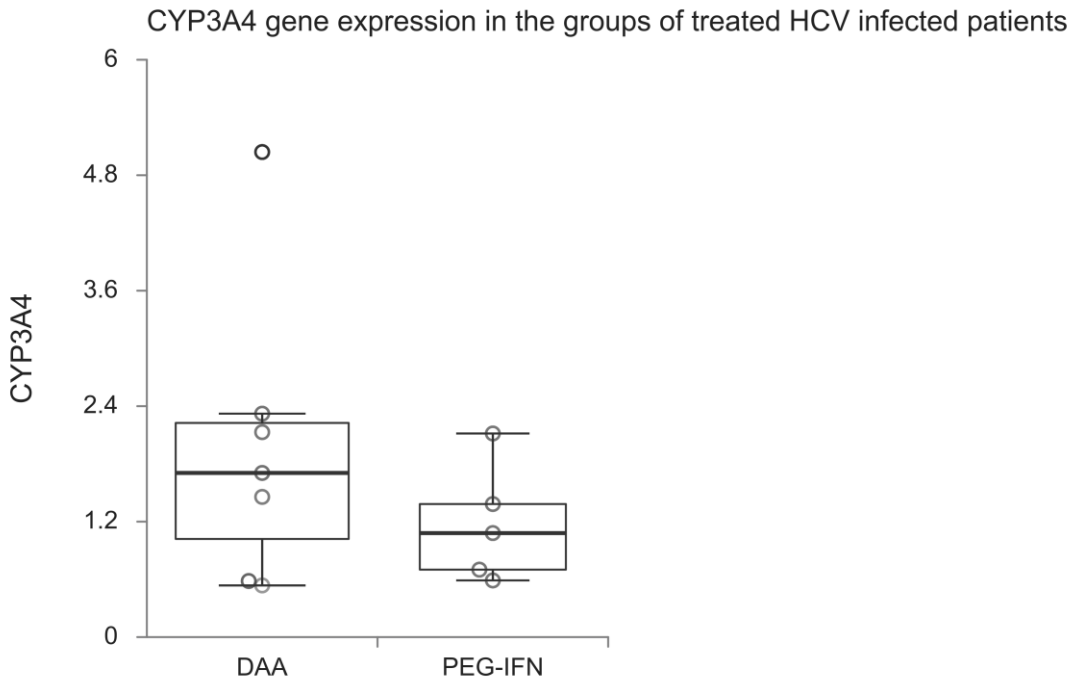

Figure 19. *CYP3A4* gene expression in the groups of HCV infected treated patients

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals;

ABCB1 gene expression in the study population

Expression of *ABCB1* in the groups of HIV, HCV, and HIV/HCV co-infected patients

Less than a 2-fold increase of expression for ABCB1 was observed in all cases of untreated HCV and HIV infected patients, while over 2-fold expression increase was found in 25% of HIV/HCV co-infected patients (Table 34).

Table 37. Frequency of increased *ABCB1* expression in the groups of HIV, HCV, and HIV/HCV co-infected patients

Groups	HCV without therapy	HIV	HIV/HCV
ABCB1	0%	0%	25%

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus;

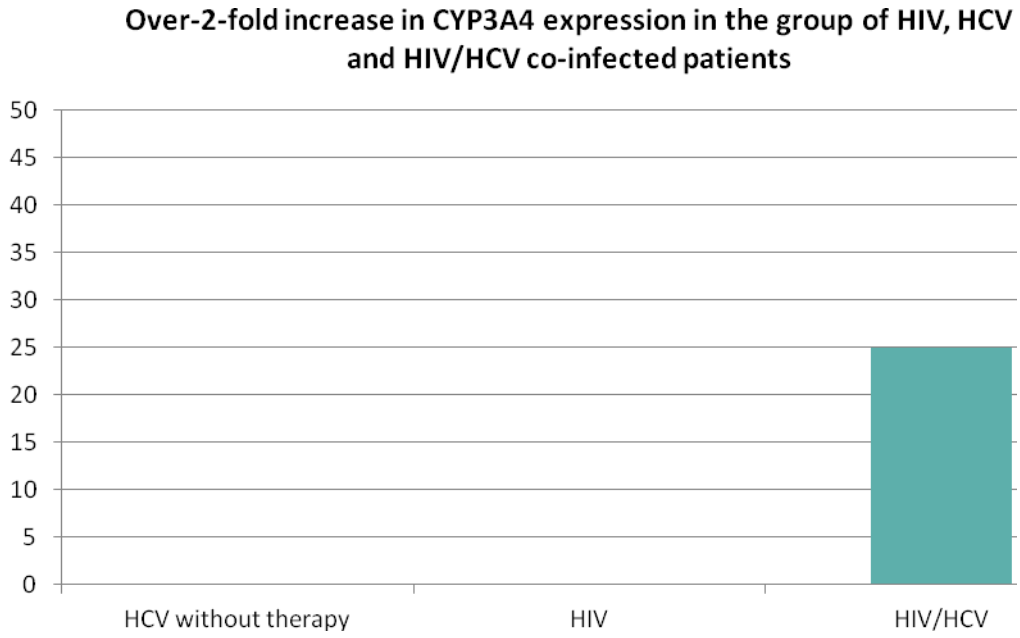

Figure 20. Frequency of increased *ABCB1* expression in the groups of HIV, HCV, and HIV/HCV co-infected patients

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus;

Analysis has not shown a significant difference ($p > 0.05$) in *ABCB1* expression between groups of HIV, HCV, and HIV/HCV co-infected patients. Although a trend was observed when comparing

differences in gene expression between groups of HCV patients and HIV/HCV co-infected patients, there was no significant correlation ($p > 0.05$) found (Table 35, Figure).

