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TESI DI LAUREA

PATHOLOGICAL ROLE OF MIR-21 IN HUMAN HEPATOCYTES OF OBESE PATIENTS

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ABSTRACT

Background

MicroRNAs, molecules involved in gene expression, are frequently deregulated in many human diseases and cancers. In recent years, many studies have shown a deregulation of these molecules in chronic liver diseases and hepatocellular carcinoma, suggesting a possible diagnostic and prognostic role. One of the most expressed microRNAs in chronic liver diseases is miR-21.

Purpose

The aim of the project is to understand the impact of miR-21 modulation on hepatic triglycerides during diabesity.
In particular our team purpose is to understand molecular mechanisms underlying the pathogenic role of microRNA-21 in hepatocytes of obese patients.
It includes three steps
1) Identify the direct target RNAs of mir21 by using the iCLIP2-seq technique and RNA-seq.
2) Validate in cells the most promising, and those releted to liver metabolism, dysregulation target RNAs of miR21 by several biochemical and molecular techniques, such as:

- western Blot
- qPCR
- Gene Reporter Assay

3) Investigate in cells the physiological and pathological role of miR-21 targets by performing a global bioinformatic analysis, such as IPA analysis.

Methods

Regarding the first step, we transfected the HepG2, Aml-12 and primary cells with the AS of miR-21 to obtain a knock-down.
Then we extracted the RNA from the cell lines to do the Northern Blot to see the results of the knock-down.
Instead to identify the direct targets of miR-21 we used the iCLIP2-seq technique.
Before doing the first test, we performed the RIP on the aml-12 and hepG2 cell lines to see if the chosen antibodies worked for Ago2 precipitation, as RIP is an important step of the iCLIP.

Results

From the Northern Blot results we have seen that the knock-down of miR-21 worked in HepG2, Aml12 and primary cell.
From the RNA immunoprecipitation we have seen that the antibodies chosen are working.

1.Introduction

1.1 MicroRNAs

1.1.1 The canonical microRNA biogenesis pathway

MicroRNAs (miRNAs) are small, non-coding RNAs that post-transcriptionally regulate gene expression by binding to specific 3′ untranslated regions (3′ UTRs) of target mRNAs and promoting their degradation and/or translational inhibition. miRNAs are predicted to target over 50% of all human protein-coding genes post-transcriptionally, enabling their regulatory roles in physiological and pathological processes. [4]

They have evolutionary preserved sequences about 18-25 nt long. miRNAs were discovered in 1993 by Lee and colleagues in the nematode *Caenorhabditis elegans*. In these organisms, the downregulation of LIN-14 protein was found to be essential for the progression from the primary larval stage (L1) to L2. Furthermore, the downregulation of LIN-14 was found to be dependent on the transcription of a second gene called lin-4. Curiously, the transcribed lin-4 was not translated into a biologically active protein. Instead, it gave rise to 2 small RNAs approximately 21 and 61 nucleotides in length. The longer sequence had a stem-loop structure and served as a precursor for the shorter RNA. The smaller RNA had antisense complementarity to multiple sites in the 3′ UTR of lin-14 mRNA. The binding between these complementary regions reduced LIN-14 protein expression while not inflicting any important modification in its mRNA levels. These studies together brought forth a model wherein base pairing occurred between multiple lin-4 small RNAs to the complementary sites in the 3′ UTR of lin-14 mRNA, thereby causing translational repression of lin-14 and subsequent progression from L1 to L2 during *C. elegans* development. [5]

In 2000, separate teams discovered that a small RNA, let-7, was essential for the development of a later larval stage to adult in *C. elegans*. Loss of let-7 gene activity causes reiteration of larval cell fates during the adult stage, whereas increased let-7 gene dosage causes precocious expression of adult fates throughout larval stages. [6]

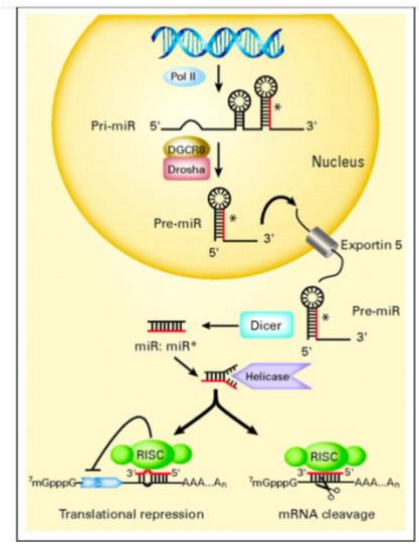


Figure 1. microRNA biogenesis[1]

Afterwards, hundreds of other short RNAs, characterized by highly conserved sequences, were found in worms, flies, plants and mammals, and they were called microRNAs (miRNAs). The human miRNA family comprises 1733 mature miRNAs.

Typically, miRNA-encoding genes are transcribed by RNA Polymerase II. miRNAs are genomically encoded and transcribed as individual genes or as clusters of several miRNA genes under control of the same promoter. Transcription of miRNA genes produces primary microRNA (pri-miRNA) transcripts. Within the nucleus, pri-miRNA transcripts are processed by the Microprocessor complex to form individual stem-loop miRNA precursors (pre-miRNAs). That are typically 70 nucleotides long. The Microprocessor complex comprises the RNAse

III- type endonuclease Drosha and its partner protein DGCR8. Processed pre- miRNA molecules are then exported from the nucleus via Exportin-5 (XPO5). The pre-miRNA is further processed by a second RNAse III-type endonuclease called Dicer after it enters the cytoplasm. Dicer cleaves the loop structure to release a miRNA duplex. The miRNA duplex is composed of two RNA strands bound together by Watson-Crick base pairing. Each strand of the miRNA duplex in animals is typically 21–22 nucleotides long. The miRNA-Induced Silencing Complex is created by loading the miRNA duplex onto an Argonaute protein. Based on sequence complementarity between the miRNA and the target mRNA's 3'UTR, one strand of the miRNA duplex is specifically anchored onto the Argonaute protein and determines the specificity of the miRISC. The miRNA may originate from the 5' side of the pre-miRNA or the 3' side of the pre-miRNA. While either strand can be chosen for miRISC loading, this rarely happens because of the dominance of one miRNA strand.

