ROMANIAN ACADEMY
School of Advanced Studies of the Romanian Academy
Institute of Biochemistry

PhD THESIS SUMMARY

Endoplasmic Reticulum signaling pathways induced by human Hepatitis B virus (HBV) infection

SCIENTIFIC COORDINATOR
CS I Dr. Norica Nichita

PhD CANDIDATE
Alina-Veronica Ghionescu

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Table of contents (Thesis *IN EXTENSO*)

Study aim

Worldwide, 1.5 million new hepatitis B virus (HBV) infections are reported annually (Hsu et al., 2023). This pathogen is the causative agent of acute or chronic hepatitis B, which can lead to liver cirrhosis or hepatocellular carcinoma (HCC). Current treatments for patients having chronic HBV infections do not provide a complete cure. Still, research into the virus and host cell interactions may lead to the development of new therapeutic targets (Jiang et al., 2021). The genome of the viral particle is represented by relaxed circular DNA (rcDNA), packed inside a nucleocapsid, surrounded by a lipid bilayer and envelope glycoproteins (S, M, L). Before being packaged into viral particles, envelope glycoproteins are translocated to the endoplasmic reticulum (ER), undergoing N-glycosylation, folding, and oligomerization processes (Tsukuda and Watashi, 2020).

Previous studies have demonstrated that the accumulation of these viral proteins induces stress in the ER and activates the Unfolded Protein Response (UPR) and increases the expression level of mannosidases from the Endoplasmic Reticulum (ER) Degradation Enhancing Alpha-Mannosidase Like Protein (EDEM) family. Incomplete or misfolded glycoproteins are processed by these mannosidases that act on N-linked glycans, producing substrates for ER-associated degradation (ERAD). Viruses can acquire mechanisms to manipulate the UPR to their advantage. It has been observed that the activation of EDEM proteins causes the degradation of surface proteins (S, L) and increased secretion of M protein in HBV infection (Lazar et al., 2012, Lazar et al., 2017). Also, previous data from our group have shown that overexpression of EDEM3 protein increases viral replication and subviral particles (SVPs) secretion (PhD Thesis-PhD. Mihaela Uță).

This study investigates the mechanisms by which ERAD, mainly the EDEM3 protein, interferes in the HBV life cycle. Thus, we have used cell lines susceptible to HBV infection, liver tissue samples, and transcriptomic and proteomic data from public databases. Gene expression of EDEM3 has been measured in HBV-infected and non-infected liver tissue samples and the results have been confirmed by analysis of public databases. In addition, molecular processes at the ER level, involved in the HBV life cycle, have been investigated in cell lines with modulated expression of EDEM3.

These results have led to another aim of this study, to investigate the role of ERAD in HCC by analyzing transcriptomic databases. The identified ERAD-associated genes have been correlated with the most critical factors involved in HCC management.

Chapter 1. Introduction

HBV is a hepatotropic virus from the *Hepadnaviridae* family, discovered by Blumberg et al. as the "Australia antigen" (Blumberg et al., 1967, Seeger and Mason, 2000).The viral genome has a compact structure, consisting of a molecule of relaxed circular DNA (rcDNA), with a length of approximately 3.2 kb (kilobases), composed of an incomplete sense strand (positive strand) and a complete antisense strand (negative strand). It is enveloped inside a nucleocapsid, surrounded by a lipid bilayer and surface proteins (Tsukuda and Watashi, 2020).

The HBV life cycle has several unique and complex stages, each critical for virus replication and persistence. These steps include internalization of viral particles into host hepatocytes, intracellular transport of the viral nucleocapsid to the host cell nucleus, conversion of rcDNA to a covalently closed circular form (cccDNA), transcription and translation of viral proteins, reverse transcription of pregenomic RNA into rcDNA, assembly and secretion of viral particles (Seeger and Mason, 2015).

The cccDNA molecule is associated with viral proteins and host cell factors and is organized into minichromosomes. It is maintained in the nucleus over a long period of time and functions as a template for viral transcription. The persistence and localization of this intermediate form of viral DNA in infected hepatocytes is the major challenge for antiviral therapies (Xia and Guo, 2020).

According to the latest statistics, more than 50% of HCC cases worldwide are caused by HBV infection. This pathogen can promote the malignant transformation of liver cells by activating various pro-tumor mechanisms such as regulation of genome instability in the host cell, and epigenetic remodeling (methylation status, histone post-translational modification or onco-microRNA induction) (Jiang et al., 2021). Moreover, the integration of viral DNA into the genome of the host cell leads to the activation of the oncogenes expression (TERT - Telomerase Reverse Transcriptase, TP53 - Tumor Protein P53, MYC - MYC Proto-Oncogene, BHLH Transcription Factor (Peneau et al., 2022). HBx also activates the transcription of genes involved in several pro-tumor signaling pathways, including JUN - Jun Proto-Oncogene, AP-1 Transcription Factor Subunit, FOS - Fos Proto-Oncogene, AP-1 Transcription Factor Subunit, MYC and TP53 (Jiang et al., 2021). The immune system plays a vital role in the development of HCC. Thus, the HBV inhibitory effect upon cytokines secretion and upon NK (natural killer) or regulatory T cell activation leads to chronic inflammation and the development of HCC (Jiang et al., 2021).

Liver cells synthesize an increased amount of secretory proteins (about 13 million secretory proteins per minute), given the high metabolic activity of the liver. Thus, it presents a high susceptibility to the disturbance of RE mechanisms (Duwaerts and Maiers, 2021). According to several studies, stress at this level causes a cascade of reactions that affect the liver (Maiers and Malhi, 2019, Zhang et al., 2022). On the other hand, during the regeneration of this organ, hepatocytes are in an active state of proliferation, which is associated with an increase in protein synthesis, leading to ER stress and UPR activation to support cell survival (Rutkowski and Kaufman, 2007).

Glycosylation is the most common post-translational modification (50% of synthesized proteins are glycosylated). It is based on the covalent attachment of polysaccharide chains (glycans) to proteins, modulating their structure and function (Dwek, 1996, Lin and Lubman, 2024). The most known forms of glycosylation involve linking N-glycans to asparagine residues and O-glycans to serine or threonine residues. N-glycosylation involves three essential steps: glycan synthesis on a lipid skeleton called dolichol-pyrophosphate (Dol-PP), transfer to the nascent polypeptide chain and process at the ER-Golgi level (Lin and Lubman, 2024).