Table 38. *ABCB1* gene expression in groups of HIV, HCV and HIV/HCV co-infected patients

Groups	*ABCB1* expression (mean±SD)	*p-value*	Posthoc
HCV without HCV therapy	0.934 ± 0.379	0.116	1* vs 3* $p > 0.05$
HIV	0.903 ± 0.339		1* vs 2 * $p > 0.05$
HIV/HCV without HCV therapy	3.652 ± 5.325		2* vs 3* $p > 0.05$

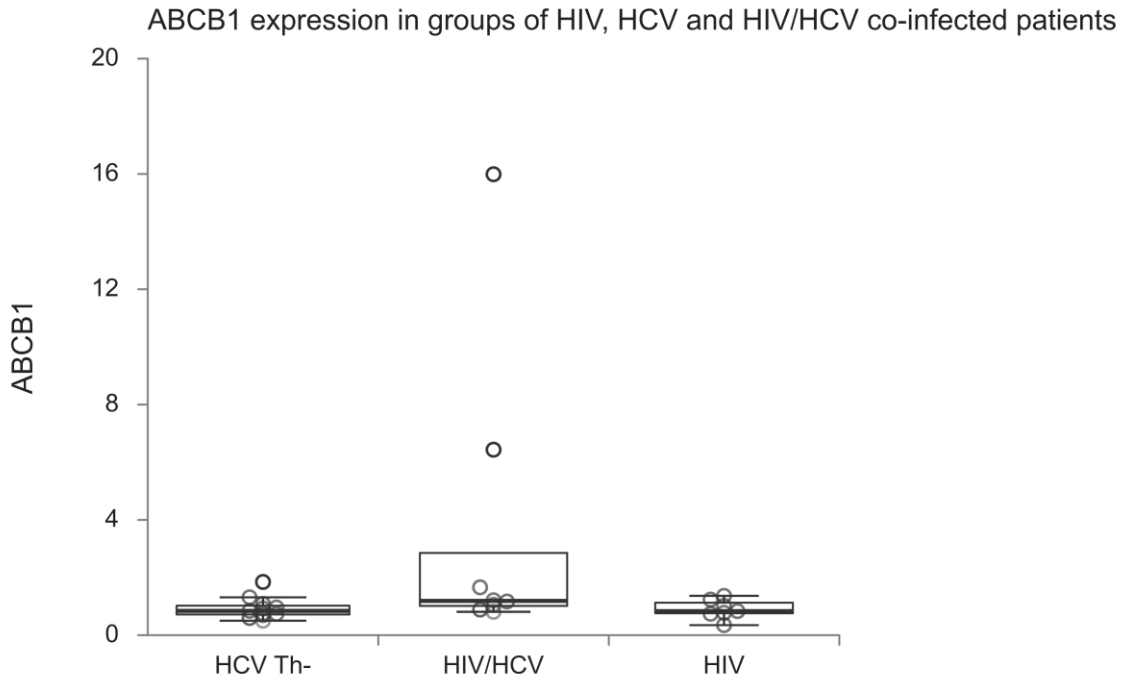

Figure 21. *ABCB1* gene expression in groups of HIV, HCV and HIV/HCV co-infected patients

HCV-hepatitis C virus; HIV-human immunodeficiency virus; HIV/HCV-human immunodeficiency virus patients co-infected with hepatitis C virus; 1*, HCV+ without HCV therapy; 2*, HIV+; 3*, HIV+/ HCV+ without HCV therapy;

In the group of HCV, none showed an over 2-fold increase of expression for *ABCB1*, both in groups of treated and untreated patients HCV patients (Table 36).

Table 39. *ABCB1* gene expression in the groups of HCV infected treated and untreated patients

Groups	HCV without therapy	HCV with therapy		
		Total	PEG-INF	DAA
ABCB1	0%	0%	0%	0%

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals;

Results of this study have shown no significant difference (p = 0.466) in the expression of genes encoding *ABCB1* in a comparison of groups of treated and untreated HCV patients (Table 40).

Table 40. *ABCB1* gene expression in the groups of HCV-infected treated and untreated patients

;

Groups		*ABCB1* expression (mean±SD)	*p* value	Posthoc
HCV without HCV therapy		0.934 ± 0.379	0.466	1* vs 4* PEG IFN
HCV with HCV therapy	Total	1.060 ± 0.398		*p* > 0.05
	PEG IFN	0.986 ± 0.588	0.657	1* vs 4* DAA
	DAA	1.110 ± 0.267		*p* > 0.05

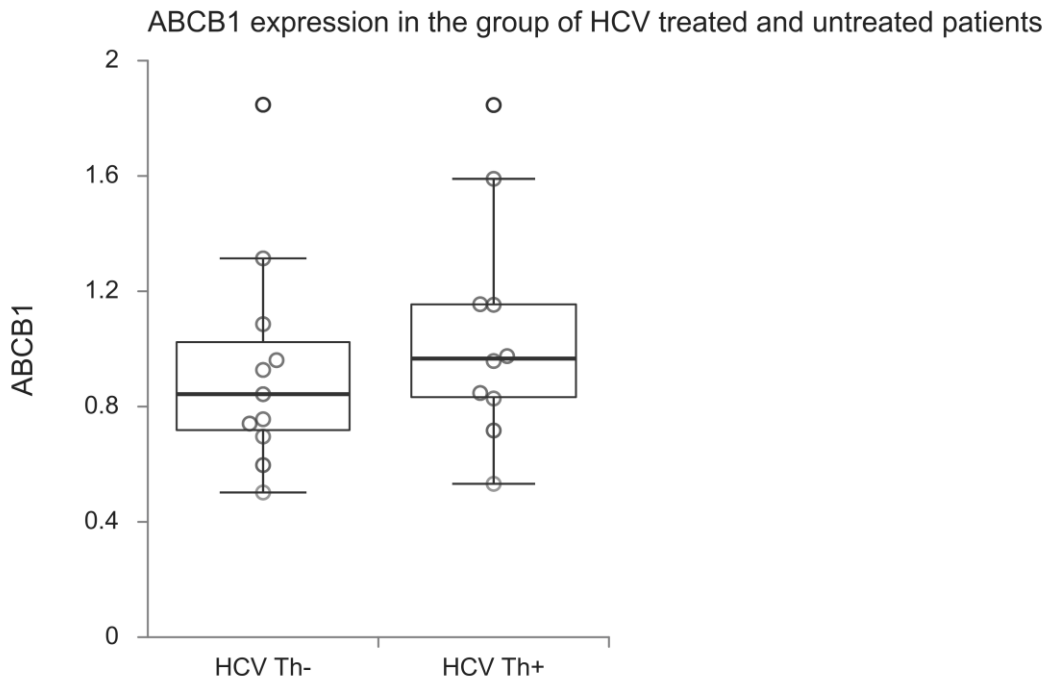

Figure 22. *ABCB1* gene expression in the groups of HCV-infected treated and untreated patients