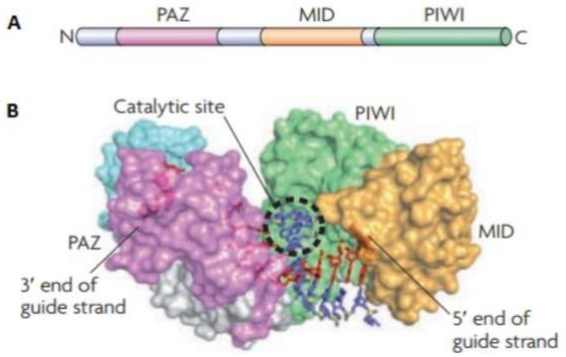


Figure 2 argonaute protein domains[3]

The Argonaute protein releases the other strand of the miRNA duplex, which is then broken down. To control gene expression, Argonaute proteins are sometimes loaded with functional mature miRNAs that are produced by both arms of the duplex. [6]

In both humans and mice, four functional Argonaute proteins (AGO1-4) have been identified that have a significant amount of structural similarity. It's interesting to note that, in contrast to the other AGOs, only AGO2 is strongly expressed during early development in humans and mice. The most heavily researched endonuclease is AGO2. AGO1 and AGO3 lack AGO2's endonucleolytic cleavage activity. AGO4 also lacks this activity.

AGO2 is therefore essential for early development both in vivo and in vitro. Although the four human Argonaute proteins share a lot of structural similarities, their functional domains only comprise a small number of non-conserved amino acids. The N-terminal domain (N), the PIWI/Argonaute/Zwille (PAZ) domain, the MID domain, and the P-element-induced whimpy tested (PIWI) domain are the four conserved domains of the Ago proteins.

The MID domain binds the 5′ phosphate of guide RNAs, whereas the PAZ domain anchors the 3′ end of the RNA. The four AGOs share a lot of similarities in these categories. [7]

In general, the so-called seed region at the 5′ end of the miRNA, which consists of nucleotides 2–7, is essential for canonical target binding specificity. As a result, seed-matching is frequently used to predict miRNA targets. The actual interactions of miRNAs with their targets, however, can depend on the cell type and be regulated by RNA-binding proteins.

The effect is also heavily influenced by the region of the miRNA when it binds to the target mRNA. For instance, miRNAs are more likely to silence their mRNA target when they interact with mRNAs close to the poly-A tail.

Unexpectedly, certain interactions with mRNAs can destabilize some miRNAs. These transcripts have sequences that are almost exactly centered matches with miRNAs. Destabilization of the miRNA's 3′ end and miRNA-unloading from AGO are the outcomes of this kind of interaction. Target-directed miRNA degradation is another name for this miRNA post-transcriptional regulation (TDMD). The miRNA 3′-end can be altered by unidentified enzymes because of the geometry of the AGO2 central cleft and the centered mismatches in the miRNA targets. These alterations cause 3′-end remodeling, which ultimately causes miRNAs to decay.[8]

1.1.2 Non-canonical miRNAs

Some miRNAs have been identified that are generated by a different biogenesis pathway and are called non-canonical miRNAs.

- miRtrons

  MiRtrons are non-canonical pri-miRNAs that are encoded in coding gene introns. Like conventional introns, all miRtrons undergo initial processing by the nuclear splicing machinery before forming stable hairpins with a shorter stem than canonical pri-miRNAs. These shorter hairpin structures must instead be lariat-debranched by the debranching enzyme 1 (DBR1) as DROSHA/DGCR8 cannot handle them. Pre-miRNAs produced by miRtrons are bound by XPO-5, moved to the cytoplasm, and then cut by DICER. [9]

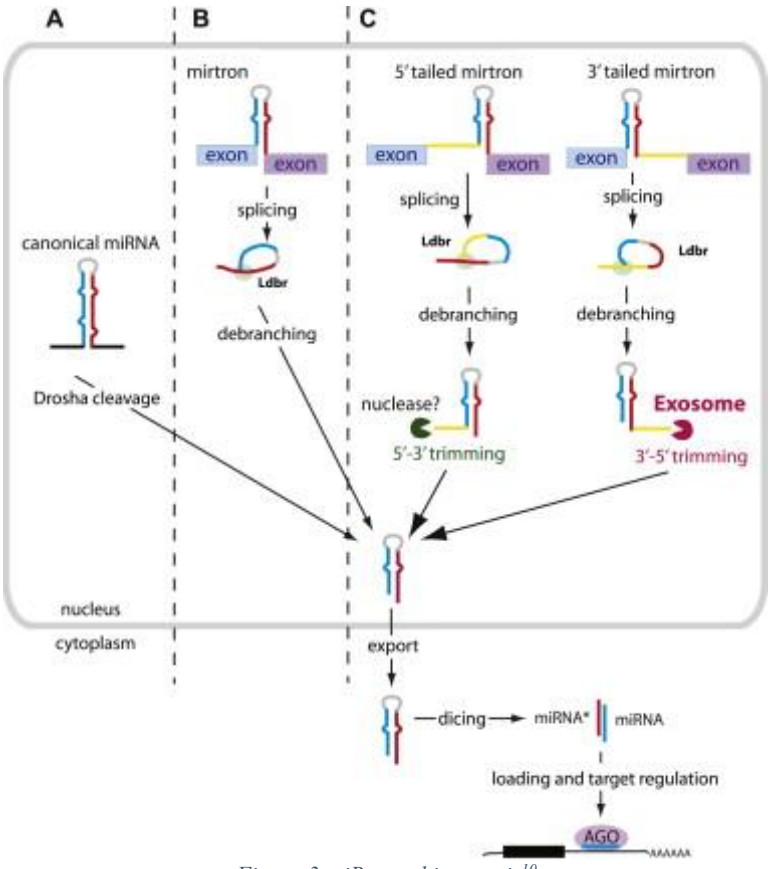


Figure 3 miRtrons biogenesis[10]