After newly synthesized protein translocation to the ER lumen, the oligosaccharide-transferase complex recognizes the specific Asn-X-Ser/Thr (where X represents any amino acid except Pro) sequence and covalently attaches the Glc3Man9GlcNAc2 chain (Zhang and Kaufman, 2006). Subsequently, the glycoprotein is remodeled by a series of enzymes in its secretory pathway (Guay et al., 2023). It is rapidly and sequentially cleaved by glucosidases I and II (GI and GII) to generate a monoglucosylated form (GlcMan9GlcNAc2), which is a substrate for the chaperone lectins such as calnexin (CNX) and calreticulin (CRT). The reduction of terminal glucose residue by GII causes dissociation from the CNX/CRT cycle. Thus, if the released glycoprotein is correctly folded, it will be transported to the Golgi apparatus. Otherwise, it is firstly recognized by UDP-glucose glycoprotein-glycosyl transferases (UGGT1 and UGGT2) and then, it re-enters the CNX/CRT cycle (Adams et al., 2019).

If the protein remains unfolded, EDEM2 catalyzes the removal of the first mannose from glycoproteins N-glycans after the glucose chains elimination, while both EDEM1 and EDEM3 process the oligosaccharide fragment to Man5-7GlcNAc2. It has previously been reported that these mannosidases are associated with oxidoreductases. Thus, all EDEM mannosidases associate with thioredoxin domain containing 11 (TXNDC11), EDEM1 and EDEM2 with Protein disulfide isomerase (PDI); EDEM3 with thioredoxin domain containing 5 (ERp46/TXNDC5), while EDEM1 with DnaJ heat shock protein family (Hsp40) member C10

(DNAJC10/ERdj5). Osteosarcoma amplified 9, ER lectin (OS-9) and endoplasmic reticulum lectin 1 (XTP3-B/ERLEC1) process the Man5-7GlcNAc2 form and interact with suppressor of Lin-12-like (Sel1L) lectin and then, with synoviolin (Hrd1/ SYVN1), an essential protein of retrotranslocation channel (dislocon) (Christianson et al., 2008, Carvalho et al., 2010, Tax et al., 2019).

The substrate retrotranslocation into the cytosol process implies several proteins, such as Derlin 1, Derlin 2, Derlin 3, Sec61, valosin-containing protein-interacting membrane protein (VIMP), ancient ubiquitous protein 1 (AUP1), ubiquitin recognition factor in ER associated degradation 1 (UFD1/Npl4) or autocrine motility factor receptor (AMFR/gp78) (Olzmann et al., 2013). The ERAD pathways (ERAD-M for ER membrane substrates or ERAD-L for lumen substrates) are associated with cytosolic AAA-ATPase (VCP/p97), which mediates the cytosol transport (Radhakrishnan et al., 2014).

In the last step, the E1 enzyme activates ubiquitin through an ATP-dependent reaction, resulting in an active E1-ubiquitin intermediate; active ubiquitin is transferred to the E2-conjugating enzyme, forming a covalent bond with it. Also, the substrate to be degraded forms a complex with E3 ligase, which has a high affinity for E2. Activated ubiquitin is transferred to the E3 enzyme, which catalyzes covalent attachment to the target substrate, which is ubiquitinylated and recognized by the proteasome for degradation. To maintain a sufficient level of free ubiquitin in the cell, polyubiquitin chains are removed by the action of deubiquitinases (DUBs) (Glickman and Ciechanover, 2002, Gong et al., 2016).

Many ERAD components, particularly Hrd1 and its cofactor Sel1L, are conserved in mammals (Vembar and Brodsky, 2008). In hepatocytes, Hrd1/Sel1L regulates fasting and feeding responses through the transcription factor CREBH, which, in turn, activates the transcription of FHF21 - Fibroblast growth factor 21, a key regulator of liver metabolic pathways (Bhattacharya et al., 2018). Complete deletion of the Hrd1 or Sel1L genes in mice leads to embryonic lethality, highlighting their essential role in embryogenesis (Francisco et al., 2010). In cancer cells, tumor suppressors (PTEN - phosphatase and tensin, FOXO - forkhead box protein O, WNT5A - Wingless-Type MMTV Integration Site Family, Member 5A or TP53) are degradation substrates of Hrd1 (Yamasaki et al., 2007, Liu et al., 2018, Bhattacharya et al., 2022). The degradation of these molecules promotes liver cancer cell proliferation both *in vivo* and *in vitro*, vascular invasion, angiogenesis and metastasis (Ji et al., 2021). Another ERAD component reported in HCC is tripartite motif-containing 25 (TRIM25), which causes ERAD activation (Zhang et al., 2020) and inhibits the signaling pathway through type I transmembrane

protein inositol requiring 1 (IRE1α), promoting tumor cells survival and growth (Liu et al., 2020).

Overexpression of OS-9 has been observed in tumor and adjacent tissues from patients with recurrence, compared to those without recurrence. *In vitro* studies further demonstrate that positive modulation of OS-9 expression enhances the migration and invasion capacity of HCC cells (Wei et al., 2022). In contrast, depletion of the HRD1 upstream molecule, gp78 (Olzmann et al., 2013), inhibits ERAD, leading to chronic stress and promoting conditions such as non-alcoholic steatohepatitis (NASH) and HCC (Zhang et al., 2015).

In addition, to ensure fast and efficient metabolism, tumor cells regulate their polyubiquitinylation or monoubiquitinylation processes during tumorigenesis since the assembly of ubiquitin chains requires an increased amount of energy. Thus, an increase in global monoubiquitinylation has been observed in metastatic cells, which is sufficient for cell functions (Ji et al., 2021).

Several studies have shown that HBV infection triggers increased expression levels of several ERAD markers. Transient overexpression of EDEM proteins has also been observed to reduce intracellular levels of envelope proteins S and L and to decrease secreted SVPs (Lazar et al., 2012, Lazar et al., 2017). Wang et al. have reported the role of Sel1L in HBV infection. Thus, the amounts of viral RNA, DNA and proteins have been significantly reduced by Sel1L overexpression in Huh7 cells, transiently transfected with a plasmid containing the viral genome. Modulation of Sel1L expression by transient silencing led to ERAD inhibition and ER quality control (ERQC) and autophagy processes activation (Wang et al., 2019).

Due to a high proliferation rate, cancer cells often experience impaired ATP generation, hypoxia, and specific mutations that can disrupt ER homeostasis and trigger UPR, ERAD or autophagy (Tsai and Weissman, 2010). Modulation of components of these pathways represents important therapeutic strategies for activating apoptotic pathways in malignant cells (Clarke et al., 2014).