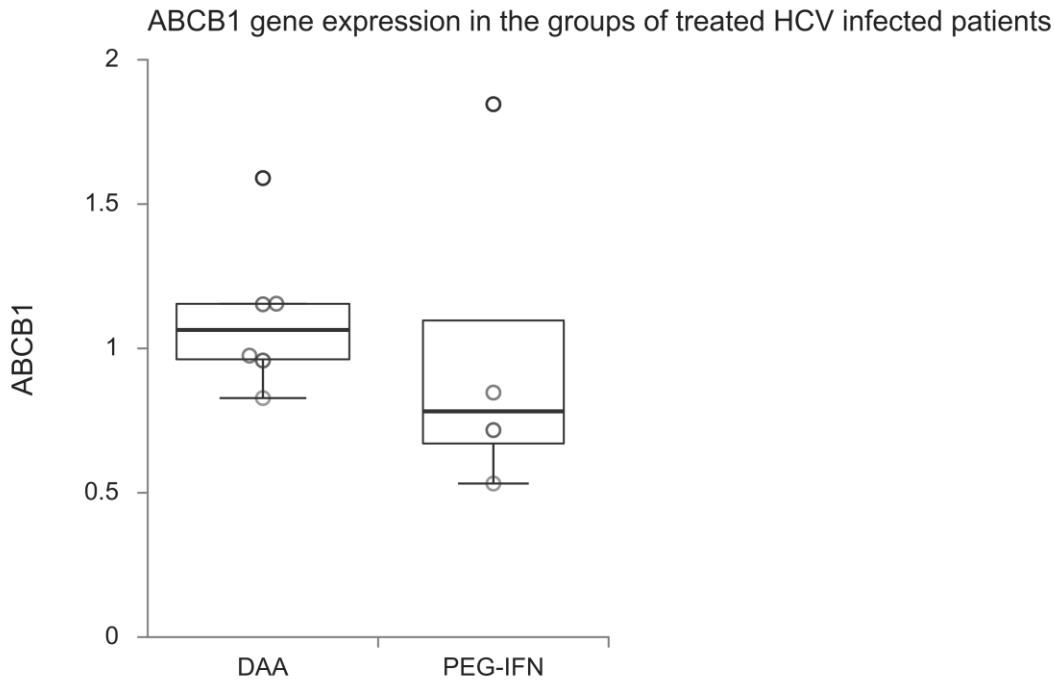

Figure 22. *ABCB1* gene expression in the groups of HCV-infected treated patients

HCV-hepatitis C virus; PEG-IFN-pegylated interferon; DAA-direct-acting antivirals;

Table 41. Correlation of *ABCB1* gene expression and number of CD8+lymphocytes per field of view x200 in groups of HIV, HCV and HIV/HCV co-infected patients

Parameters	**CD8 count**	***p value***
ABCB1	Rho=0.442; N=23	0.035

CD8 count-number of CD8+ lymphocytes per field of view x200

5. DISCUSSION

Metabolic enzymes and transporters play crucial roles in the processing and disposition of various numbers of medications. Metabolic enzymes are responsible for catalyzing different sets of chemical reactions that transform medications, facilitating their elimination or modifying their activity. These enzymes catalyze reactions such as oxidation, reduction, and hydrolysis, which can either activate or inactivate drugs, making them more soluble and easier to eliminate from the body. One of the most important group of metabolic enzymes is the CYP P450 enzymes and it is well-known that they are involved in the metabolism of a wide range of drugs. CYP enzymes are predominantly expressed in the liver but may be found in other tissues as well, such as intestine, brain, placenta, peripheral blood cells etc.

Transporters, on the other hand, are proteins that facilitate the movement of substances across cell membranes. They are present in various tissues, including the liver, intestine, kidney, and brain. Transporters are classified into different families such as the ABC and SLC transporter, both of them involved in drug transport. The ABC transporter family includes *ABCB1*, also known as P-gp, which is located in the membranes of cells lining various organs and acts as an efflux pump, actively removing drugs and toxins from cells and tissues. ABCB1/P-gp plays a significant role in drug absorption, distribution, and elimination and its activity can affect the bioavailability and efficacy of drugs.

Both metabolic enzymes and transporters can exhibit interindividual variability due to genetic factors, environmental influences, and disease states. This variability can lead to differences in drug response and susceptibility to adverse effects. Therefore, understanding the expression and function of metabolic enzymes and transporters, such as *CYP2B6*, *CYP3A4*, and *ABCB1*, is crucial in the field of pharmacology and personalized medicine to optimize drug therapy and minimize the risk of drug interactions or toxicity. In this analysis, we have conducted research of gene expression of the genes encoding *CYP2B6*, *CYP3A4* and *ABCB1* transporter on the samples of human liver in the cohort of HIV mono-infected, HCV mono-infected and HIV/HCV co-infected patients. Each group was also analyzed for correlation of gene expression with liver inflammation and liver fibrosis as well as factors such as applied therapy or absence of the same, smoking and alcohol consumption as well as biochemical characteristics such as ALT, AST and CRP levels, platelets level and BMI.