- Dicer-Independent miRNAs

Pre-miR-451 is the sole miRNA whose stem-loop shape is too short for DICER to cleave; as a result, miRNA maturation depends on AGO2 slicer activity. AGO2-mediated processing of pre-miR-451 and subsequent RISC loading are determined by the length of the stem-loop structure, the incorrect base pairing in the stem, and the low GC content in the distal stem. [11]

- snoRNA-Derived miRNAs

Small nucleolar RNAs (snoRNAs), which range in size from 60 to 300 nt, are a subclass of non-coding RNAs. Recent research demonstrates that snoRNAs regulate gene expression in a variety of ways, including by altering ribosomal RNA to regulate the ribosome (rRNA). The 2′-O-methyltransferase Fibrillarin is directed to certain rRNA sites by snoRNAs, and Dyskerin is directed to rRNAs that pseudouridylate RNA substrates by snoRNAs. These are the roles of snoRNAs that are best understood. [12]

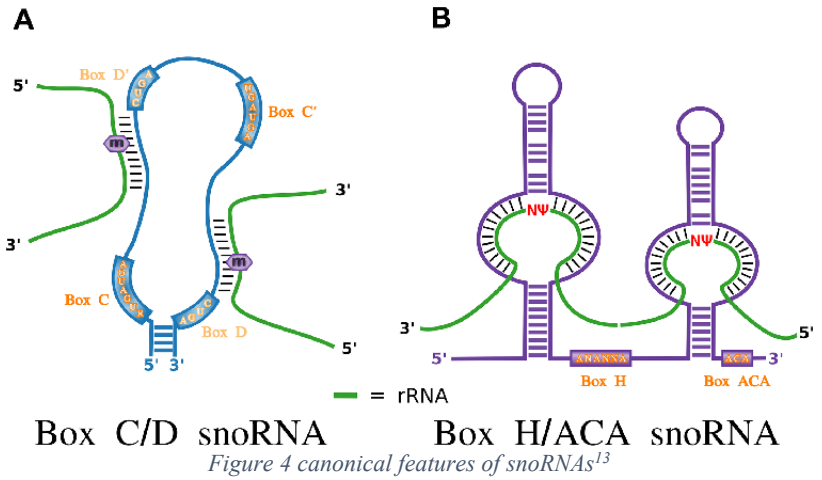


Figure 4 canonical features of snoRNAs[13]

- tRNA-Derived miRNAs

Although numerous and plentiful short RNAs may be produced from tRNA according to sequencing studies of many model systems, the purpose of these molecules is yet unknown. One of these tRNA-derived fragments, known as CU1276, was cloned from mature human B cells and has been shown to have the properties of a microRNA, including DICER1-dependent biogenesis, physical association with Argonaute proteins, and the capacity to repress mRNA transcripts in a sequence-specific manner. This tRNA-derived microRNA, which is down-regulated in B cell lymphom, specifically regulates proliferation and the DNA damage response. These studies define a class of genetic entities with potentially significant

biological significance by showing that functionally active microRNAs can be produced from tRNA.[14]

1.1.3 Mechanism of action

mRNA metabolism depends on RBPs, which control splicing, transport, translation, and destruction. The K-homology domain (KH), RNA recognition motif (RRM), Zinc finger domain (ZNF), Pumilio homology domain (PUM), cold shock domain (CSD), double stranded RNAbinding domain (dsRBD), and other RNA binding domains (RBDs) are examples of RBDs that RBPs use to bind RNAs.
They can distinguish between typical mRNA characteristics like the 3′ poly(A) tail and sequence motifs or secondary structures found in mRNA 3′ UTRs. [15]
Only in collaboration with Argonaute proteins (AGOs) can miRNAs attach to their intended target mRNAs; after loading mature miRNAs into AGO, the passenger strand is released, and the guide strand leads the AGO complex to complementary sites on mRNAs. Trinucleotide repeat-containing gene 6A–6C (TNRC6A–TNRC6C) and the carbon catabolite repressor 4-negative on TATA (CCR4–NOT) complex, which mediate translational repression, deadenylation, or decapping of target transcripts, are downstream factors that are recruited by all AGOs to silence their target mRNAs. [16]
By matching the target RNAs' sequence to the miRNA stored on AGO, the target RNAs can be identified. Target recognition sequences, or "miRNA responsive elements" (MREs), are precisely referred to as this and are typically found in the 3'-UTR. A region of 6 nucleotides called the MRE is complementary to the miRNA seed sequence.