Several studies have shown that HBV infection induces ER stress and stimulates the early stages of autophagy, which support viral replication and secretion. Also, the activation of UPR, cell proliferation and carcinogenesis processes have been observed in the infection. The UPR activates associated signaling pathways, including ERAD. Thus, cell homeostasis is restored (Lin et al., 2020, Chen and Cubillos-Ruiz, 2021, Wang et al., 2022) by removing accumulated or misfolded proteins, by increasing folding capacity or by reducing protein translation (Cao and Kaufman, 2012).

UPR promotes the cell's adaptive response, which favors tumor growth and chemoresistance but also triggers apoptosis in persistent stress conditions (Stefani et al., 2012). This fact represents a notable challenge in using it for HCC therapy, as it depends on the characteristics of the tumor microenvironment. Therefore, this study aims to understand the role of the ERAD degradation pathway in HBV infection and HCC development.

Chapter 2. MATERIALS AND METHODS

The gene expression of EDEM3 was quantified in HBV-infected and uninfected liver tissues. These findings were corroborated by analyzing a transcriptomic database that included infected primary hepatocytes. Additionally, several molecular mechanisms affecting HBV, such as UPR, ERAD, and autophagy, were investigated in EDEM3-overexpressing hepatic cell lines. To validate these results, an EDEM3 knock-out line was generated using CRISPR/Cas9 technology. Due to phenotypic changes in these cells and the difficulty in maintaining the line over several passages, apoptosis was examined. Finally, to explore the role of ERAD in HCC, the correlation between this signaling pathway and clinicopathological data from HCC patients was analyzed using several public transcriptomic datasets.

Chapter 3. RESULTS

To explore the intricate interactions in ER-associated signaling pathways in HBV infection, we selected and analyzed public datasets from the GEO platform (Gene Expression Omnibus, https://www.ncbi.nlm.nih.gov/geo/), which included gene expression data obtained through whole transcriptome sequencing. The GSE183156 dataset comprised six primary hepatocyte samples, three of which were infected with HBV and three uninfected, collected 28 days post-infection. Data analysis using clustering methods in R revealed a significant increase in genes associated with the UPR, ERAD, and autophagy mechanisms in HBV-infected cells compared to uninfected cells. Furthermore, the high expression levels of EDEM3 were consistent with previously reported results from our department (Lazar et al., 2012, Lazar et al., 2017).

To validate the *in vitro* results, we quantified the expression level of the EDEM3 gene by RT-real-time qPCR in liver tissue samples: 10 normal (N) samples and 50 non-tumor adjacent to tumor tissue, of which 25 were infected with HBV (NAT+) and 25 were uninfected (NAT-). The results revealed that EDEM3 was highly expressed in NAT samples, both with and without HBV, compared to normal (N) samples. Additionally, the EDEM3 transcript level was

higher in HBV-infected tissues (Figure 1.A), which suggests that EDEM3 may play a role in HBV infection and the development of chronic liver diseases.

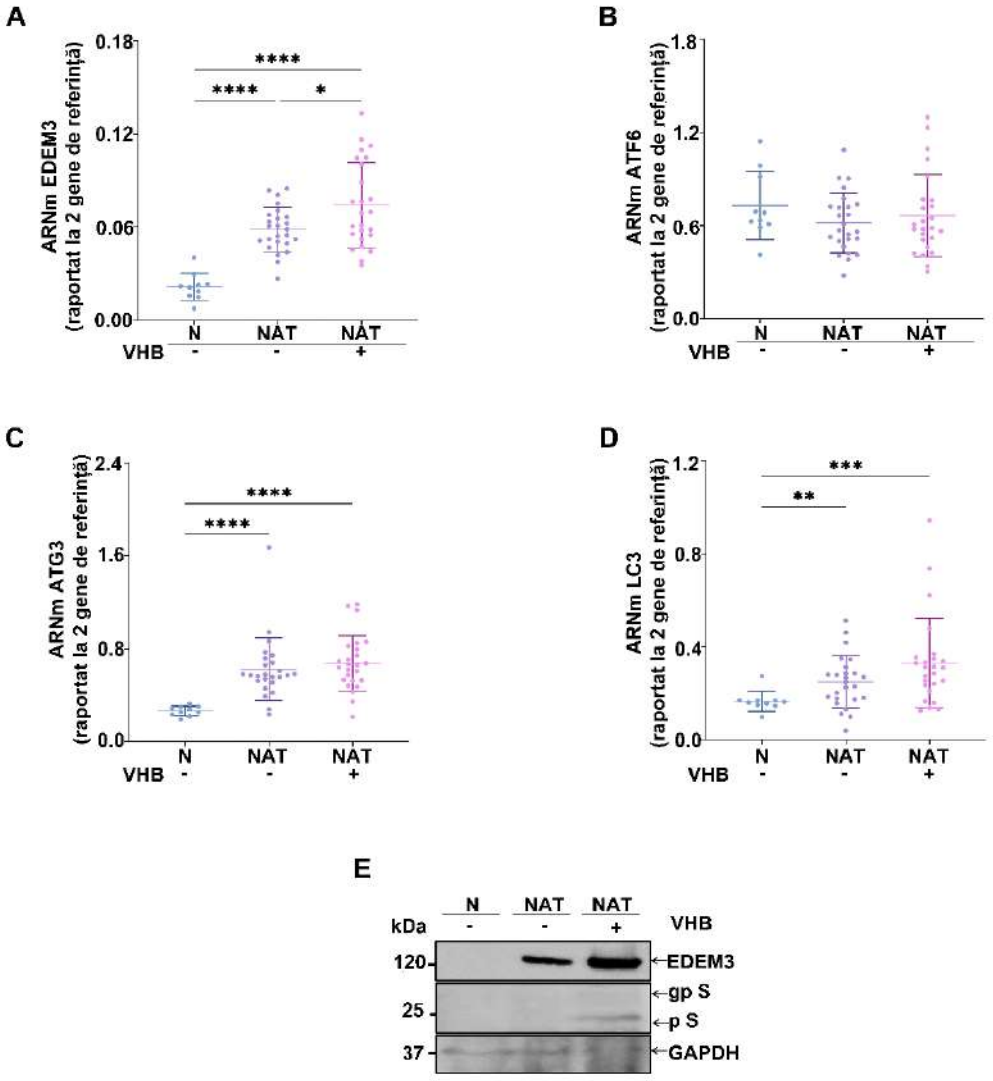


Figure 1. Analysis of UPR and autophagy markers in non-tumor tissue samples from HCC patients with/without HBV infection. The expression of (A) EDEM3, (B) ATF6, (C) ATG3 and (D) LC3 genes was quantified in the following types of samples: normal liver tissue – N (n=10) and non-tumor liver tissue adjacent to the tumor – NAT from HBV-infected HCC patients (n=25) and uninfected patients (n=25), by RT-real-time qPCR and using the $2^{-\Delta Ct}$ method. Transcript expression was normalized to the reference genes, GAPDH and TBP. Each scatter plot illustrates the median and standard deviation of gene expression values. Statistical analysis was performed using the unpaired Student t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). (E) EDEM3, HBV-S -nonglycosylated form (pS), and HBV-S -glycosylated form (gpS) proteins were evaluated by Western blot. N, NAT HBV (−) and NAT HBV (+) liver

tissue samples were lysed, processed, and detected with specific antibodies. GAPDH was used as a loading control.