CYP2B6 is predominantly expressed in the liver, and its activity can vary significantly among individuals. Several factors contribute to the interindividual variability in CYP2B6 expression and function, including age, gender, race/ethnicity, and lifestyle factors such as cigarette smoking and alcohol consumption (Zanger UM et al., 2013.). This enzyme accounts for a relatively small proportion, ranging from 2% to 10%, of the total cytochrome P450 content in the liver. Despite its relatively low abundance compared to other CYP enzymes, it plays a crucial role in the metabolism of various drugs, including antiretroviral medications (Wang PF. et al., 2015.).

Efavirenz is one such antiretroviral drug metabolized by *CYP2B6*. Efavirenz is one of the NNRTI drugs used for decades now in the treatment of HIV infection. CYP2B6 is the major enzyme responsible for the metabolism of efavirenz, converting it into its primary metabolite, 8-hydroxy efavirenz (Apostolova N. et al. 2015.). The interindividual variability in *CYP2B6* activity, determined in part by genetic factors, can influence the metabolism of efavirenz. Genetic polymorphisms in the *CYP2B6* gene can result in different enzyme activity levels. For instance, the *CYP2B6*6* variant is associated with reduced *CYP2B6* activity, leading to decreased efavirenz metabolism. As a result, individuals carrying this variant may have higher plasma concentrations of efavirenz and its metabolites, which can increase the risk of side effects or toxicity (Apostolova N. et al. 2015.).

Understanding the role of *CYP2B6* in the metabolism of efavirenz and other antiretroviral drugs is crucial for optimizing drug therapy in individuals with HIV infection. By considering factors such as genetic variations in *CYP2B6* and potential drug-drug interactions, healthcare professionals can tailor the dosage and treatment regimens to ensure optimal drug efficacy, minimize the risk of adverse effects, and improve patient outcomes.

Limited studies have investigated the gene expression of metabolic enzymes and transporters in patients with HIV, HCV, and HCV/HIV co-infection, particularly in human liver tissue. Previous studies have indicated that gene expression levels, which serve as indicators of enzyme activity, demonstrate relatively lower variability when compared to factors such as protein abundance, enzyme catalytic activity, or the presence of genetic variations. However, it is worth noting that only a limited number of studies have explored this area, with a few reports available in the existing literature (Kap M. et al., 2015; Klomp F. et al., 2020; Lenoir C. et al., 2021.).

Initially, using variance test, we analyzed gene expression of all three genes of interest, *CYP2B6, CYP3A4* and *ABCB1* in between groups of HIV and HCV mono-infected patients as well in comparison with HIV/HCV co-infected patients. This initial analysis showed a statistically significant correlation between increased gene expression of *CYP2B6* and the group of co-infected patients ($p = 0.017$). A further detailed analysis comparing the expression of *CYP2B6* in between groups of HIV, HCV mono-infected and HIV/HCV co-infected showed a significant difference ($p = 0.007$) for comparison made between co-infected patients and HCV mono-infected but also when comparing co-infected patients with HIV-mono-infected ($p = 0.030$). However, there was no rise in *CYP2B6* gene expression in individuals with mono-infections of either HIV or HCV.

This finding of increased *CYP2B6* expression in co-infected individuals, which seemed to be unrelated to other probable causes, was particularly intriguing (Obradovic et al., 2023). These results are consistent with those of Pereira et al., who found that patients with HIV/HCV co-infections had lower plasma concentrations of efavirenz than HIV mono-infected individuals, indicating decreased *CYP2B6* activity (Pereira SA. et al., 2008.). We have also analyzed *CYP2B6* gene expression in correlation with other known factors that may influence this enzyme gene expression but in our results neither the usage nor selection of cART had any impact on this increased gene expression. The same results were shown for alcohol and tobacco consumption, liver enzymes and CRP values

As mentioned previously, a number of different methods may be used to evaluate the activity of metabolic enzymes and transporters. As roughly 90% of efavirenz is metabolized by CYP2B6-mediated 8-hydroxylation, with a modest contribution from other CYPs (e.g., CYP2A6, CYP3A, etc.), efavirenz plasma concentration has frequently been employed as a measure to evaluate CYP2B6 metabolic capacity (Apostolova N. et al. 2015.). Efavirenz concentrations in peripheral blood have frequently been used in studies as a proxy for testing CYP2B6 metabolic activity. Elevated efavirenz plasma levels were seen in the majority of these researches, which suggests that CYP2B6's metabolic activity was for some reason, decreased (Apostolova N. et al. 2015). However, recently a study has been done in Kenya measuring efavirenz plasma concentration in HIV population and correlating it to different genotypes (Klomp F. et al., 2020.). Results of this study have shown that a subpopulation of HIV-infected patients may have lower efavirenz concentrations due to certain CYP2B6 polymorphisms, indicating decreased activity of *CYP2B6*. It is significant to highlight that whereas polymorphisms can correlate with gene expression, other findings imply that this may not always be the case for *CYP2B6* (Hesse LM. Et al.,2008.).

When considering efavirenz as a model for determining *CYP2B6* activity, it is crucial to acknowledge that efavirenz can exhibit both states, acute inhibition and chronic induction of this enzyme, and both processes are determined in a genotype-dependent manner. This was demonstrated in a study involving healthy volunteers that investigated the interaction between efavirenz and bupropion

(Ngayo MO. et al., 2022). Enzyme induction can lead to increased expression of genes responsible for encoding the enzyme in question.

It is crucial to recognize that several research looking at *CYP2B6* activity have used correlate or substitute endpoints, such as efavirenz plasma concentration, or have used samples from peripheral blood cells or cell cultures (Pereira SA. et a., 2005; Calza L., 2005; Gufford BT. et al., 2022.). Some of these approaches utilize models or techniques that are not specific to the liver or indirectly measure *CYP2B6* activity.