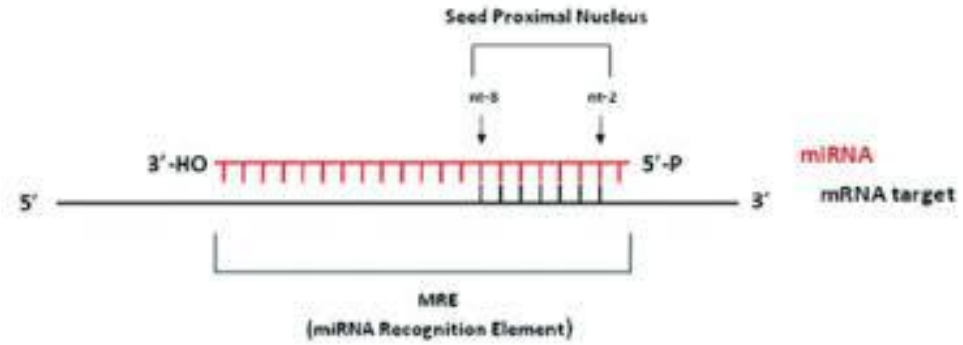


Figure 4 microRNA binfing to the target mRNA[17]

Target recognition may also benefit from the miRNA's 3′ end, and centrally matched targets have been found. Ago2's endonuclease activity can cleave the mRNA target if complementarity occurs in the miRNA's core region (nucleotides 9–11). [18]
Atypical canonical sites, which involve an additional pairing of the miRNA 3'-region containing nucleotides 13–16, can occasionally occur.
Non-canonical sites have also been highlighted in a similar way. These locations feature substantial pairing to the 3' of the miRNA at nucleotides 13–16 and incomplete pairing to the seed region. However, the majority of human miRNA targets do not have this core sequence match and are not immediately cleaved by Ago2. Argonaute is instead drawn to a complex that includes GW182 (TNRC6A/B/C) in the cytoplasmic P bodies, which is where translational repression takes place. The removal of the poly(A) tail and eventual

degradation of the mRNA target are facilitated by the recruitment of the CCR4-NOT deadenylase complex to RISC.
Translational repression, mRNA deadenylation, and decapping are all brought about by the target RNA and miRNA recognizing one another. Gene expression is silenced by miRNA binding to the 5′ UTR and coding areas, whereas transcription is claimed to be induced by miRNA contact with the promoter region.

- MicroRNA-Mediated Gene Silencing via miRISC

A fully complementary miRNA:MRE interaction induces AGO2 endonuclease activity and targets mRNA cleavage. However, this interaction destabilizes the association between AGO and the 3′ end of the miRNA promoting its degradation.[19]
Most miRNA:MRE interactions in animal cells are not entirely complementary (50). Most MREs have at least one central mismatch to their guide miRNA, which inhibits the function of the AGO2 endonuclease. As a result, AGO2 mediates RNA interference in a manner similar to that of the non-endonucleolytic AGO family members (AGO1, 3, and 4 in humans).
PAN2/3 start target mRNA poly(A)-deadenylation, and the CCR4-NOT complex finish it. Effective deadenylation is facilitated by the interaction of the tryptophan (W)-repeats of GW182 with poly(A)-binding protein C (PABPC) (50). Subsequently, decapping takes place facilitated by decapping protein 2 (DCP2) and associated proteins (52), followed by 5′−3′ degradation by exoribonuclease 1 (XRN1).[20]

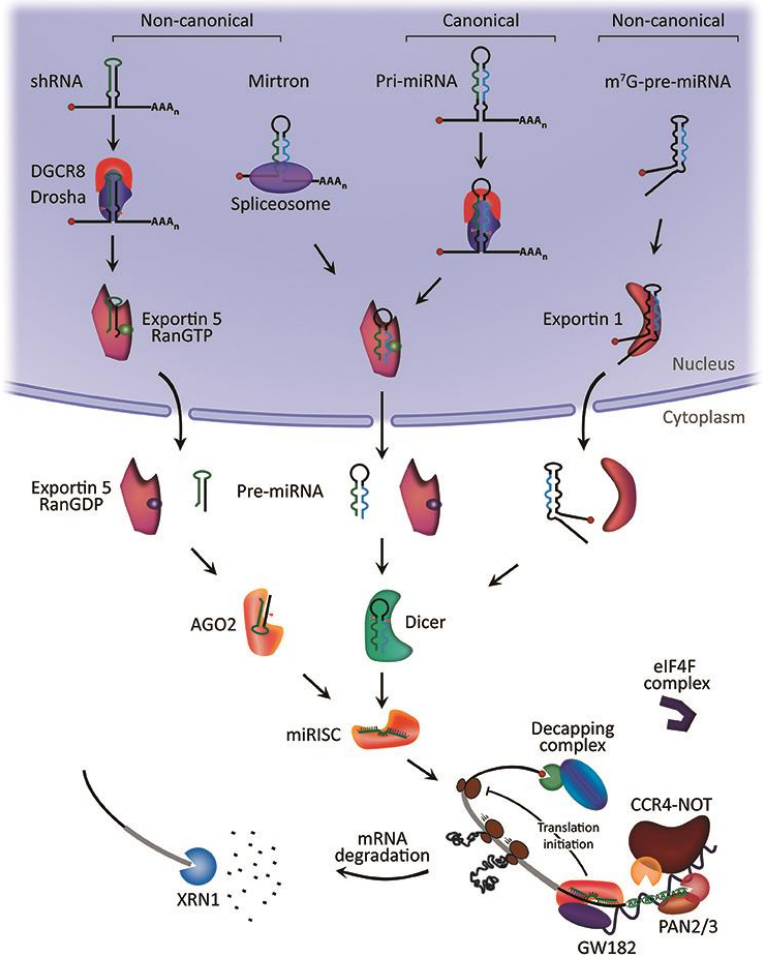


Figure 5 -MicroRNA-Mediated Gene Silencing via miRISC[21]