In the same tissue samples, we quantified the expression levels of genes involved in the UPR (ATF6) and autophagy (LC3, ATG3). While these genes showed a significant increase in infected hepatocyte samples collected shortly after infection (28 days), no statistically significant change in transcript levels was observed between HBV-infected and uninfected liver tissues. However, for the two autophagy markers, gene expression was elevated in the non-tumor adjacent tissue (NAT) samples compared to the normal (N) samples (Figure 1.B-D).

Furthermore, EDEM3 protein levels were assessed using Western blot on liver tissue samples (N, NAT-, NAT+), confirming the findings at the transcriptional level (Figure 2.E). These findings emphasize the differences in ER mechanisms between chronic and acute HBV infections and indicate a potential role for EDEM3 in developing chronic liver disease.

Based on these observations, a stable line lacking EDEM3 (HepaRG^{EDEM3KO}) was generated using CRISPR/Cas9 technology to investigate the function of this protein in HepaRG cells. The EDEM3 overexpressing cell line was established prior to this study.

The consequences of EDEM3 modulation on the HBV life cycle in liver cells were previously examined by Dr. Mihaela Uță in the PhD program, that showed the HBs antigen and viral DNA levels isolated from immunoprecipitated enveloped virions were increased in the EDEM3-overexpressing line in HBV infection compared to the control line. Moreover, we verified if the observed effects were caused by an increased level of the HBV receptor (NTCP). The results showed no difference between HepaRG^{EDEM3} and HepaRG^{C} cell lines. Additionally, analysis of intracellular viral DNA after 24 hours post-infection revealed no significant differences between the two HepaRG lines. This suggests that EDEM3 is not involved in the viral internalization phase, which is consistent with previous findings from our laboratory.

Following the findings that viral infection enhances EDEM3 expression, we examined the opposite effect on HBV. Despite multiple optimization efforts, the HepaRG^{EDEM3KO} cell line could not resist the 14-day differentiation period as phenotypic alterations and apoptosis emerged. Several studies have reported that HBV can acquire mechanisms to manipulate the UPR to its advantage. Thus activation of ER signaling pathways has been observed both in chronic HBV infection (Wang et al., 2022) and in tumorigenesis (Wei and Fang, 2021). To understand how EDEM3 affects the HBV life cycle, we investigated the effects of its overexpression on the UPR. Thus, IRE1α, ATF6, and PERK signaling pathways were analyzed in HepaRG cells by Western blot.

The results show that while the expression levels of the ATF6 and BiP markers remained unchanged, the GRP-94, PERK, eIF2α and IRE1α expressions were decreased in HepaRG^{EDEM3} cells compared to HepaRG^{C} cells (Figure 2).

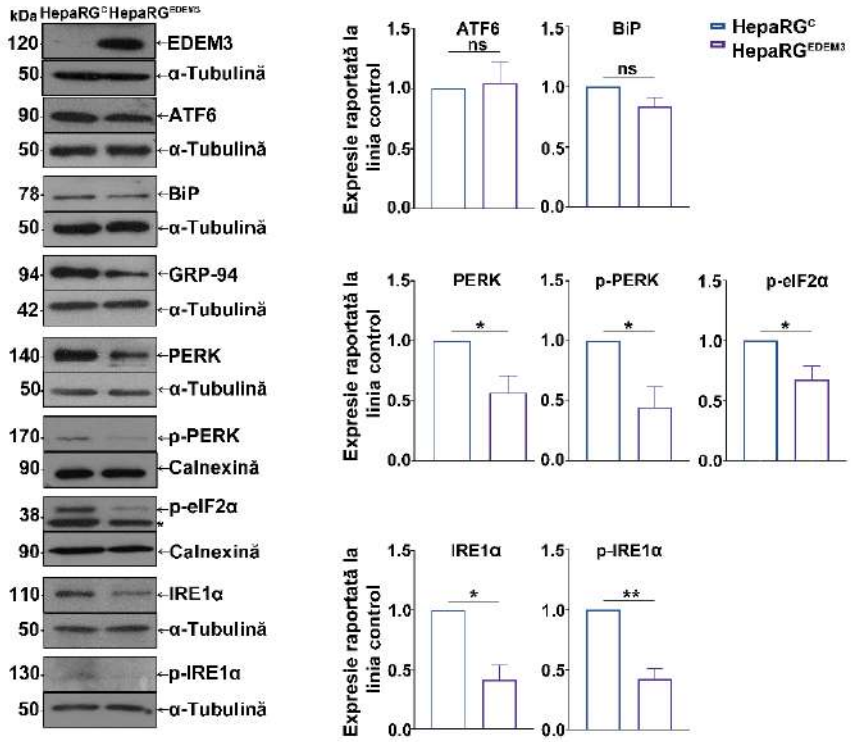


Figure 2. Expression of UPR markers in EDEM3-overexpressing HepaRG cells.

HepaRG^{C} and HepaRG^{EDEM3} cell lysates were probed by Western blot with the appropriate antibodies. α-tubulin, β-actin, or calnexin was used as a loading control. The band sizes were estimated using the ImageJ program. The *asterisk* indicates a non-specific band of the anti-β-eIF2α antibody. The right image shows the quantifications of UPR markers, and the graphs represent the median and standard deviation of the relative expression levels from 3 independent experiments. Statistical analysis was performed using the unpaired Student t-test. (*, $p < 0.05$; **, $p < 0.01$).

Moreover, we investigated the effect of EDEM3 overexpression on the ERAD signaling pathway using Western blot analysis. The expression levels of OS-9, XTP3-B, and Sel1L were similar between the two cell lines, while Hrd1 was significantly elevated in the EDEM3-overexpressing line (Figure 3). This protein has previously been linked to the degradation of Rheb, an activator of mTOR, and its increased expression in the HepaRG^{EDEM3} line suggests a potential inhibition of the mTOR signaling pathway.