Among the few investigations on human liver tissue samples one of the recent study was done on a group of individuals with HCV mono-infection by Drozdzik et al. Results of this study showed significant downregulation of a couple of metabolic enzymes such as *CYP1A1, CYP2C8, CYP2C9*, and *CYP2D6* in the liver tissue, however, *CYP2B6* levels were curiously unaffected. This result implies that, given the particular circumstances examined in HCV mono-infected individuals, CYP2B6 expression is not necessarily down-regulated and may be quite stable.

Although higher *CYP2B6* expression was unexpectedly found in our HIV/HCV co-infected patients, there are a number of other potential factors that can offer an explanation for this phenomenon. Our study stands out as one of the very few researches performed on human liver samples from this particular cohort with chronic inflammation and chronic usage of therapy (Obradovic et al., 2023.). To address the issue of other potential factors that may influence gene expression of *CYP2B6* we have performed univariate analysis as we wanted to investigate the possible effects of several parameters, including gender, smoking, alcohol use, liver fibrosis, and inflammation on the gene expression of *CYP2B6*. However, we found no statistically significant correlation between *CYP2B6* expression and these well-known variables as shown in the result section. This may be a unique feature of our findings since the result of increased gene expression of *CYP2B6* in HIV/HCV co-infected patients, appear to be unaffected by other contributing variables while chronic inflammation associated with HIV/HCV co-infection could potentially contribute to this unique pattern of *CYP2B6* expression. Further research is needed to elucidate the underlying mechanisms that contribute to the observed increase in *CYP2B6* expression and to explore potential interactions between *CYP2B6* and other factors in this specific population. Understanding these intricate relationships can enhance our knowledge of drug metabolism and guide personalized treatment approaches for patients with HIV, HCV, and co-infections.

As with comparison with previous groups of patients, we performed analysis looking for a correlation between *CYP2B6* gene expression, increased or decreased, between two groups of patients, HCV mono-infected with or without therapy. Treated patients were taking DAA drugs (sofosbuvir and veltapasvir in combination or as mono-therapy for sofosbuvir) or PEG IFN. There was initially no statistically significant difference between untreated and treated HCV patients after evaluating the variations in *CYP2B6* gene expression in these two groups. However, a greater expression of *CYP2B6* was seen in individuals treated with PEG IFN as opposed to those treated with sofosbuvir and veltapasvir. Additionally, a rise in *CYP2B6* gene expression was seen when comparing the PEG IFN treated group with a group of untreated HCV patients.

There are very few and contradictory available studies on the impact of PEG IFN on *CYP2B6* expression, although, some of the *in vitro* studies have revealed that certain people may have reduced expression of *CYP2B6* and higher levels of *CYP3A4* (Brennan BJ. et al., 2014.). From previous studies, it is well known that *CYP2B6* expression varies greatly between individuals in response to PEG IFN therapy, which may be a possible contributing factor in our results as well. This variability may also be influenced by additional variables such as CYP2B6 polymorphisms, age, gender, and liver injury.

However, it is necessary to mention that our study had a substantial limitation because of the

small sample size that included patients receiving PEG IFN. The current movement toward interferon-free therapy approaches in the treatment of HCV infection, however, is in alliance whit these data.

Our study utilized univariate analysis to evaluate the effect of several known variables on the expression of the *CYP2B6* gene in these two groups as well. These variables included smoking, alcohol intake, liver fibrosis, liver inflammation, gender, and use of other medications. The expression of *CYP2B6* was not correlated significantly with any of these variables, though.

However, to further comprehend the effect of PEG IFN therapy on *CYP2B6* expression including its interactions with other variables, more studies with bigger sample sizes and in-depth analyses are required. Such research will be very helpful in improving HCV treatment plans and personalized care for patients based on each patient's unique genetic and clinical traits.

Understanding the factors that influence CYP2B6 expression is crucial for optimizing treatment outcomes, as *CYP2B6* plays a significant role in the metabolism of various drugs, including antiretrovirals. By gaining insights into the unique regulation of *CYP2B6* in this population, we can potentially refine treatment strategies and improve therapeutic efficacy.

CYP3A4 is a member of the cytochrome P450 enzyme superfamily, which plays a crucial role in the metabolism of a wide range of endogenous compounds and exogenous substances, including many different groups of drugs. It is primarily expressed in the liver, but it is also found in various extrahepatic tissues, such as the intestines, lungs, and kidneys. CYP3A4 is responsible for the metabolism of approximately 50% of clinically used drugs, making it one of the most important drug-metabolizing enzymes in humans (Chen Q. et al., 2015.). Also, it metabolizes a wide array of drugs belonging to different therapeutic classes including many antiretroviral and DAA drugs. Due to its involvement in the metabolism of numerous drugs, understanding the substrate specificity and metabolic pathways of CYP3A4 is crucial for predicting drug interactions and optimizing drug therapy. The CYP3A4 gene is highly polymorphic, with several genetic variants identified that can influence its expression and activity. Moreover, the expression of CYP3A4 can be influenced by various factors, including endogenous regulators, environmental factors, and drug-drug interactions (Chen Q. et al., 2015.).

The significant role of CYP3A4 in drug metabolism has important clinical implications. Pharmacogenetic studies have provided insights into the impact of CYP3A4 genetic polymorphisms on drug efficacy and toxicity, allowing for personalized medicine approaches.