- MicroRNA-Mediated Translational Activation

AGO2 and another protein connected to the miRNA-protein complex (microRNPs), fragile x-related protein 1 (FXR1), were linked to AU-rich elements (AREs) at the 3 ′ UTR to stimulate translation in serum-starved cells. In order to promote translation during cell cycle arrest, a number of miRNAs, including let-7, were discovered to be linked to AGO2 and FXR1. However, in proliferating cells, these miRNAs restrict translation. GW182 is not required for the miRNA-mediated stimulation of translation; AGO2 and FXR1 are involved. [22]

- MicroRNA-Mediated Transcriptional and Post-transcriptional Gene Regulation Within the Nucleus

Through Importin-8 or Exportin-1, human AGO2 shuttles between the nucleus and cytoplasm. Nuclear localized miRISC was found to regulate both transcriptional rates and post-transcriptional levels of mRNA and associate with euchromatin at gene loci with active transcription.[23]

It has been reported that low molecular weight miRISC can interact with mRNAs within the nucleus and induce nuclear mRNA degradation, although the mechanism behind this is unclear.

miRISC may interacts with target mRNA co-/post-transcriptionally. The involvement of AGO and Drosha in mRNA splicing further supports co-transcriptional miRISC:mRNA interactions. miRISC may also regulate transcription directly.

A study found nuclear miR-522 interacting with a DNA cruciform structure (a stem-loop on sense and antisense DNA strands) within the promoter of CYP2E1 and suppressing its transcription**.**

miRNAs have also been shown to interact at genomic loci, where enhancer-derived RNA (eRNA) are transcribed, and increase mRNA levels of adjacent genes by promoting a transcriptionally active chromatin state while altering alternative splicing profiles. [24]

1.1.4 microRNA and clinical application: diagnosis, prognosis and new treatments

The experimental evidence collected to date shows that microRNAs can represent valid diagnostic and prognostic markers in human tumors. The aberrant expression of miRNAs in different types of cancer was in fact related to specific bio-pathological characteristics, the outcome of the disease and the response to drug treatments.

- Diagnostic role of microRNAs
  It has been almost two decades since the first link between microRNAs and cancer was established.
  The diagnostic potential of microRNA molecular signatures in identifying a particular tumor phenotype has recently been demonstrated by the study of miRNA expression profiles in a large number of tumor samples. For instance, in one study, samples from individuals with acute leukemia were examined, and diverse miRNA expression patterns matching to various tumor transformation pathways were found. Therefore, in this investigation, miRNA expression profiles were a great tool for correctly diagnosing individuals with poorly differentiated cancers who would otherwise be impossible to diagnose by histological analysis alone. [25]

- Prognostic role of microRNAs
  The value of miRNA expression profiles is currently clear for the prognosis of various kinds of neoplasia; for instance, distinct expression patterns have been linked to a favorable or poor prognosis in chronic lymphatic leukemia (CLL) and lung cancer. It was demonstrated in a study that 143 instances of lung cancer may be divided into two major groups based on the expression of let-7 microRNA, and it was also discovered that low levels of let-7 are significantly correlated with a shorter patient survival time. [26]
  Unexpectedly, it was discovered that let-7 expression levels were a more accurate predictor of age, tumor histology, and smoking. A study that revealed a link between let-7 and the oncogene RAS, a well-known prognostic marker of individuals with lung cancer, offers a potential mechanistic explanation for this association. [27]

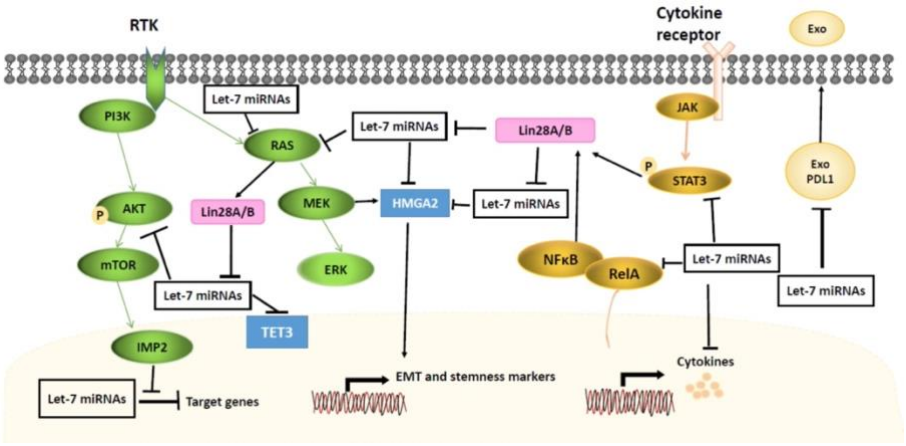


Figure 6 The Roles of the Let-7 Family of MicroRNAs in the Regulation of Cancer Stemness[28]

- Role of microRNAs in resistance to drug treatments

  Resistance to therapeutic treatments is one of the most significant variables impacting the likelihood that cancer patients will survive, and in this regard, the development of efficient pharmacological techniques is a critical step for the management of many cancer kinds.

  Different TRAIL (TNF-related apoptosis-inducing ligand) recombinant versions are among the most promising anti-tumor therapies based on the induction of apoptosis in cancer cells because of their unique toxicity against malignant cells.

