UNIVERSITY OF MEDICINE AND PHARMACY
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DOCTORAL SCHOOL
MEDICAL FIELD

CLINICAL RELEVANCE OF MIRNA IN CUSTOMIZING DIAGNOSIS AND PROGNOSIS FOR PATIENTS WITH CHRONIC HBV INFECTION

DOCTORAL THESIS SYNOPSIS

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2024

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Abbreviations encountered in this synopsis

ALT- alanin-aminotransferase

AST- aspartat-aminotransferase

AUC- aria under ROC curve

DNA- dezoxiribonucleic acid

DNAds- dezoxiribonucleic acid double stranded

FAMD- mixed data factor analysis

FIB-4- fibrosis-4 index

FOXO- forkhead box transcription factors

GGT-gamma glutamil-transferase

HBsAg- s antigen for Hepatitis B Virus

HBx- X protein for Hepatitis B Virus

HBV-Hepatitis B Virus

HCC- hepatocellular carcinoma

HCV- Hepatitis C Virus

IL- interleukine

MAPK- mitogen activated protein kinase

PT- prothrombine time

PTEN- phosphatase and tensine homologue

RNA- ribonucleic acid

RT-PCR- Real Time-PCR

TGF- Tumour Growth Factor

TP53- tumour protein 53

WHO- World Health Organization

WNT- wingless integration MMTV family

β- beta

Fundamental problem

HBV has distant origins, and this was proven by Suh et al. using tests on prehistoric nucleic acid traces coming from modern birds' ancestors [1]. Despite many preventive measures, WHO draws focus on the lack of efficient viral elimination policies, leading to the need for communication, diagnosis, and treatment improvements [2].

On the other hand, despite the developments of health systems, HBV diagnosis can have limitations in certain situations, such as „occult infection" [3]. Some countries have, according to WHO, a problem in dealing with and treating chronic infection, because of the costs and the lack of therapeutic and diagnostic efficient means [2]. According to researchers' requests inactive carriers should be constantly evaluated for the assessment of viral reactivations, or complications [4]. The spending associated with repeated evaluations cannot be, in WHO's opinion, maintained by every governmental policy. Furthermore, curative treatment is a priority [2]. According to some authors, another problem of the assessment is related to the evaluation of the evolution of fibrosis through liver biopsy, because this is an invasive technique, with the possibility of having important complications [5]. To avoid unpleasantries in the strategy for the diagnosis of fibrosis staging, researchers chose to investigate non-invasive tests without reaching an ideal method [6].

On the other hand, microRNA detection has been developed, because it is of interest to know their participation in the replicative [7] and pathogenic [8] HBV pathways. Furthermore, these molecules might be versatile biomarkers with diagnostic [9] or prognostic roles [10] during several phases of the viral condition [9, 10]. According to some, modern therapies might be developed using microRNA [11]. On the other hand, one has not discovered the participatory pathogenic pathways of the non-coding molecules in the picture of chronic HBV infection and its complications. There are discordant opinions among researchers, some considering that the microRNA expression decreases in a certain pathology [12], while others find that non-coding molecules are stimulated in the same illness [13]. This problem, of the lack of uniformity in different detection protocols, appears from divergent ideas related to the correct normalization in experiments focused on the expression of microRNAs. Some authors underline the importance of internal control in such research because, without the optimal reference, the expression result becomes uncertain [14]. To improve normalizers, one uses several stability assessment techniques, as some

research papers describe [15]. Furthermore, superior RT-PCR usage improves the results of the microRNA analysis [16].

Despite deterrents, studies related to non-coding molecules have flourished in the sense of HBV pathology. Some researchers base themselves on the predictive role of miR-122 in the evaluation of antiviral treatment response in patients with multiple concomitant viral infections [17]. Others bring into discussion the connections between this microRNA and viral loads [18]. On the other hand, miR-146a is also important in viral replication during HBV infection, through regulatory proteins which he influences [19]. Another interesting fact is the microRNA intervention in oncogenic processes during the infection evolution, through interactions with HBx and TGF-β [20]. Furthermore, some authors hope to find curative therapies in chronic viral infections, aiming for some molecules with microRNA help [21].

Therefore, the technology for microRNA exists and it can be improved, and finding the interactions between non-coding molecules and HBV could bring an efficient patient evaluation and treatment.

Concerning Romania, HBV infections persist among people, having still a percentage of undiagnosed persons [22] and a vaccination rate of only 85% amongst children [23]. Until the conception of the present thesis, large studies have not been made on Romanian patients for the understanding of the microRNA involvement in disease mechanisms, or for studying their diagnostic and prognostic potential.

The current paper has complexity, with a theme situated in the meeting point between biomolecular, immunological, and virology disciplines while searching for microRNAs as diagnostic and prognostic elements in chronic HBV infection. The novelty of this thesis is based on an elaborate methodology, built upon theoretical insights, and the first clinical studies on Romanian subjects concerning miR-122, miR-146a, and HBV replication. Therefore, new diagnostic methods microRNA-based have emerged for high viral loads and high ALT values. Furthermore, new studies have been done related to the associations between fibrosis, antiviral treatment, and microRNA in Romanian subjects chronically infected with HBV, thus leading to new prognostic perspectives based on miR-122 and miR-146a. The modern and practical approach has been reflected in the last chapters, where the author has illustrated the first large bioinformatics analysis based on molecular mechanisms between miR-122 and miR-146a, impacting the retrieval of signalizing pathways involved in the immunological activities.

This research brings new study perspectives related to the association of microRNAs with biological markers for establishing prognosis, or with future therapies, based on the associations between non-coding molecules and immune elements.

I. The general part

1. Hepatitis B Virus- a new approach

According to the latest published international reports, HBV deaths persist in the disease evolution picture throughout the world [2]. Official records in Romania show almost a million HBV-infected people [24].