It has been shown that people with HIV as well as HCV infections have down-regulated levels of CYP3A4, just like other metabolic enzymes. However, it is essential to remember that CYP3A4 expression regulation might differ between people, and the underlying processes causing these decreased levels are not entirely known (Vo T. et al., 2016.). In a study by Jones et al. ten HIV - positive males and seven HIV - positive women who were taking drugs that were known to influence the activity of CYP enzymes were included in study. Over the course of two months, the subjects received phenotyping evaluations four times. Caffeine, dextromethorphan, and midazolam were used as the probe substrates for the phenotyping. Urinary metabolite ratios of caffeine and dextromethorphan were tested to gauge the activity of certain enzymes. These ratios were used to measure the activity of the enzymes CYP1A2, NAT2, XO (xanthine oxidase), and CYP2D6. Midazolam clearance in plasma was also evaluated to gauge CYP3A activity. This study design allowed for repeated measurements over a two-month period, which helps capture any potential variability in enzyme activity within individuals over time. By employing these phenotyping methods, the researchers aimed to gain insights into the impact of HIV infection and medication use on CYP enzyme activity, which can have implications for drug metabolism and treatment outcomes in this population. The researchers were able to show significantly lower metabolic activity of CYP3A4. In a similar study conducted by Jetter et al.,

CYP3A4 activity was analyzed in a group of 30 HIV-infected patients in comparison to a control group of healthy volunteers. The researchers used *in vivo* phenotyping tests using drugs such as midazolam, dextromethorphan, and digoxin to assess CYP3A4 activity. The study findings indicated a significant reduction in overall CYP3A4 activity in the HIV-infected patient group when compared to the healthy volunteers. Specifically, the researchers reported an approximate 50% decrease in CYP3A4 activity in the HIV-infected patients.

Antiretroviral medications that are largely metabolized by CYP3A4 may be affected by any degree of inflammation in terms of their pharmacokinetics. The expression and activity of CYP3A4 enzymes can be affected by inflammation processes within the organism, potentially altering medication metabolism. This may happet due to the generation of pro-inflammatory cytokines and other mediators. Also, since this enzyme is involved in the metabolizing process of many antiretrovirals, changes in enzyme activity brought on by inflammation may have an influence on the levels of the medicine in the body. This in turn may have an impact on the medication's effectiveness and chance of drug interactions.

However, the availability of data on CYP3A4 modulation in HIV-infected individuals using human liver samples is limited in the existing literature. Despite the importance of understanding the effects of HIV infection on CYP3A4 activity for ensuring safety and efficacy of antiretroviral therapy, the current body of research presents conflicting and inconsistent results. The conflicting findings may stem from various factors, including differences in study design, patient populations, sample sizes, and methodologies used to assess CYP3A4 modulation. Additionally, the complexity of HIV infection itself, which involves dynamic interactions between the virus, immune response, and inflammatory processes, contributes to the challenges in understanding its specific impact on CYP3A4.

In our research, we found an intriguing finding regarding the expression of CYP3A4 in HIV-infected individuals, whether patients had mono- or co-infections with HCV. We discovered a positive connection, which suggests that these individuals had higher CYP3A4 expression. It is important to note that all of the HIV-positive individuals in our research were on antiretroviral treatment (ART), which may have influenced this result.

The majority of our patients were using a combination containing either efavirenz, lamivudine, or dolutegravir as part of their ART regimen. It is known that these drugs are predominantly metabolized by CYP3A4, which suggests a potential link between their use and the observed increase in CYP3A4 expression. It is important to mention that dolutegravir is only partially metabolized by CYP3A4, indicating that other factors might also contribute to the observed correlation.

Some studies have reported decreased CYP3A4 activity in HIV-infected individuals, suggesting potential alterations in drug metabolism and potential drug-drug interactions. On the other hand, other studies have failed to find significant changes in CYP3A4 activity or have even observed increased enzyme activity in certain patient populations.

The discrepancies in findings could be attributed to the heterogeneity of the HIV-infected population, including variations in disease progression, concomitant infections, antiretroviral therapy regimens, and individual genetic differences. Moreover, the influence of other factors such as inflammation, co-morbidities, drug-drug interactions, and lifestyle choices (e.g., smoking, alcohol consumption) on CYP3A4 modulation further complicates the interpretation of study results.

Atazanavir, a medication used in antiretroviral treatment, serves as one example. Atazanavir levels have been shown to be lower in HIV-positive individuals than in healthy volunteers (Venuto C. S., 2018.).This shows that atazanavir's metabolism and clearance may be impacted by variables related to HIV infection, such as the inflammation and modified enzyme activity. Further research, done on caffeine metabolism revealed no discernible variations in caffeine metabolism between HIV-positive individuals and healthy subjects. However, a reduction in caffeine metabolism was seen when HIV-

infected patients with AIDS were compared to healthy people. This shows that the degree of inflammation and the development of a disease may have an impact on the activity of enzymes engaged in metabolism, such as those involved in the metabolism of caffeine. (Jetter A. et al., 2010; Venuto C. S. et al., 2018).

It is still unclear whether the observed alterations in enzyme expression in the context of CYP3A4 regulation in HIV-infected patients are predominantly attributable to HIV infection itself or to the administration of antiretroviral treatment (ART). It might be difficult to separate the particular contributions of HIV infection and ART from those of CYP3A4 activity and expression. Immune activation and systemic inflammation brought on by HIV infection have been linked to changes in a number of physiological systems, including medication metabolism. HIV- associated inflammation may impact CYP3A4 expression and performance directly or indirectly. Additionally, liver disorders caused by HIV, such as cirrhosis or liver fibrosis, might affect CYP3A4 activity. On the other hand, cART medications such as ones used in our study population can affect CYP3A4 in different ways and can affect the expression and activity of this enzyme.

Regrettably, in our study, we did not observe a correlation between the specific choice of drug combination in antiretroviral therapy (ART) and CYP3A4 gene expression. This finding suggests that the selection of a particular drug combination may not directly influence CYP3A4 expression levels.

The absence of a control group made up of HIV - positive individuals who weren't receiving treatment was a significant research drawback. It should be noted that the inclusion of such a control group would have given important insights into the possible effects of HIV infection on the expression of the CYP3A4 gene. However, when effective treatment alternatives are available, ethical constraints frequently prevent the inclusion of untreated persons in research. Many studies examining the effects of ART on CYP3A4 and other metabolic enzymes have the common constraint of not having a control group of HIV-infected individuals who are not receiving treatment. Although it would be interesting to compare CYP3A4 expression between treated and untreated persons, it is difficult to carry out such research due to the ethical need to offer appropriate treatment to HIV-infected patients. Despite this limitation, our study contributes to the existing literature by examining the relationship between ART and CYP3A4 expression in HIV-infected patients. By analyzing different drug combinations, we aimed to identify any potential associations between specific ART regimens and CYP3A4 gene expression. While we did not find a significant correlation, our findings provide valuable information on the complexity of CYP3A4 modulation in the context of HIV infection and ART.