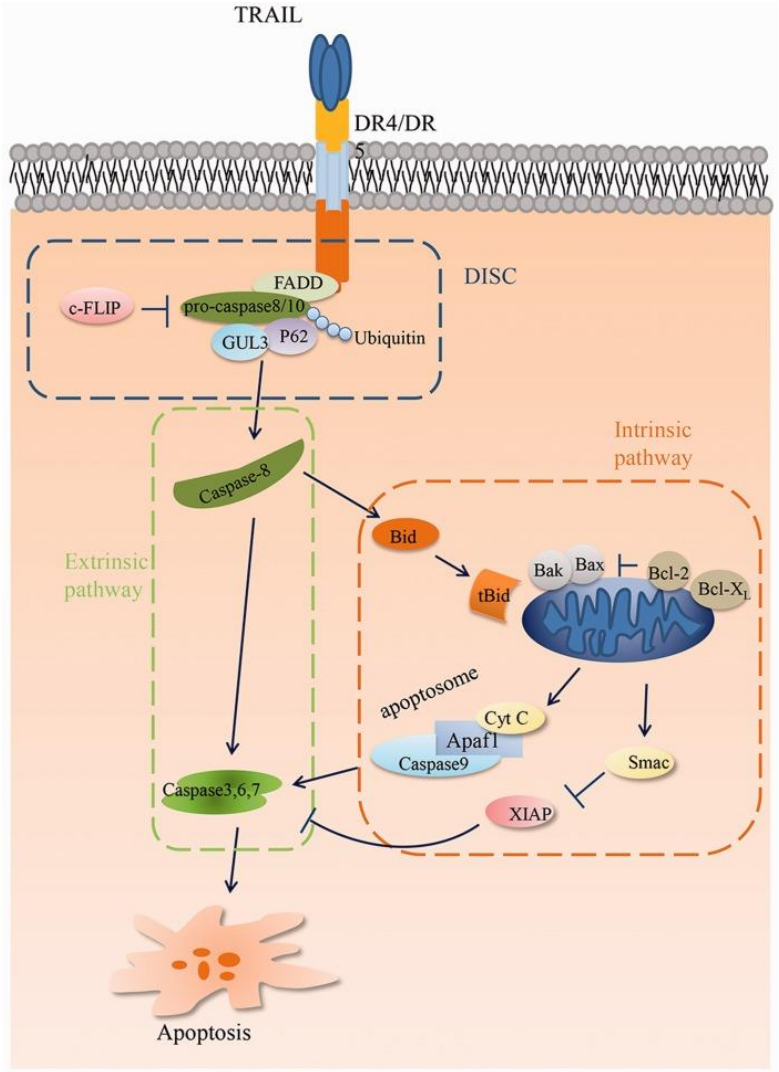


Figure 7 a schematic diagram of TRAIL-induced apoptotic signaling cascades.[29]

When TRAIL binds to TRAIL death receptors, the FADD and caspase-8 are drawn together to create DISC. Caspase-8, which can activate caspase effectors and the BH3-only protein Bid, is cleaved and activated by DISC. Caspase-8 activation in the extrinsic route is sufficient to trigger the downstream caspases-3, -6, and -7, resulting in cell death. To cause cell death, however, through the intrinsic pathway, Bid must be cleaved into its truncated form (tBid). tBid can quickly move to the mitochondria and cause the outer mitochondrial membrane to permeabilize (via Bax and Bak), releasing mitochondrial cytochrome c and Smac. Bcl-2 and Bcl-xL overexpression can stop this process. When cytochrome c enters the cytosol, it attaches to the adaptor Apaf-1 to bring the initiator caspase-9 into the apoptosome, where it can activate both the effector caspases and caspase-9. By counteracting XIAP's inhibitory action on the multiple effector caspases, Smac release increases apoptosis.

Despite this, a large number of human cancer cells are resistant to TRAIL-induced apoptosis, and the exact mechanism underlying this resistance is still a mystery.

According to a study, miR-221/222 increases cell migration after activating the AKT and metallopeptidases pathways by controlling the two molecular targets PTEN and TIMP3. This increases resistance to treatment with recombinant TRAIL. When compared to the corresponding healthy tissues, these microRNAs are overexpressed in many cancer forms, including hepatocellular carcinoma and lung cancer (NSCLC). This work demonstrates that TRAIL resistance in these two kinds of cancer is correlated with high expression of miR-221/222. In particular, miR-221/222 expression level-based tumor stratification might be a helpful predictive tool for determining resistance or sensitivity to treatment with TRAIL. [30]

Additionally, it has been demonstrated that some microRNAs play a role in radiotherapy sensitivity, according to research on prostate cancer (PCa, the second-most frequent malignancy in men, and roughly 50% of PCa patients had radiotherapy) (RT). It has already been established that 23 miRNAs have a role in the genetic control of PCa cells' response to RT. Although it has been proposed that miRNAs play a significant role in cell signaling pathways, the mechanisms of radioresistance remain poorly understood.
MiRNAs can be employed in the diagnosis of cancer to enhance therapeutic sensitivity and planning, avoid the toxicity and side effects of various drugs, and even monitor treatment.