HBV, from Hepadnaviridae, has a characteristic genome with incomplete, circular DNAds [25]. Several factors act on his replication process, amongst which one finds the glycosylation of coating proteins [26-27].

Chronic HBV infection passes through various progression steps and, among the problematic phases, one can mention „the occult infection", because it brings diagnosis difficulties [28]. Immunosuppressed patients belonging to such a phase could experience viral reactivations, so careful monitoring should be performed [29]. Some authors think that a difficult evaluation emerges in patients with non-progressing pathology, as defined following the guidelines [30].

On a molecular level, the researcher's attention is directed at the lymphocytes' depletion, also reflected in many cellular categories [31-32]. This phenomenon has been studied recently by some authors to approach new techniques for defining HCC prognosis [33].

On the other hand, researchers claim that cytokines are the main molecules involved in the inter-cellular communication between cells involved in HBV infection [34]. According to recent data, some viral proteins influence cytokine production, concerning, for instance, IL-6 and IL-10 [35]. Researchers such as Gu et al. underline connections between the interleukine secretion and the HBV replicative cycle [36]. Others promote cytokine usage in establishing prognosis [37].

Regarding the means used in the diagnosis of HBV infection, the literature mentions progress through the discovery of HBsAg quantification techniques [38], superior RT-PCR methods [39], and viral RNA detection tests [40]. On the other hand, combinations between viral loads and specific HBV proteins are under study by some researchers, for the development of prognostic panels [41]. WHO supports rapid tests for diagnosis and prognosis because of economic reasons [2].

Literature data underlines the observed limitations of current antiviral treatment, and researchers choosing to support actions for the development of curative therapies [42].

Candidates for this purpose are, according to some, modern therapies based, for instance, on microRNAs [43], or immunological treatments [44].

2. MicroRNAs- a new approach related to Hepatitis B Virus

MicroRNAs are represented by molecules that do not directly codify proteins, their roles being centred on the influence exerted over messenger RNA [45]. Some researchers believe that microRNAs can travel to the infected body through exosomes produced by various cells [46].

The roles of these non-coding molecules are multiple. For instance, miR-511-5p was studied by Fei et al. in HCC metastasis-promoting processes [47]. According to some, miR-195a-5p is involved in lesional processes through the generation of autophagy signals [48]. Some researchers focused on the study of fibrogenesis in cirrhosis, especially regarding the interaction between miR-541 and TGF-β [49]. According to some researchers, some non-coding molecules show a therapeutic potential, this being the case of the effect of the effect exerted by miR-143-3p on hepatic regeneration [50], or the case of antitumor actions of miR-223-3p [51]. Furthermore, studies have shown that HBV has its microRNAs, and one of these, HBV-miR-3, acts on the activity of the host's immune system [52].

Concerning the associated pathology of chronic HBV infection, one could underline that microRNA studies are under continuous development. A modern approach to this theme has investigated the potential of miR-143 and miR-145 in the development of a non-invasive testing procedure in HCC [53]. On the other hand, according to some studies, miR-30e was associated with HBx in the development of fibrosis [54]. As research shows, some microRNAs, such as miR-17-92, relate to HBV replication [55]. On the other hand, some authors show the involvement of microRNAs in the development of HCC, through the interaction with cytokines such as TGF-β [56].

The elements mentioned above are theoretical focus points proven by researchers while studying connections made between microRNAs and HBV. In the conception of this doctoral thesis, there were multiple opinions related to the concept of non-coding molecular study for the understanding of the pathogenic mechanisms in chronic HBV infection, but one could not define precisely the molecular pathways. Because of the large volume of information obtained throughout the years, researchers have created bioinformatics analysis platforms meant to process data for the creation of molecular interaction maps in chronic HBV infection. One of these platforms, which is under continuous and recent development, has signalized connections between miR-122, miR-146a, and other microRNAs, in the course of the chronic infection [57-58].

II. Personal contributions

3. Work hypothesis and general objectives

The main theme of this thesis was connected to the investigation of the diagnostic and prognostic potential of miR-122 and miR-146a in chronic HBV infection. The scientific effort was concentrated on fulfilling the general objectives focused first on the associations between microRNA and viremia, and then between non-coding molecules and ALT; predictive elements based on the expression of microRNA in fibrosis and treatment were also searched; the mechanisms behind the connections between the currently studied microRNAs were also searched through a large bioinformatics analysis.

The specific objectives were centred on establishing diagnostic patterns based on the expression of miR-122 (in those with high viral loads, and then, with high ALT), establishing the prognostic potential of miR-122, and detecting the connections between the researched non-coding molecules and lymphocytes in chronic HBV infection.

The main questions for this research were:

1. Can one find correlations between viral loads, miR-122, and miR-146a in Romanian patients with chronic HBV infection?
2. Is there any association between ALT, miR-122, and miR-146a in Romanian patients with chronic HBV infection?
3. Could treatment influence the expression of some serologic variables connected with microRNA?
4. Do microRNAs bring changes in the serological values of some classical biomarkers?
5. Could fibrosis be diagnosed with a non-invasive test based on microRNA?
6. Are there molecular connections between cytokines, miR-122, and miR-146a in Romanians with chronic HBV infection?

To answer all these, several secondary work hypotheses were made, assuming that the connection between miR-122, miR-146a, and viremia, or ALT was true and that it had a possible effect on interactions based on the alterations of some proteins. Furthermore, treatment and fibrosis were considered influencers of the serological characteristics of positive HBV patients, and, consequently, on the expression of non-coding molecules. Furthermore, the hypothesis of a predictive potential of microRNAs was launched, related to the evolution of biochemical and haematological serological tests.