In our study, we observed a significant association between CYP3A4 gene expression and platelet count in all HIV - infected patients. Specifically, patients with higher expression of CYP3A4 tended to have elevated platelet counts. This finding suggests a potential link between platelet count and CYP3A4 activity, although the underlying mechanisms for this association remain unclear.

On the other hand, we did not find any significant correlations between changes in CYP3A4 expression and other biochemical or clinical parameters that were analyzed. These parameters encompassed various factors such as liver function tests, lipid profiles, and markers of inflammation. The lack of significant associations indicates that these specific factors may not exert a significant influence on CYP3A4 expression in our study population.

Interestingly, we did find a positive correlation between CYP3A4 expression and higher grades of liver inflammation. This finding suggests that the extent of liver inflammation may play a role in modulating CYP3A4 expression levels. However, we did not observe any significant correlation between CYP3A4 expression and the stage of liver fibrosis, indicating that fibrotic changes in the liver may not be directly linked to CYP3A4 expression in our study.

It is important to note that our study had certain limitations, including a relatively small sample size and the cross-sectional design. These limitations should be considered when interpreting our findings. Additionally, further research is needed to investigate the underlying mechanisms and potential clinical implications of the observed associations between CYP3A4 expression, platelet count, liver inflammation, and fibrosis.

Understanding the factors that influence CYP3A4 expression is crucial for optimizing the efficacy and safety of antiretroviral therapy, as well as other medications metabolized by this enzyme. Future studies with larger sample sizes and longitudinal designs may provide more comprehensive insights into the complex interplay between CYP3A4 expression, HIV infection, liver disease, and other relevant clinical factors. Such knowledge can potentially guide treatment strategies and improve patient outcomes in individuals with HIV and other conditions requiring CYP3A4 - metabolized medications.

ABCB1, also known as P-glycoprotein or MDR1, is a membrane - bound efflux transporter that plays a crucial role in the transport of various endogenous and exogenous substances across cell membranes. It is primarily expressed in tissues with barrier functions, such as the intestines, liver, kidneys, and blood-brain barrier. ABCB1 is involved in the efflux of a wide range of drugs including antiviral drugs as well. The expression and activity of ABCB1 can be influenced by various factors, including genetic variations, environmental factors, and disease states. Understanding the function and regulation of ABCB1 is of great importance in clinical practice and pharmaceutical research. It provides insights into drug absorption and distribution, drug resistance mechanisms, and the development of strategies to optimize drug therapy and overcome multidrug resistance.

In the present study, we found a notable association between the expression of the drug transporter gene ABCB1 and lamivudine use in HIV patients. In particular, we found a positive correlation, suggesting that lamivudine usage was associated with reduced expression levels of the ABCB1 transporter genes. This conclusion is consistent with the findings of earlier research that looked at related interactions (Lee LS. Et al., 2010.). Previous research has suggested that certain drugs, including lamivudine, may influence the expression and function of ABCB1. Specifically, lamivudine usage has been associated with a downregulation of ABCB1 expression, indicating a potential impact on the transport of drugs and other substances across cell membranes (Ceckova M. et al., 2016.).

The regulation of ABCB1 gene expression is a complex process influenced by multiple factors, including viral infections, inflammation, and the administration of antiretroviral therapy. However, the precise mechanisms and consistent patterns of regulation remain a subject of debate and investigation.
Numerous research have investigated the connection between ABCB1 gene expression and viral infections including HIV and HCV (Ahmed Juvale II. et al., 2022; Agrawal N. et al., 2020.). According to some research, these infections resulted in a down regulation of the ABCB1 gene's expression, which might have an effect on how drugs are transported and disposed of (Calza L., 2015; Chen Q et al., 2015). On the other hand, studies demonstrating up regulation or no discernible alteration in *ABCB1* gene expression in the setting of viral infections have also been described, producing inconsistent results (Ebert C. et al., 2016; Whyte-Allman SK et al., 2020).

Individualized differences in *ABCB1* gene expression have been noted in addition to viral infections. It is significant to highlight that a complex interaction of many variables, including viral factors, host factors, and treatment-related factors, is probably responsible for the control of ABCB1

gene expression. Different study designs, patient groups, and methods for measuring gene expression may be to blame for the variety of study results.

The current study has great importance since it adds to the scant body of knowledge on human liver tissue samples, concentrating primarily on those with HIV and HCV infections. This work uses human liver tissue analysis to directly examine the expression of genes in a relevant population, enabling a thorough knowledge of the variables affecting the expression of metabolic enzymes and transporters.

The use of human liver tissue samples permits the analysis of all potential confounding variables that may affect gene expression, unlike *in vitro* or animal models. The complicated interactions between many variables influencing the expression of metabolic enzymes and transporters in human liver tissue are better reflected by this method. Utilizing this particular population of chronic HIV, HCV and HIV/HCV co-infection and tissue type offers insightful information on the variables affecting the gene expression of metabolic enzymes and transporters. Future research with bigger sample sizes will assist validate and extend these preliminary findings, which will ultimately contribute to a more thorough knowledge of gene expression in the setting of HIV and HCV infections, despite the small sample size being a restriction.