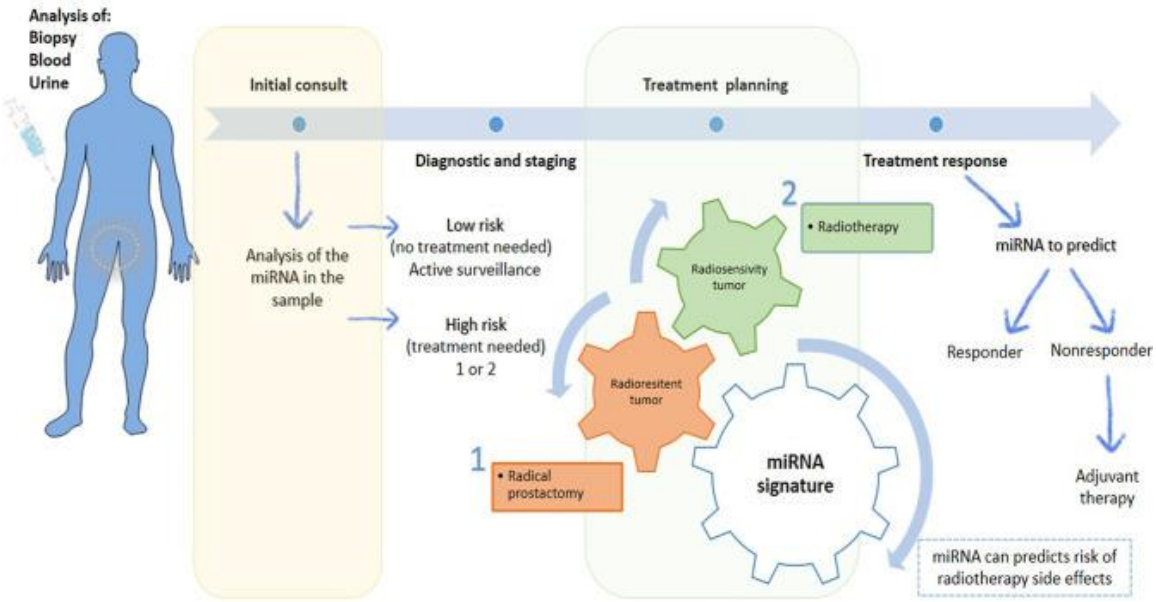


Figure 8 Scheme of the potential of miRNAs in personalized prostate cancer.

Tumor radiation response includes DNA repair, epithelial-to-mesenchymal transition (EMT), and stemness, all of which are regulated by miRNAs. However, because radio resistance is a complicated phenomenon, further research is required to fully comprehend these processes.
MiRNAs can be used in therapeutic methods to imitate or inhibit translational gene expression. If the miRNA is underexpressed in the first method, it can be restored by adding

miRNA. The second method involves the addition of synthetic anti-miRNAs to block miRNA if it is overexpressed. [31]

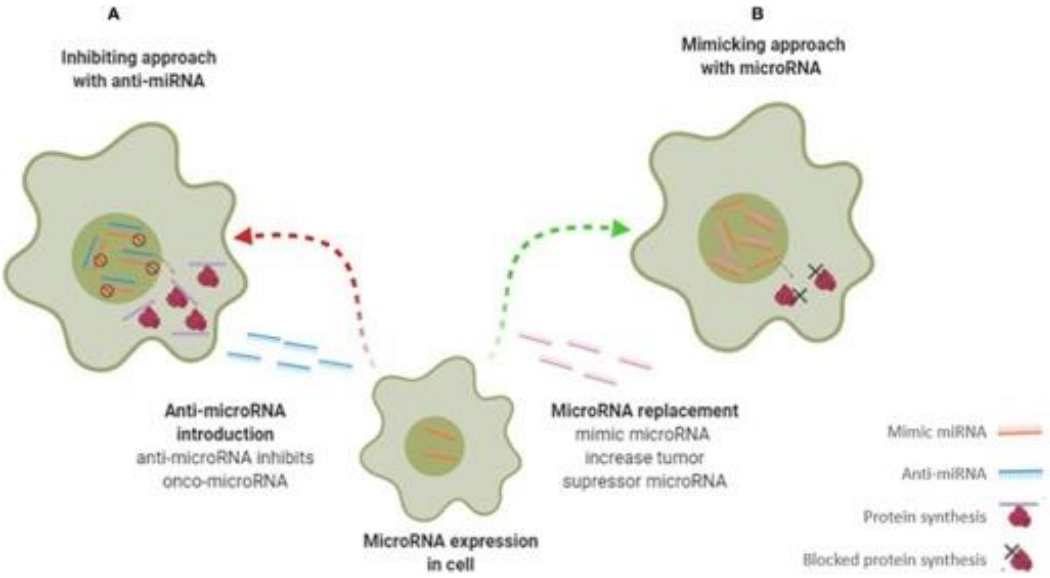


Figure 9 Scheme illustrates different microRNA therapy approaches[31]

Several studies have also demonstrated that LNCaP and PC3 cells are more sensitive to ionizing radiation when miRNA-145 is overexpressed. This miRNA inhibits DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3b), which is essential for carcinogenesis and affects PCa cells' proliferation, migration, apoptosis, and cycle. According to some research, miRNA-145 overexpression may enhance radio sensitivity by downregulating DNA double-strand breaks and specifically targeting oncogenes. [32]

Another study reported that miRNA-521 modulates the radio sensitivity of LNCaP cells by Specifically restoring DNA repair protein, Cockayne syndrome protein A (CSA) and manganese superoxide dismutase (MnSOD), an anti-apoptotic enzyme. If miRNA-521 is overexpressed, it will further sensitize cells to RT contributing to a raise in RT efficacy. [33]

In a similar manner, certain miRNAs have a role in cell cycle arrest. Chromosome 13q14 contains microRNA-16-5p, which is downregulated in LNCaP cells. This miRNA is a tumor suppressor and is involved in the onset of PCa, according to research by Wang et al. The Cyclin D1/E1-pRb/E2F1 pathway is modulated by the overexpression of miRNA-16-5p, which causes G0/G1 phase arrest following radiation and ultimately increases radiosensitivity in LNCaP cells. [34]

It is known that miRNA-301a is an oncomir and it has been proposed that miRNA-301b can act as a tumor suppressor in LNCaP, PC3 and DU145 cells. However, in a study both these miRNAs were related to hypoxia and led to a decrease in autophagy in LNCaP, PC3

and DU145 cells by targeting N-myc downstream-regulated gene 2 (NDRG2). If these miRNAs are overexpressed, they may induce radio resistance in PCa cells by decreasing NDRG2 that suppresses EMT (epithelial-mesenchymal transition). [35]
Despite the relative few numbers of studies exploiting the miRNAs relation with radiation response of PCa cells, this subject has been progressively explored and it continuous to be a challenge regarding the role of miRNAs as predictive markers for therapeutic targets.