4. General research methodology

4.1. Establishing the studies carried for the doctoral thesis

Carrying studies for the completion of the doctoral thesis was performed in stages, then all the findings were reunited in the complex picture of this current work. An element that gives weight to this research was the large volume of contained information, obtained through published meta-analyses and systematic reviews. Therefore, one of the published papers, based on the rigorous control of over 58000 articles, found a connection between IL-8, IL-9, IL-10 and viral loads in chronic HBV infection [59]. This work represented the basis for the conclusions of the bioinformatic analysis.

On the other hand, in another meta-analysis, viral loads were correlated with variations in the expression of microRNAs and the end result was centred on miR-122 [60]. This was the starting point for the idea of the chapters related to the viral replicative cycle and this microRNA. Furthermore, the author of this thesis conceived the first in vivo pilot study, from Romania, connected to the connection between microRNA and viremia [61], and continued with a validation study in this study. Another article illustrated after-therapy connections between miR-122 and lymphocytes [62], and it was continued, in the doctoral thesis, with a prognostic study based on the same microRNA and both treated and not treated patient lots. General knowledge related to the associations between fibrosis-biochemical tests was improved through a third article containing both a meta-analysis based on the variations of GGT and ALT and a pilot study of a new non-invasive test for the identification of important fibrosis [63]. The hereby obtained results led to the elaboration of a validation study, to verify the obtained data on a larger patient lot, and to develop new prognostic ideas.

4.2. Details related to the included subjects

103 chronically HBV-infected patients and 21 healthy individuals were included in this research, following the Informed Consent and the approval of the Ethical Council of Fundeni Clinical Institute, Bucharest (approval paper 46274 from 6.07.2021). All subjects had to be infection-free (this meant any other infection apart from HBV). Every patient had to be over 18 years old. No pregnant or breastfeeding persons were included.

4.3. Biological samples collection and processing

Venous blood samples were used, processed and stored separately (at -80°C). The biochemical and hematological tests were made on Versacell V2- Dimension RXL and SYSMEX-XN-1000-5 machines. ADVIA CENTAUR XPT detected viral antigens and anti-HBV antibodies, and BOSPHORE HBV QUANTIFICATION KIT established the levels of viral load. The producers of the used machines were Siemens Healthineers, Erlangen, Germany and Anatolia geneworks, Istanbul, Turkey (for viral loads). **Tabel IV.1.** shows the work principles of the used machines.

Table IV.1. The main laboratory analysis performed

Test	Machine	Detection method
Transaminases	VersaCell V2-Dimension RXL	Spectrophotometric method [64-65]
GGT	VersaCell V2-Dimension RXL	Spectrophotometric method [66]
Total bilirubin	VersaCell V2-Dimension RXL	Spectrophotometric method [67]
Hematological values	Sysmex-XN-1000-01	Flow-cytometry with fluorescence [68]
PT	Sysmex-CS-2500	Coagulometric method, chromogen [69]
Antigen and viral antibody detection	Advia Centaur XPT	Chemiluminescent method, sandwich-based [70-73]
Viral load quantification	BOSPHORE HBV QUANTIFICATION KIT	TaqMan multiplex RT-PCR [74]

* GGT-gamma-glutamil-transferase, PT-prothrombin time, RT-PCR- real-time PCR.

MicroRNA detection was based on Thermo Fisher kits, using total ARN extracted from 100µL of serum sample, followed by a series of steps and ending with the binding of total ARN on magnetic beads. The reverse-transcription cycles and miRNA quantification were done using an Applied Biosystems 7300 machine (Thermo Fisher Scientific, San Francisco, California, United States of America) with an RT-PCR technique. MiR-21 was the used internal control, its selection being based on its greatest stability, according to RefFinder

[75]. The $2^{-\Delta\Delta CT}$ method was chosen for microRNA expression computation, depending on the amplification of the internal control. The selection of the microRNAs used was made after documented data (such as [17-19]). The producer's site gave a great part of the necessary information for the selection of the internal control [76].

4.4. Statistical tools

Every scientific work was performed after estimating the necessary patient number using Open Epi [77]. Then the statistical scientific analysis tool was represented by R 4.2.2 [78]. After assuring data normality, Pearson correlations were performed, together with comparisons calculated by Mann-Whitney or Kruskal-Wallis methods. Patient characteristics were evaluated in detail using FAMD, and the connections between variables were observed using mediation and JSmediation packages. Youden's method was the basis for cut-off value calculations. Diagnostic curves needed ROC curves. The research paper included advanced statistical techniques based on regression analyses and subdistribution risk methods derived from Cox analysis [79]. Setting statistical test significance meant a *p* score< 0,05.

5. Purpose 1. Connections between miRNAs and viremia

5.1. Introduction

The chapter aimed to validate preliminary studies related to the association of microRNA-HBV replication in Romanian patients with chronic infection (a new diagnostic formula for high viral loads was discovered). A mediation analysis was performed to explore potential links between miR-122-viral load, this analysis being the first of its kind in Romania.

5.2. Materials and method

This part of the doctoral study followed the general methodology previously described, and it included 57 subjects (chronically infected with HBV, chosen in order, by admission time, during 2021-2023) and 11 healthy individuals.

5.3. Results

Patients were classified into two categories after viral loads (subjects with a viral load above 2000 IU/mL and individuals below this value). Viral loads were selected using literature benchmarks [80]. 57 individuals were compared with 11 healthy subjects, obtaining a stimulated expression of both microRNAs. Both sexes were represented. Among microRNAs, only miR-122 varied significantly between groups with different viremia (p= 0.002).

The diagnostic pattern identified in the preliminary pilot study (see Chapter 4 of the synopsis and the PhD thesis for details) was found to be statistically related to a high viral load. The AUC of this test was 0.778, placing it in the category of useful methods for viral load detection. MiR-122 was significantly correlated with viral load, and a relationship between the non-coding molecule, age and HBV-DNA value was observed during mediation analysis.