Higher values od area percentage of fibrous tissue in liver biopsies positively correlated with clinical data about consumption of alcohol, as well as with use of tobacco. Also higher levels of serum ALT have shown positive correlation with higher area percentage of fibrous tissue in liver biopsies per field of view of magnification 200x. These correlations are valuable to us in two main reasons. They potentialy show intuitivly logical continuum of level of liver fibrosis as the histological parameter of liver damage with levels of ALT values as established clinical parameter of liver function. In this way showing potential pathway or direction for further investigation to deepen the correlation observed in the persue of limiting the need for invasive procdures for objective assesment of liver damage. These tendencies are in line with trends of developing, and more and more depending, on non invasive technicks for these assesments as it is the case with more frequent use of FibroScan. Also, we shoud consider the findings of our meassurements in positive light, because these results have shown better correlations to clinical parameters in our group, then values of METAVIR score, for semiquantitative evaluation, while consistency betwen four fields of view selected was high. This furthers our confidence, in the first place, in the quality and precision of the method used, and also in its reroducibility as potential additional tool in assesment of liver tissue fibrosis. Also these findings correlation with known risk factors, that accelerate liver damage alcohol consumption and smoking, are in line with the results of previus studies (Safdar K & Schiff ER 2004; Dev A. et al, 2006).

6. CONCLUSIONS:

1. When examined for differences in gene expression of *CYP2B6, CYP3A4* and *ABCB1* between groups of HIV mono-infected, HCV-mono-infected and HIV/HCV co-infected there was a significant increase in gene expression of *CYP2B6* in the group of HIV/HCV co-infection. There was also found increased expression of *CYP3A4* in all patients infected with HIV, including both, mono and co-infected.

2. There was no correlation found between changes in gene expression of *CYP2B6, CYP3A4* and *ABCB1* and liver fibrosis between these groups of patients. However, there was a positive significant correlation found between increased expression of *CYP3A4* and higher grade of liver inflammation.

3. There was a significant correlation found between increased expression of *CYP3A4* and the use of pegylated interferon in a group of HCV mono-infected patients. There was also a significant correlation found between lamivudine usage and a decrease of genes encoding *ABCB1*. However, there was no correlation found between antiretroviral and DAA treatment and gene expression of *CYP2B6, CYP3A4* and *ABCB1*.

4. There was a significant correlation found between increased levels of gene expression of *CYP3A4* and high platelets count. There was no other significant correlation found between gene expression of *CYP2B6, CYP3A4*, and *ABCB1* and other examined biochemical or environmental factors.

7. LITERATURE

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Scientific papers published from this doctoral dissertation:

1. Obradovic, B.; Roberts, O.; Owen, A.; Milosevic, I.; Milic, N.; Ranin, J.; Dragovic, G. Expression of CYP2B6 Enzyme in Human Liver Tissue of HIV and HCV Patients. Medicina 2023, 59, 1207. doi.org/10.3390/medicina59071207.

Biografija kandidata

Dr Božana Obradović je rođena u Prištini 25.11.1987., gde je završio osnovnu i srednju školu. Medicinski fakultet je upisala 2006., a diplomirala 2012. godine sa prosečnom ocenom 8.87. Od 2015. godine je zaposlen na Institutu za Farmakologiju, kliničku farmakologiju i toksikologiju Medicinskog fakulteta, Univerziteta u Beogradu, gde je deo tima za oblast farmakokinetike i farmakogenomike. U zvanje saradnika u nastavi za užu naučnu oblast farmakologija izabrana je 2015. godine, a 2017. godine je izabrana u zvanje asistenta. Za svoj pedagoški i nastavni rad je dobio najviše ocene studenata. 2018. godine je u okviru naučno-stručnog usavršavanja boravila Katedri za farmakologiju Univerziteta u Liverpulu, gde je njenv rad bio usmeren na farmakokinetiku antivirusnih lekova i uticaj različitih faktora na aktivnost metaboličkih enzima i transportera. Specijalistički ispit je položila u aprilu 2023. godine sa najvišom ocenom, stekavši zvanje specijalista kliničke farmakologije. Učesnik je medjunarodnih i domaćih projekata od 2014. godine. Autor je ili koautor 10 naučnih publikacija koje su štampane u celini u časopisima indeksiranim u Journal Citation Reports listi.

Изјава о ауторству

Име и презиме аутора др Божана Обрадовић

Број индекса МФ 01/14

Изјављујем

да је докторска дисертација под насловом

АНАЛИЗА ЕКСПРЕСИЈЕ ГЕНА КОЈИ КОДИРАЈУ СИНТЕЗУ ЕНЗИМА CYP3A4, CYP2B6, И ABCB1 ТРАНСПОРТЕРА КОД БОЛЕСНИКА СА ХИВ И ХЦВ ИНФЕКЦИЈОМ ЛЕЧЕНИХ АНТИВИРУСНИМ ЛЕКОВИМА

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Број индекса МФ 01/14

Студијски програм Медицинска фармакологија

Наслов рада АНАЛИЗА ЕКСПРЕСИЈЕ ГЕНА КОЈИ КОДИРАЈУ СИНТЕЗУ ЕНЗИМА CYP3A4, CYP2B6, И ABCB1 ТРАНСПОРТЕРА КОД БОЛЕСНИКА СА ХИВ И ХЦВ ИНФЕКЦИЈОМ ЛЕЧЕНИХ АНТИВИРУСНИМ ЛЕКОВИМА

Ментори Проф. др Гордана Драговић Лукић; проф. Др Ивана Милошевић и проф. Andew Owen

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањивања у **Дигиталном репозиторијуму Универзитета у Београду**.

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду

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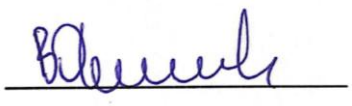

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Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

АНАЛИЗА ЕКСПРЕСИЈЕ ГЕНА КОЈИ КОДИРАЈУ СИНТЕЗУ ЕНЗИМА CYP3A4, CYP2B6, И ABCB1 ТРАНСПОРТЕРА КОД БОЛЕСНИКА СА ХИВ И ХЦВ ИНФЕКЦИЈОМ ЛЕЧЕНИХ АНТИВИРУСНИМ ЛЕКОВИМА која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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