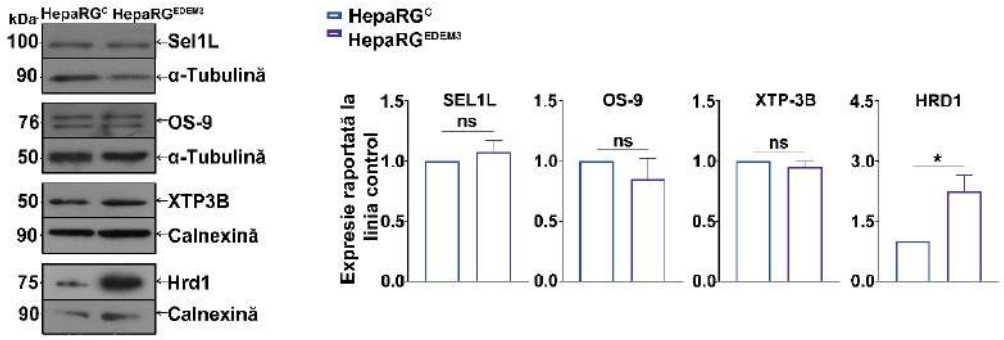


Figure 3. Expression of ERAD markers in EDEM3-overexpressing HepaRG cells. The protein level of ERAD markers (Sel1L, OS-9, XTP3-B and Hrd1) was analyzed by Western blot with specific antibodies. α-tubulin or calnexin was used as a loading control. The band sizes were estimated using the ImageJ program and normalized to the corresponding loading controls. The right image shows the quantifications of ERAD markers, and the graphs represent the median and standard deviation of the relative expression levels from 3 independent experiments. Statistical analysis was performed using the unpaired Student t-test (*, $p < 0.05$).

Secretory autophagy is a key process in the HBV life cycle, promoting nucleocapsid assembly, viral replication and viral particle secretion (Chu et al., 2022, Wang et al., 2022). To investigate whether the modulation of EDEM3 expression affects the autophagy process, HepaRG^{C} and HepaRG^{EDEM3} cell lines were transfected with the plasmid pEGFPC1-LC3, which encodes the autophagy marker LC3. Treatment of cells with chloroquine (CQ), an inhibitor that blocks autophagosome-lysosome fusion (Mauthe et al., 2018), was included as a positive control in this experiment. The results showed an increased number of autophagosomes labeled with LC3 in EDEM3-overexpressing cells, while they were barely detectable in the HepaRG^{C} cell line. Moreover, EDEM3 and LC3 weren't co-localized (Figure 4).

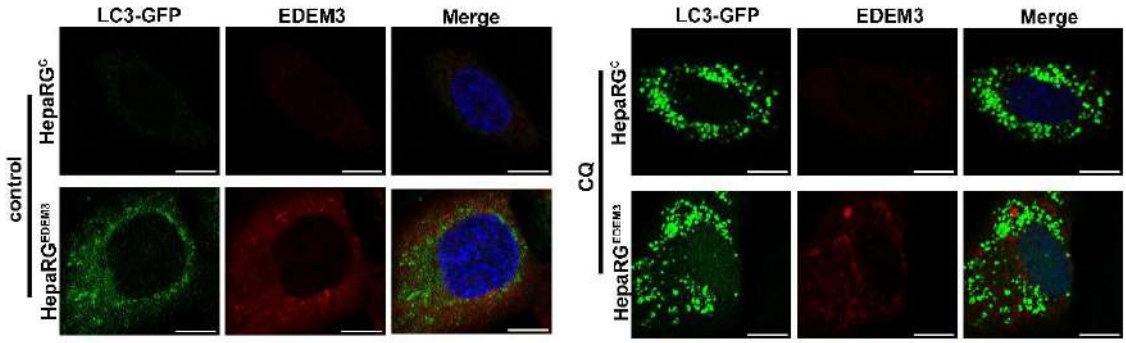
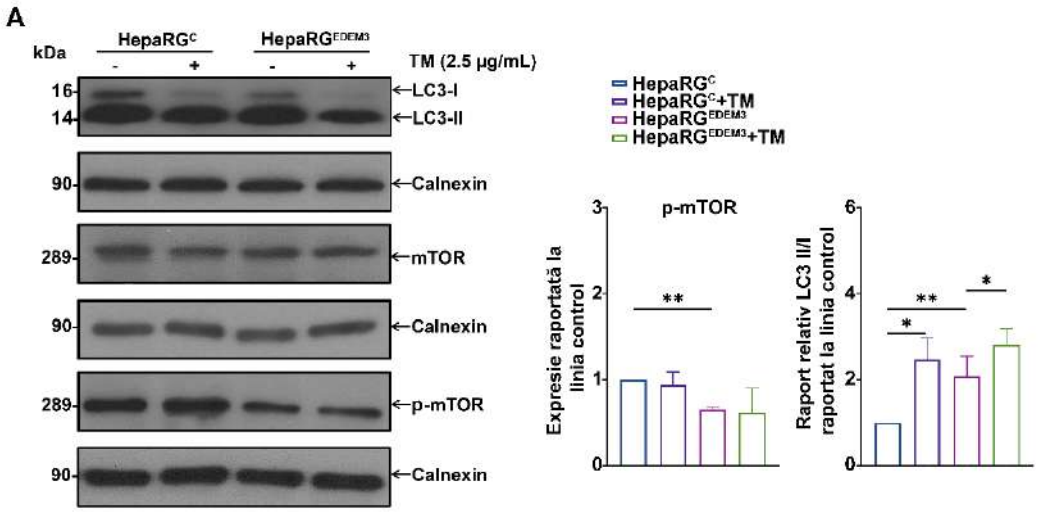


Figure 4. The expression level of LC3 is increased in cells overexpressing EDEM3. HepaRG^{C} and HepaRG^{EDEM3} liver cells were transfected with pEGFPC1-LC3 plasmid. 6 h after transfection, cells were incubated with 10 µM chloroquine (CQ) for 24 h. EDEM3 protein expression (red) was highlighted with specific antibodies, and nuclei were labeled with DAPI. Scale bar: 10 µm.

This result was confirmed by Western Blot analysis, where an elevated LC3-I to LC3-II transition was observed in HepaRG^{EDEM3} cells and in TM treatment, used as a positive control (Wang et al., 2022) (Figure 5.A).