5.4. Discussions

The main goal, related to the connections between microRNAs and viral loads, was fulfilled by miR-122, which, although it has been previously associated with the viral load [81], its association with the level of HBV-DNA has not been investigated before in Romanian patients with chronic HBV infection.

The mediation phenomenon between miR-122 and age was explained by the link between this non-coding molecule and aging [82]. The miR-122-age-viral load connection was discovered for the first time in this study.

The main limitation of the research was related to the absence of participant groups, formed according to the stage of HBV infection, and this was due to the small number of subjects. However, the complexity of the statistical computations performed, as well as the rigor of several types of regressions used, led to valuable results, unique so far, with the potential to promote new rapid monitoring tests, performed at the patient's bedside. Thus, the associations found could pave the way for new future studies related to the interaction between the risk factors encountered in chronic HBV-infected patients.

6. Purpose 2. Connections between miR-122 and ALT

6.1. Introduction

This chapter investigated the connections between miR-122, miR-146a and ALT, while being the first work with this purpose in Romania. The novelty is based on a high-accurate, discovered test formula, for establishing high ALT values. The research is important for the understanding of the progress towards complications (arising from liver injury) and for the promotion of new studies related to the molecular aspects involved in the evolution of transaminase variations.

6.2. Materials and method

68 patients were divided into two categories, according to the ALT value, and then comparisons were made between them and 11 healthy subjects. The choice of the transaminase cut-off level was performed according to current guidelines [80]. The rest of the methodology, as well as the statistical computation, was carried out as described in chapter 2 of the synopsis.

6.3. Results

It was observed that the expression of miR-122 was higher in subjects with high transaminases by comparison to others (p= 0.0004).

After univariate and multivariate regression tests, it was emphasized that the variables sex, AST, GGT, PT and the expression of miR-122 might be potential candidates for diagnostic tests performed for high viral load identification. Thus, four diagnostic models were outlined, of which the one based on the association between miR-122, AST and GGT had the best profile, with a sensitivity of 100% and a specificity of 82.2%.

6.4. Discussions

The existence of associations between microRNAs and transaminases has been mentioned before in scientific documents [83]. ALT value evaluations are important, especially for disease staging [80] or for patient prognosis [84]. The model assay discovered, based on the expression of miR-122, is useful in detecting high ALT values, and it is the first

one with this formula in Romania. The main limitation of the study was due to the absence of patient group partitions (performed after the evolutive stages of chronic HBV infection), and this was due to the small number of participants. However, the rigorous use of the highly developed statistical computations helped in achieving the purpose of this chapter, by finding a valid, high-accurate, diagnostic test. It would be possible to use this assay for the conception of future point-of-care analyses, focused on molecular mechanisms, which might be effective in monitoring.

7. Purpose 3. MiRNA implications in prognosis

7.1. Introduction

This chapter aimed to evaluate the predictive effects of the researched microRNAs (as a continuation work of the pilot study related to the impact of antiviral therapy - see details in chapter 4). Moreover, this chapter pursued the prognostic capabilities of miR-122 and miR-146a related to the decrease of ALT and viral load. This is the first study of its kind in Romania, which succeeds in identifying connections between the in vivo, time-dependent variations of the expression of miR-146a and the number of lymphocytes. Furthermore, this is also the first research to demonstrate the predictive potential of the diagnostic models illustrated in previous chapters.

The implications of the findings are important in future studies aimed at conceiving new prognostic tests based on the patterns identified in this thesis. Moreover, associations with immunological processes are outlined, and these might be the basis of further discoveries focused on miR-146a, in Romanian patients chronically infected with HBV.

7.2. Materials and method

The methodology of this study followed the pattern previously described in Chapter 4. It should be emphasized, however, that advanced statistical analyses, such as FAMD, linear regressions, as well as subdistribution models derived from the Cox method (the latter being important in prediction) were used [79]). Statistical significance was chosen for p values below 0.05.

7.3. Results

41 chronically infected HBV-positive individuals, some treated and some untreated with antiviral agents (interferon derivatives or nucleoside/nucleotide analogs) were included. The FAMD plots showed that the main elements that could predict the group distribution of subjects, by common characteristics, were the previously identified diagnostic patterns (in Chapters 5 and 6), the expression of miR-122 and the variation in time of ALT levels. Furthermore, for the construction of predictive models to assess the characteristics of the treated patients, one would need the variations in the number of lymphocytes and leukocytes and the expression values of microRNAs (miR-122 and miR-146), associated with sex and age.

The temporal projections built on one hand, using the diagnostic models found in the previous Chapters, and on the other hand, with the expressions of miR-122, illustrated a series of newly identified aspects among Romanians chronically infected with HBV. Thus, higher, as opposed to lower, miR-122 values had a higher probability of predicting decreases in both ALT level and viral loads (at both six months and one year). The diagnostic model found in Chapter 6 (linked to high ALT) had a better predictive ability than miR-122 alone in establishing the decline of viral loads.

In a smaller group of HBV-positive patients (9 subjects) a statistically significant, negative correlation was found between the variation of miR-146a over time and the basal level of patient lymphocytes (r= -0.7).

7.4. Discussions

International guidelines emphasize the need for ALT monitoring over time in HBV-positive patients [80]. The presented study confirms the impact of the temporal variations of the ALT level in the characterization of the parameters of the chronically infected. The novelty of the work arises from the fact that miR-122 and the diagnostic models derived from it are emphasized in the general picture of potential prognostic factors of HBV-positive patients.

The impact of lymphocyte variations, along with the expressions of miR-146a and miR-122, are also important for the prognosis of treated patients. These elements were highlighted for the first time. Some authors have illustrated the impact of antiviral therapy on variations in the expressions of microRNAs (such as miR-122 [18] and miR-146 [85]), as well as on the number of lymphocytes [86]. But, to the author's knowledge, all these elements have not been so far studied together, in patients chronically infected with HBV.

Some researchers have outlined in animal studies, certain immunological connections between lymphocytes and miR-146a [87]. For the first time in HBV-positive Romanian patients, the study highlights a correlation between the variation in the expression of miR-146a and the basal number of lymphocytes. Thus, a new research direction is outlined, centred on the explanation of the association between lymphocytes and microRNAs in chronic HBV infection.

Predictive projections illustrated the prognostic potential of the test formula based on the expression of miR-122, AST and GGT in chronically HBV-infected patients. This is another novelty element of the thesis.

Limitations were identified, related to the number of patients and the short period during which the follow-up was performed. Nevertheless, the work is important because, through the complexity of the statistical models, it manages to emphasize new predictive models based on the expression of miR-122 expression, as well as on the associations between the number of lymphocytes and the expression of microRNAs.

8. Purpose 4. Associations between microRNAs and fibrosis degree

8.1. Introduction

This chapter was intended to introduce a new potential test for fibrosis identification in HBV-positive patients. In addition, for the first time in Romania, associations were sought between the degree of fibrosis and the expressions of miR-122 and miR-146a.

The diagnostic algorithm found together with miR-122 had the role of promoting a new perspective, fibrosis-related, over prognostic evaluation related to fibrosis. In this way, future explanatory studies can be developed, which deepen, at the molecular level, the discoveries hereby made.

8.2. Materials and method

The methodology was based on the aspects illustrated in Chapter 4. The evaluation of the diagnostic test for significant fibrosis was performed on 60 HBV-positive patients with chronic infection, selected according to the previously explained criteria (see Chapter 4). In addition, the FIB4 score was used to highlight the degree of fibrosis, and this calculation required the usage of free software [88]. Furthermore, the FibroScan method, based on elastography techniques, was also used to determine liver fibrosis. The threshold for significant fibrosis was F2.

To increase the complexity of the results, the FAMD method was again used, and the correlation computations were adjusted by Holm (statistical significance was established at $p< 0.05$).

8.3. Results

The new test for detecting the degree of fibrosis (based on a combination of GGT and ALT values) was statistically significantly higher in patients with significant fibrosis (as determined by the FibroScan method), by comparison to others. There was also a significant correlation between this new diagnostic model and FIB4 values ($r= 0.42$).

After FAMD graph analysis, it was established that, in the case of the 41 patients presented in Chapter 7, the new fibrosis diagnostic test, together with miR-122, lymphocyte and platelet variation, influences the group characteristics of the subjects.

8.4. Discussions

The hereby study confirmed the correlation between the new fibrosis identification test with the values calculated by the FibroScan method and the FIB4 score.

The main limitation of this research was based on the small number of patients with significant fibrosis, which did not allow further tests to evaluate the diagnostic accuracy of the newly discovered model. However, the potential of the discovered test for future usage exists, because the results obtained had a superior statistical precision (given by the usage of highly accurate mathematical correction elements).

Some authors have included miR-122 in diagnostic scores for detecting high-grade fibrosis in HBV-positive patients with chronic infection [89]. The novelty of this work is related to the connections between miR-122 and the newly analysed diagnostic test, which might be used for evaluating the prognosis of chronically HBV-infected subjects.

The present thesis thus establishes new research directions focused on the fibrosis-generating mechanisms involving both miR-122 and the variations of biochemical parameters of interest such as ALT and GGT. Also, a new study idea could be focused on combining the newly discovered diagnostic test with the miR-122 expression values, in the discovery of point-of-care methods for detecting fibrosis.

9. Purpose 5. Bioinformatics analysis

9.1. Introduction

For a complex analysis of molecular connections, bioinformatics methods are needed, because they can give a multidimensional perspective to a large volume of data, using interpretation tools from fields such as physics, mathematics or biology [90]. For microRNAs, bioinformatics software is continuously improving, and increasing performance through sorting algorithms [91].

This chapter aimed to deepen previously obtained results through an extensive bioinformatics analysis, using stored data from four freely available software.

The novelty of the approach arises from the fact that this is the first complex interpretation of its kind of the connections between miR-122 and miR-146a. Furthermore, new perspectives for the interpretation of the microRNA connections in the HBV infection are designed, thus establishing future study ideas.

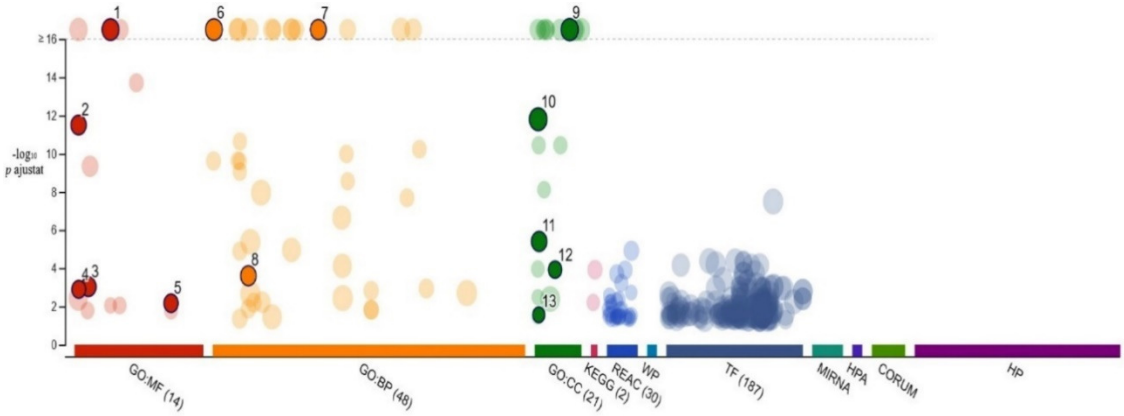
9.2. Materials and method

To carry out the study it was necessary to access four databases: miRWalk databases [92-93], g: Profiler [94-95], UniProt [96-97] and STRING [98-99]. First, the research steps involved the identification of the target genes of miR-122 and miR-146a with the help of miRWalk [92-93], followed by complex expression analyses performed by the g:Profiler algorithms [94-95]. Another step was the identification of the proteins encoded by the studied microRNA's target genes through the UniProt software [96-97]. Finally, STRING helped in creating complex interconnection graphs of the proteins found in the previous step for the understanding of molecular interactions [98-99].