Since autophagy is strongly inhibited by mTOR (Rabanal-Ruiz et al., 2017, Saxton and Sabatini, 2017), the expression of the total protein, but also of the phosphorylated form, in HepaRG^{EDEM3} cells was investigated. As expected, phospho-mTOR decreased in these cells, while total mTOR was unaffected (Figure 5.A). In addition, Rheb showed a low level in the HepaRG^{EDEM3} cell line (Figure 5.B). Thus, it can be assumed that the higher Hrd1 expression causes a Rheb decrease. Moreover, the degradative autophagy marker, ApoE, was increased in the HepaRG^{EDEM3} compared to the HepaRG^{C} cell line (Figure 5.B). These results suggest that EDEM3 overexpression increases secretory autophagy by the Hrd1/Rheb/mTOR axis.



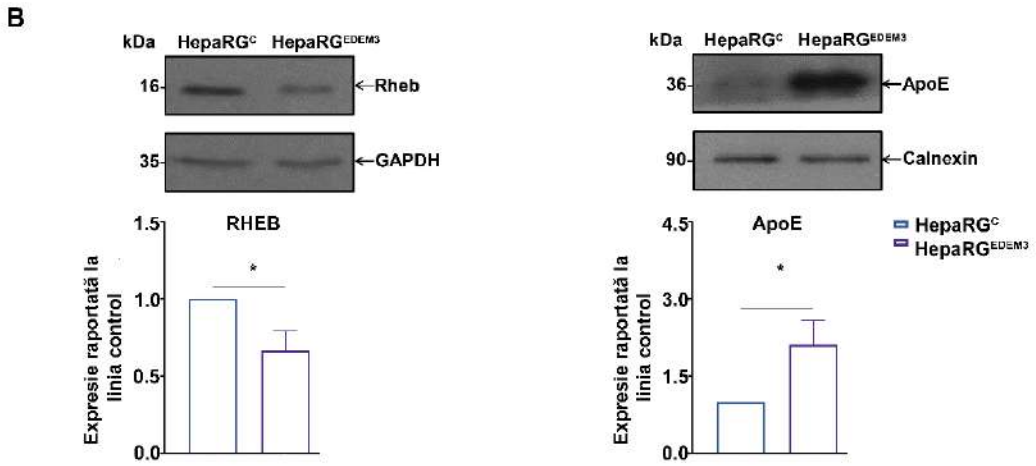


Figure 5. EDEM3 expression promotes secretory autophagy in HepaRG liver cells. HepaRG^{C} and HepaRG^{EDEM3} cell lysates treated/untreated with 2.5 µg/mL TM for 6 h were investigated by Western blot. (A) LC3, mTOR, p-mTOR; (B) Rheb and ApoE proteins were detected using specific antibodies. For normalization, calnexin or GAPDH were used as internal control. The band sizes were estimated using the ImageJ program. The image shows the quantifications of the markers from 3 independent experiments in the form of bar charts representing the median and standard deviation. Statistical analysis was performed using the unpaired Student t-test (*, $p < 0.05$; **, $p < 0.01$).

To confirm the role of EDEM3 on ER signaling pathways, UPR and autophagy mechanisms were investigated in HepaRG^{EDEM3KO} cells. Throughout the HepaRG^{EDEM3KO} cell expansion, a notable decline in viability was observed once the number of passages exceeded 10. Consequently, experiments were carried out on these cells at early passages (4–5), either in the presence or absence of TM, revealing an increase in IRE1α and PERK signaling.

Li et al. reported that chronic ER stress activates pro-apoptosis (Li et al., 2019). Therefore, the increase in UPR markers and the presence of an altered phenotype led us to investigate apoptosis in EDEM3 knockout cells. Through Western Blot analysis, we observed a higher expression of pro-apoptotic proteins, p53 and Bax (Stefani et al., 2012) , in HepaRG^{EDEM3KO} cells (Figure 6). Also, by flow cytometry, we highlighted the presence of apoptosis in the HepaRG^{EDEM3KO} cell line.

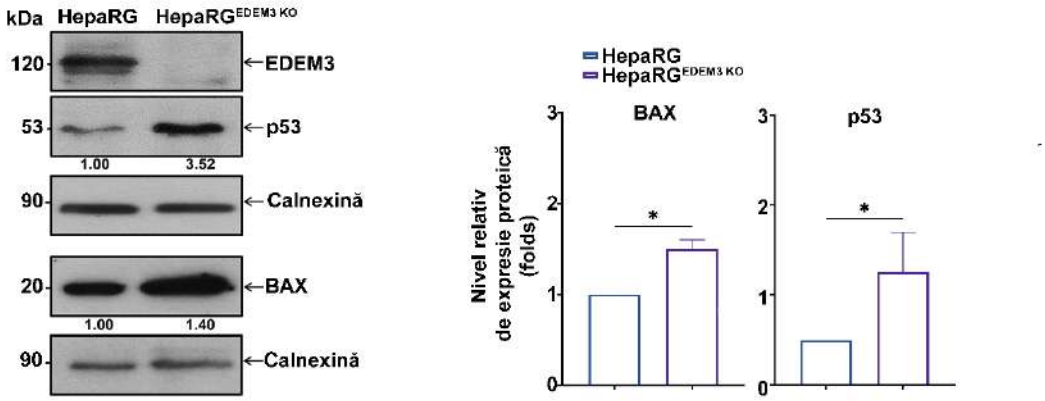


Figure 6. EDEM3 depletion activates molecules involved in the process of apoptosis in the HepaRG cell line.

HepaRG and HepaRG^{EDEM3KO} cell lysates were investigated by Western blot. Apoptosis markers p53 and BAX were detected with specific antibodies. For normalization, calnexin was used as a loading control. The band sizes were estimated using the ImageJ program. The bar charts illustrate the median and standard deviation from 3 independent experiments. Statistical analysis was performed using the unpaired Student's t-test. (*, $p < 0.05$).

Chronic HBV infection is a risk factor for HCC (Arbuthnot and Kew, 2001). The increased gene expression level of EDEM3 in non-tumor tissue samples adjacent to the tumor compared to normal liver samples (Figure 7.A) suggests that this molecule also contributes to the development of HCC. To investigate this hypothesis, the gene expression profile of EDEM3 was evaluated in 50 HCC tumor samples and in 50 adjacent non-tumor samples by RT-real-time qPCR. The results indicated a higher expression of the EDEM3 transcript in tumor samples than in non-tumor ones and a significant decrease in the autophagy marker LC3 in tumor tissue samples. In contrast, ATG3 and ATF6 gene expression levels remained unchanged in the two types of samples (Figure 7.A). EDEM3 protein expression was detected in 12 liver tissue samples (6 non-tumor and 6 paired tumor samples). The results confirmed that EDEM3 shows an increased level in tumor samples from HCC patients (Figure 7.B) and may be implicated in HCC development. To investigate this hypothesis, we investigate the clinical significance of the EDEM3 transcriptional profile in HCC. The Kaplan-Meier curve showed that patients with elevated levels of EDEM3 have a poorer prognosis, but is not an independent prognostic biomarker. The correlation between the expression profile of EDEM3 and the response of liver cells to sorafenib was investigated in several transcriptomic databases. The results showed a

decrease in EDEM3 expression after the treatment, which confirmed the observations obtained *in vitro*, according to which EDEM3 negatively correlates with apoptosis.