Platforms were selected by performance, all having recent database improvements [93, 95-96, 99]. The decision to use these large databases was also influenced on one hand, by the fact that they presented validated experimental resources [92, 94, 97, 99], and on the other, by the superior statistical models used by automatic analyzers [92, 95-96, 98]. Thus, the provided results had a highly computer-controlled precision, doubled by the possibility of an even more rigorous control through Benjamini-Hochberg-type statistical corrections (p significantly below 0.05) [94, 99]. By combining all these techniques, the level of confidence in the final interrelationship graphs increased.

9.3. Results

8634 gene targets for miR-122 and miR-146a were found by miRWalk search [92–93]. Their functionality was then analysed by g:Profiler [94-95], using complex algorithms to identify over- and under-representations. The results contained a series of molecular pathways in which the retrieved genes took part. Among these, we can mention those associated with antigenic activation, the functionality of the T receptor complex, as well as those involved in the regulation of peptidase activity (see **Figure 9.1.**).



Număr	Sursă	ID	Numele termenului	p ajustat
1	GO:MF	GO:0017069	legarea snRNA	8.072×10^{-80}
2	GO:MF	GO:0003735	constituentul structural al ribozomului	3.138×10^{-12}
3	GO:MF	GO:0004984	activitatea receptorului olfactiv	9.262×10^{-4}
4	GO:MF	GO:0003823	legarea antigenică	1.262×10^{-3}
5	GO:MF	GO:0061134	activitatea reglatorie a peptidazelor	6.753×10^{-3}
6	GO:BP	GO:0000365	legarea mRNA prin spliceozom	4.978×10^{-84}
7	GO:BP	GO:0035195	suprimarea expresiei genelor mediată post-transcriere de miRNA	1.183×10^{-22}
8	GO:BP	GO:0007606	percepția senzorială la stimuli olfactivi	2.468×10^{-4}
9	GO:CC	GO:0097525	complexul spliceozom-snRNA	6.564×10^{-91}
10	GO:CC	GO:0005730	nucleol	1.573×10^{-12}
11	GO:CC	GO:0005840	ribozom	3.933×10^{-6}
12	GO:CC	GO:0042101	complexul receptorului T	1.187×10^{-4}
13	GO:CC	GO:0005791	reticul endoplasmatic rugos	2.733×10^{-2}

Figure 9.1. g:Profiler enrichment analysis of the genes associated with miR-146a and miR-122. Results obtained through the analysis of the g:Profiler platform [92-93] (GO- Gene Ontology; MF- molecular functions; BP- biological processes; CC- cellular components; REAC- abbreviation of the REACTOME database; WP- WikiPathways; TF- TRANSFAC; HPA- Human Protein Atlas; HP- Human Phenotype Ontology).

Then, 367 proteins with validated structures were assigned to previously found genes by UniProt software [96-97]. These participated in the STRING analysis [98-99]. The experimentally proven results of high statistical confidence were connected by a large network with 51 points (see **Figure 9.2.**). The presence of ubiquitination and messenger and

ribosomal RNA translation and regulation pathways has been noticed [100-101].

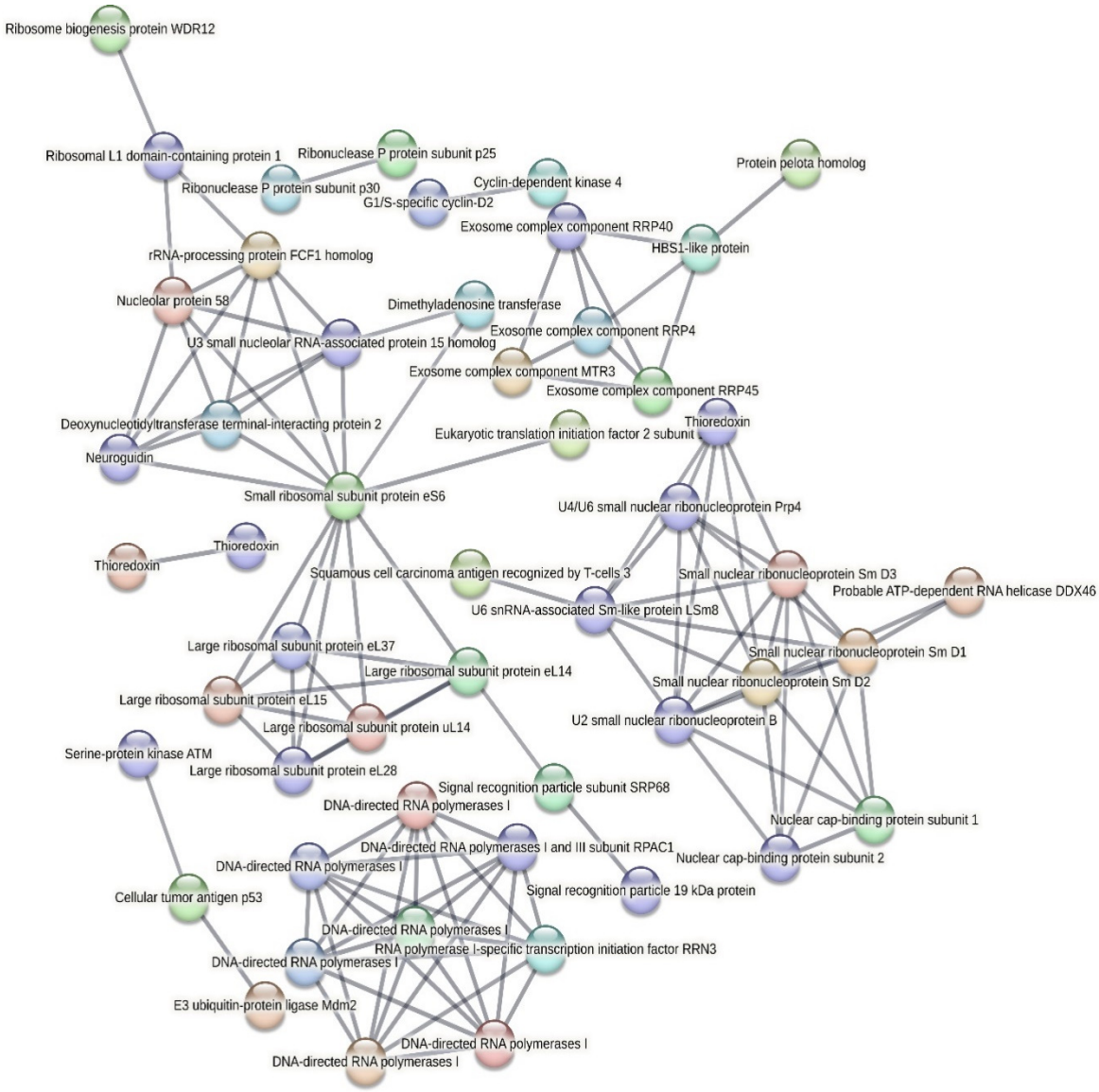


Figure 9.2. The STRING analysis of the proteins associated with genes influenced by miR-146a and miR-122. Results obtained from the STRING platform [98-99].

After enriching the results with information collected from the databases automatically connected to the STRING platform [100-105], the molecular pathways were divided into clusters, the predominant one containing various molecular pathways such as: T cell proliferation in the immune response, miRNA processing [100-101], p53 signaling pathway, FOXO signaling pathway, viral carcinogenesis, cellular senescence, microRNA in cancer [102-104], MAPK6/MAPK4 signaling pathway, viral messenger RNA translation, PTEN

regulation, WNT-independent signaling, NOTCH signaling, molecular pathways associated with infectious diseases [105].

9.4. Discussions

To analyse the obtained results, some theoretical benchmarks should be highlighted. First, according to some authors, ubiquitination can be a method of establishing and maintaining chronic HBV infection in the body, because of its connection to viral replication [106]. Related to the latter is also TP53, according to other scientists [107]. On the other hand, according to published studies, several molecular elements found by protein network analysis are involved in carcinogenic processes, such as PTEN [108], WNT [109] and TP53 [110]. Furthermore, researchers have shown possible involvements for MAPK1 [111] and FOXO1 [112] in the IL-10 production. The latter is studied by some scientists related to lymphocyte production [112].

Therefore, combining information from preliminary studies (see Chapter 4) as well as from previous chapters and because all the above-mentioned molecules are involved in the results obtained on bioinformatics platforms, it was decided that the analysis of proteins related to miR-122 and miR-146a might bring new insights into the picture of interactions in chronic HBV infection. Thus, the study brings novelty, by issuing the possibility of interactions between ubiquitination processes, TP53 and miR-122, respectively miR-146a in HBV replication. Moreover, related to the influence of microRNAs on the cell cycle, previous research started during the PhD work (see Chapter 4) illustrated the influence of IL-10 in viral replicative processes. Considering what this bioinformatics analysis shows, one can bring the novelty of the possible association between miR-122, miR-146a, lymphocytes and IL-10 in immunological processes during chronic HBV infection, some even related to HBV replication. Finally, the study promotes the novelty of the connections between miR-122, miR-146a, PTEN, WNT and TP53 in HBV-associated carcinogenesis. Following the research undertaken in the previous chapters, it can be hypothesized that these pathways could be related to fibrogenic processes or the increase of transaminases, considering that some associate fibrosis with the occurrence of cancer [41], as well as the increase of ALT [113]. Although there were limitations related to the use of in vitro studies (without in vivo results) in the accessed databases, the complexity of the statistical apparatus helped to surpass them.

Therefore, the present research emphasizes, for the first time, the possibility of molecular pathways that could explain previously proven results. All this converges towards the trend

of conducting future studies for the development of microRNA-based, anti-HBV therapies.

10. Conclusions and personal contributions

10.1. Conclusions

1. The work achieves an important objective related to the validation of some associations between miR-122 and viremia, in patients with chronic HBV infection (see chapter 5). The last chapter, of the bioinformatics analysis, reinforces the results obtained in vivo (see chapter 9), thus achieving yet another goal of the research. The demonstrations made have practical applicability, as they can substantiate the development of point-of-care tests related to miR-122 expression. Their benefit would be a financial one, but also one related to the speed of the response. There is also potential for future studies to investigate other microRNAs associated with viral load, possibly connected to miR-122.

2. The study illustrated in Chapter 5 also shows the utility of a diagnostic model based on miR-122 and age for high viral load detection. This test is the only one of its kind till now. This is a novelty of the research for its usefulness in the development of future diagnostic and monitoring methods, based on microRNA-type molecular elements.

3. Another important goal achieved was to find a high ALT detection model using the expression of miR-122. This is the first such model, designed after observations conducted on Romanian subjects. Its utility stands out, as a test as such, with high accuracy, could be a starting point for non-invasive strategies for HBV-positive patient evaluations. These would give a faster and more accurate result on the events leading to complex mechanisms in liver injury.