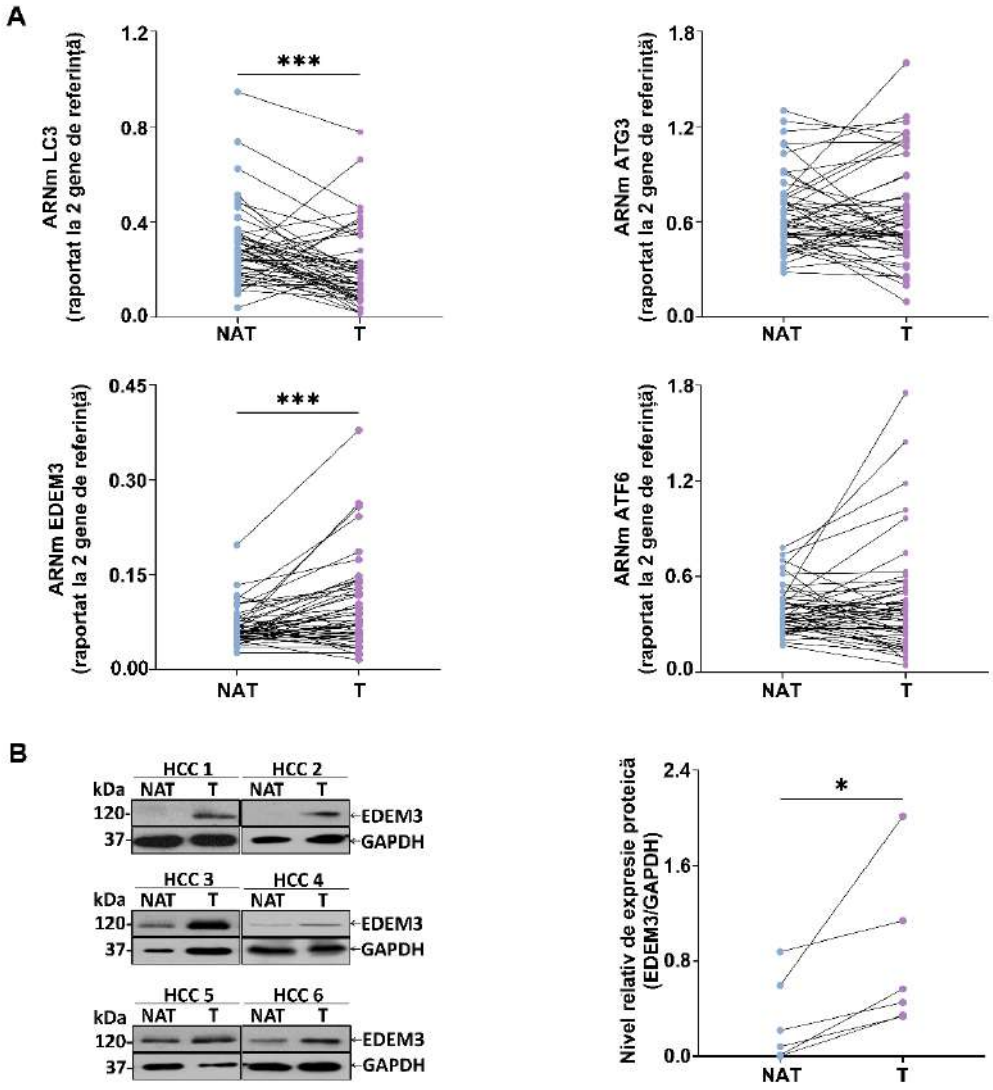


Figure 3.7. The expression level of EDEM3 is increased in tumor tissue samples from HCC patients.

(A) The gene expression levels of LC3, ATG3, EDEM3, and ATF6 were analyzed in non-tumor liver tissues adjacent to the tumor – NAT (n=50) and tumor liver tissues -T (n=50), respectively, by RT-real-time qPCR and using the calculation formula $2^{-\Delta Ct}$. The results were normalized using 2 reference genes, GAPDH and TBP. Statistical analysis was performed using the paired t-test (***, $p < 0.001$). (B) Evaluation of EDEM3 protein expression by Western blot in 6 paired samples of liver tissue, NAT and T. Samples were lysed and detected with specific antibodies. GAPDH was used as a loading control. The

quantification of protein expression is represented in the right panel, in which the statistical analysis was performed using the paired Student t-test. (*, $p < 0.05$).

To confirm these results in HCC patients as well, the GSE109211 database was analyzed, which included tumor samples from 21 sorafenib-sensitive and 46 sorafenib-resistant patients. Data analysis indicated an increase in EDEM3 transcript in sorafenib-resistant patients. This result is in concordance with the previous findings, which also reported that an increased transcriptomic profile of EDEM3 is associated with radioresistance in prostate cancer (Scott et al., 2022). These data indicate that EDEM3 could be a potential biomarker of therapy resistance, a hypothesis that requires further investigation by analyzing more tissue samples and by testing primary tumor cultures (2D or 3D models) from sensitive HCC patients /resistance to treatment.

To investigate the role of ERAD in HCC, we analyze the ERAD-associated differentially expressed genes (DEGs) belonging to multiple whole transcriptome sequencing data suggested the involvement of 10 genes (HSPA4, HSPA5, ERDJ3/DNAJB11, HYOU1, BAG2, CANX, SEC61A1, NEDD4, NPLOC4 and PSMD4), in addition to EDEM3, in the process of HCC tumorigenesis. The expression level of these ERAD-associated genes was significantly increased in tumor tissue samples compared to adjacent non-tumor tissue. Moreover, the tumor samples from the TCGA-LIHC patient group could be divided into two clusters based on the expression of the 11 genes in high-ERAD (high expression of all genes) and low-ERAD (low expression of all genes). Analysis of the clinicopathological characteristics of HCC patients based on this signature indicated that the group of patients in the high-ERAD cluster has a lower survival rate, this signature being an independent prognostic factor.

Reduced immune infiltration in the tumor microenvironment (TME) has been identified as one of the primary contributors to chemoresistance and poor survival in liver cancer (Fu et al., 2019). In this study, we examined the ERAD signaling pathway, TME, immune infiltration and found that HCC patients in the high-ERAD cluster exhibit a diminished immune response, which negatively impacts their response to sorafenib and TACE therapy.

Chapter 4. CONCLUSIONS AND PERSPECTIVES

This study investigates the ERAD signaling pathway in liver diseases, focusing on the ER-associated mechanisms through which EDEM3 is involved in the HBV life cycle and tumorigenesis. The transcriptomic expression of EDEM3 was elevated in HBV-infected NAT samples than in uninfected NAT samples. Notably, a marked increase of EDEM3 was observed

in HCC samples compared to non-tumoral samples, which suggests that this molecule promotes the development of chronic liver diseases.

Moreover, by modulating the expression of EDEM3 in the HepaRG cell line, this study highlighted the important role of EDEM3 in cell survival. Overexpression of EDEM3 led to the downregulation of ER stress and UPR. However, the data also showed a significant increase in the secretory autophagy process and inhibition of lysosomal degradation. This modulation of autophagic flux by EDEM3 may explain previous findings from our group, which demonstrated that EDEM3 promotes viral infection in HepaRG cells. At the molecular level, these mechanisms are coordinated by the EDEM3-Hrd1-Rheb-mTOR axis, which is described in this study. These findings were further confirmed in HepaRGEDEM3KO cells, where chronic ER stress and induction of apoptosis were observed. These findings suggest that EDEM3 protects liver cells, potentially promoting pro-tumor processes. Future studies will explore this hypothesis by modulating EDEM3 expression in tumor liver cells and analyzing processes such as invasion, migration, metastasis and EMT.

For the first time, this study evaluates the relationship between the clinicopathological characteristics of HCC patients and the expression levels of EDEM3 using transcriptomic databases. Thus, the results indicate that patients with increased expression of EDEM3 exhibit lower survival rates and resistance to sorafenib treatment, suggesting that EDEM3 could serve as a potential biomarker for therapy response. This hypothesis will be further investigated in a prospective cohort of HCC patients, selected based on various clinicopathological factors, including the presence of viral infections, disease staging, and the type of therapy. Additionally, another aspect of this study is to investigate the role of EDEM3 in developing HCC tumors *in vivo* by assessing the ability of tumor cells with modulated EDEM3 expression to form tumors in murine models. Based on the *in vitro* results, direct targeting of EDEM3 in infected or tumor cells could induce necrosis in the liver by activating ER stress and apoptosis mechanisms. Therefore, an alternative approach involving the restoration of EDEM3 expression to levels resembling normal conditions will be investigated. Furthermore, through transcriptomic analysis of multiple patient cohorts from public databases, this study identified a signature of 11 ERAD-associated genes implicated in HCC. These genes can distinguish tumor tissue from non-tumor tissue based on their expression profiles, suggesting their potential role in diagnosing HCC. Using unsupervised clustering methods with the 11 genes, HCC tumors were classified into high-ERAD and low-ERAD groups. These clusters were found to influence the clinicopathological characteristics of HCC patients. Specifically, the high-ERAD cluster was significantly associated with lower immune infiltration, increased expression of EMT markers,

resistance to therapy, and poor prognosis. The *in silico* findings will be initially validated at the proteomic level through *in vitro* experiments on liver tumor cell lines treated with various anti-tumor agents. Additionally, the study will explore the modulation of these gene expression levels and their effect on cell sensitivity to treatment.

In conclusion, future studies may clarify the role of these molecules in HCC and their potential as biomarkers or therapeutic targets. The investigation of the effects of 11 gene expressions or of specific inhibitors in primary liver cultures and murine models could further enhance our understanding of the ERAD mechanism in HCC pathology.

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List of published papers within the doctoral thesis

1. **Ghionescu A.-V**.*, Sorop A.*, Linioudaki E., Coman C., Savu L., Fogarasi M., Lixandru D., Dima S.O. A predicted epithelial-to-mesenchymal transition-associated mRNA/miRNA axis contributes to the progression of diabetic liver disease. *Scientific Reports*. **2024**. 14(1):27678, doi: 10.1038/s41598-024-77416-4, **IF 3.8, Q1, AIS 1.059**
2. **Ghionescu A.-V***., Sorop A.*, Dima S.O. The pivotal role of EMT-related noncoding RNAs regulatory axes in hepatocellular carcinoma. *Front Pharmacol*. **2023**. 14, 1270425, doi:10.3389/fphar.2023.1270425, **IF 4.4, Q1, AIS 0.961**
3. **Ghionescu A.-V**.*, Uță M.*, Sorop A., Lazar C., Flintoaca-Alexandru P.R., Chiritoiu G., Sima L., Petrescu S.M., Dima S.O., Branza-Nichita N. The endoplasmic reticulum degradation-enhancing α-mannosidase-like protein 3 attenuates the unfolded protein response and has pro-survival and pro-viral roles in hepatoma cells and hepatocellular carcinoma patients. In revision- *Journal of Biomedical Science*. **IF 9.0, Q1, AIS 2.372**

List of published papers in the field of the doctoral thesis

4. Constantinescu D.R.*, Sorop A.*, **Ghionescu A.-V.***, Lixandru D., Herlea V., Bacalbasa N., Dima S.O. EM-transcriptomic signature predicts drug response in advanced stages of high-grade serous ovarian carcinoma based on ascites-derived primary cultures. *Front Pharmacol*. **2024**, 6;15:1363142. doi: 10.3389/fphar.2024.1363142. **IF 4.4, Q1, AIS 0.961**
5. Uță M., **Ghionescu A.**, Popa C., Nichita N., Coriu D. Investigation of Molecular Mechanisms Involved in Hepatitis B Virus Associated B-cell Non-Hodgkin Lymphoma (B-NHL). *Documenta Haematologica -Revista Romana de Hematologie*. **2023**, 1(2), doi: https://doi.org/10.59854/dhrrh.2023.1.2.59. **BDI**
6. Bucataru I.C., Dragomir I., Asandei A., Pantazica A.-M., **Ghionescu A.**, Branza-Nichita N., Park Y., Luchian T. Probing the Hepatitis B Virus E-Antigen with a Nanopore Sensor Based on Collisional Events Analysis. *Biosensors*. **2022**, 12(8):596, doi: 10.3390/bios12080596, **IF 4.9, Q1, AIS 0.765**

*first author with equal contribution

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