4. Predictions based on the expression of microRNA led to another goal of the study (see Chapter 7). They have applicability in the more complex evaluation of the immunopathological processes involved in the evolution of chronic HBV infection.

5. A novelty for Romanian patients with HBV infection appeared through the predictive potential of miR-146a related to the quantification of serum lymphocytes (see Chapter 7). The bioinformatics analysis in Chapter 9 reinforces the results. The importance of such findings could be reflected in the discovery of antiviral therapies with an immunological basis, which might bring long-term benefits by contributing to the eradication of the infection. Future studies can be conducted based on the results of this thesis.

6. Treated patients were characterized by associations between lymphocyte count variation over time, miR-122 expression, and miR-146a expression (see Chapter 7). This is a novelty of the work, with applicability in improving existing antiviral therapies, through an in-depth

understanding of molecular mechanisms.

7. The newly found fibrosis test correlates with the expression of miR-122 (See Chapter 8). Thus, new methods can be developed to monitor the evolution of patients, by understanding the molecular mechanisms as a base for fibrosis.

8. Immunological associations are drawn (see Chapter 9), through which previously obtained results are explained. Thus, the thesis acquires depth, by the fact that highly developed statistical analyses can lead to the validation of some hypotheses drawn at the beginning. Therefore, the applicability of the discoveries made is visible.

9. All the objectives of the paper were fulfilled, and thus new research directions with impact in practice are promoted.

10.2. Personal contributions

The whole work is under the sign of novelty in the research field related to HBV infection in Romanians.

The diagnostic models designed for the first time (see Chapter 5, Subchapter 5.3. and Chapter 6, Subchapter 6.3.) have been verified by developed and complicated statistical methods, which raises their level of confidence.

The concept of the mediation analysis between miR-122, age and viral load (see Chapter 5, Subchapter 5.3.) is an innovative one, that paves the way for new explanations of the mechanisms involved in chronic infection. Thus, novel antiviral therapies as well as ways of evaluation, conducted after microRNA expression, might be envisioned. The strengthening of the concepts drawn from the results obtained in vivo through the carefully statistically controlled bioinformatics analysis (Chapter 9, Subchapters 9.3., 9.4.) brings yet another argument to trust the results.

The prognostic potential of miR-122 and miR-146a (see Chapter 7, Subchapter 7.3.) brings the evaluation of Romanian patients to another level, suggesting a possible modernization of the already existing techniques. The perspective of microRNAs in the patient's evaluation is of interest because they could introduce a new vision of pathogenic mechanisms, bringing to full sight the possibility of controlling their evolution in detail. This might bring benefits over time, as it could lead to the development of non-invasive techniques and increase their accuracy. Thus, the novelty of the association between miR-122 and a new fibrosis test (Chapter 8, Subchapter 8.3.) is useful to understand the

occurrence of CHC, on one hand, but also in starting studies related to the improvement of non-invasive tests, on the other hand.

The connection between microRNAs, lymphocytes, cytokines and viral loads is another novelty, and this is the first such work to emphasize such a finding by corroborating the results previously obtained with bioinformatics analysis (Chapter 9, Subchapter 9.3.). In the present thesis, the first thorough approach to the connections between miR-122 and miR-146a was achieved through a carefully controlled statistical technique. Thus, the discovery gained confidence, being able to be the basis for understanding some immunological elements in the evolution of the disease. The utility of such scientific connections could be realized through the development of new, curative anti-HBV therapies.

All the initial steps carried out in the doctoral thesis (see Chapter 4, Subchapter 4.1.) also brought new scientific contributions, and they were published in ISI journals.

The work entirely is, in conclusion, a collection of new complex elements that have applicability in the development of diagnostic, prognostic, and therapeutic strategies in chronic HBV infection.

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List of scientific work related to this thesis and published *in extenso*

- 1. **Manea M**, Mărunțelu I, Constantinescu I. Extended Analysis on Peripheral Blood Cytokines Correlated with Hepatitis B Virus Viral Load in Chronically Infected Patients- A Systematic Review and Meta-Analysis. *Frontiers in Medicine*. 2024; 11. doi: 10.3389/fmed.2024.1429926 (mentioned in Chapter 4, used as scientific basis for Chapter 9)
- 2. **Manea M**, Mărunțelu I, Constantinescu I. A New Assessment of Two Transferase-Based Liver Enzymes in Low- and High-Fibrosis Patients Chronically Infected with Hepatitis B Virus: A Meta-Analysis and Pilot Study. *Journal of Clinical Medicine*. 2024; 13:3903. doi: 10.3390/jcm13133903 (mentioned in Chapter 4, used as scientific basis for Chapter 8)
- 3. **Manea M**, Apostol D, Constantinescu I. A MicroRNA-Based Method for High-Viremia Detection—A New Approach on a Romanian Lot of Chronically Infected Patients with Hepatitis B Virus. *Diagnostics*. 2023; 13:3425. doi: 10.3390/diagnostics13223425 (mentioned in Chapter 4, used as scientific base for Chapters 5 and 7)
- 4. **Manea M**, Apostol D, Constantinescu I. The Connection between MiR-122 and Lymphocytes in Patients Receiving Treatment for Chronic Hepatitis B Virus Infection. *Microorganisms*. 2023; 11:2731. doi: 10.3390/microorganisms11112731 (mentioned in Chapter 4, used as scientific base for Chapter 7)
- 5. **Manea M**, Mărunțelu I, Constantinescu I. An In-Depth Approach to the Associations between MicroRNAs and Viral Load in Patients with Chronic Hepatitis B—A Systematic Review and Meta-Analysis. *International Journal of Molecular Sciences*. 2024; 25:8410. doi: 10.3390/ijms25158410 (mentioned in Chapter 4, used as scientific base for Chapters 5 and 9)