- Role of microRNAs as therapeutic targets

Considering the importance of microRNAs in the development, progression and treatment of cancer, their potential use in cancer therapy is currently being studied by numerous research groups. The aim is to modulate its expression by introducing under-expressed miRNAs into cancer or by inhibiting the expression of oncomiR through antagonist molecules.
The development of molecules similar to microRNAs but with a higher half-life and increased efficacy in vivo, such as modified oligonucleotides using ring-structured nucleic acids (LNA), anti-miR oligonucleotides (AMO) and 'antagomirs' conjugated to cholesterol molecules, it was the first step in translating scientific advances into clinical practice.
The loss-of-function modulating system of miRNAs is mainly achieved by applying miRNA sponges; miRNA-Masking Antisense Oligonucleotides; or by using antisense oligonucleotides targeting miRNAs (AMOs)
MiRNA-sponge technique involves the creation of mRNA molecules containing several target miRNA binding sites, which serve as a decoy or "sponge" to capture the targeted miRNAs. In this manner, the endogenous target mRNA will be protected and able to carry out its intended function. In vitro, this method is frequently employed to suppress functional classes of miRNAs, but its application in living organisms has been restricted so far. MiRNA sponges are specific to a miRNA target and not to a gene of interest. Using a miRNA sponge to knockdown a miRNA or a miRNA seed family could affect all target genes of its targeted miRNAs, leading to undesirable effects.[36]
MiRNA-Masking Antisense Oligonucleotides technology (miR-Mask, also known as BlockmiR, target protectors or as target site blockers) relies on an inverted approach: instead of blocking the target miRNA, these molecules shield the mRNA which function is desirable to preserve. In this way, the miR-Mask are able to block the access of the miRNA to its target mRNA, impeding its action to the target gene. Moreover, miR-mask technology targets the miRNA in a gene-specific manner, i.e. the oligonucleotides are designed to protect the mRNA, and consequently, the expression of the protein of interest, leaving intact the miRNA responsible for the repression, which might have other important roles in the system. MiR-Mask is an alternative and valuable supplement to AMOs technology.[37]
The most popular approach to correct aberrant miRNA expression is based on the synthesis of antisense oligonucleotides with a complementary sequence. Antisense oligonucleotides (ASOs) are being used to suppress dysfunctional mRNAs for more than four decades and are currently being used to target miRNAs. In antisense oligonucleotides targeting mRNAs, ASOs mostly stimulate an RNase-dependent degradation of the target RNA.
A study demonstrated that anti-miRNAs cause miRNA silencing mostly by steric blocking the target miRNA, where they tested a designed ASO sequence known to trigger the RNase H-dependent degradation to target the miR-21. The antisense oligonucleotide, despite

being effective promoting mRNA degradation, failed to inhibit miR-21 activity, suggesting that the miRNA-ASO duplex may not be accessible to the RNase H. [38]

The potential use of antisense oligonucleotides to target aberrant miRNAs (AMOs) involved in a variety of human illnesses has been demonstrated in numerous publications published over the past few decades. Endo- and exonucleases in a biological system quickly break down antisense oligonucleotides made entirely of DNA bases, preventing them from connecting with most of the target miRNA in human cells. A wide range of chemical changes have been researched and applied to antisense technologies as a result of the urgent need to get past DNA-AMOs restrictions.

Recent studies have demonstrated the effectiveness of new kinds of chemically modified AMOs, such as locked nucleic acid oligonucleotides, in silencing miRNAs.

By boosting, for example, nuclease resistance and target affinity, the proper design of a particular AMO can aid in improving its performance and potency towards the target miRNA.

It's interesting how chemically altering sugars can improve binding affinity and nuclease stability. The 2′ carbon of the ribose is where the majority of modifications made to miRNA studies are located. As opposed to DNA, AMOs are said to have higher binding affinity and nuclease resistance in the case of 2′-OMe, 2′-MOE, and 2′-F changes.

In AMO designs, locked nucleic acids (LNA) are frequently used.

There are now two medications in clinical trials that make use of LNA-AMO technology. Santaris Pharma's first medication, Miravirsen, which targets miR-122 to treat HCV infection, has entered phase 2 of clinical trials. [39]

A different organizations (miRagen Therapeutics) started phase 1 clinical trials for MRG106-11-101 LNA-antimiR, which was created to regulate miR-155 activity. Using the created AMO in in vitro testing, lymphoma cells' miR-155 activity was successfully reduced, restoring normal function and decreasing aberrant cell growth. [40]

Backbone changes are available in addition to sugar adjustments.

The naturally occurring internucleotide linkage in DNA and RNA is called a phosphodiester bond (PO). In this way, oligonucleotides that are made to contain the natural PO bonds are more tolerable in vivo and exhibit less toxicity. However, in mammalian cells, they are more likely to be cleaved by endo- and exonucleases. [41]

A phosphorothioate (PS) linkage was the first described internucleotide modification in antisense oligonucleotides. PS bonds are more resistant to the activity of exo- and endonucleases and have an affinity for serum albumin, which reduces plasma clearance and extends the AMOs lifetime in serum from hours to days. [42]

Phosphonoacetate (PACE) and thio-PACE are two further internucleotide linkage modifications that are utilized to silence miRNAs. Both changes are said to be entirely immune to nuclease activity and capable of entering cells as neutral esters on their own. [43]

Phosphorodiamidate groups, instead of phosphates, are used to bind the morpholine ring in phosphorodiamidate morphholino oligomers (PMOs), which are uncharged alternatives for PO bonds. PMO oligonucleotides have a similar or slightly higher binding affinity for target miRNAs and are more resistant to nuclease action. [44]

An uncharged synthetic DNA analog known as peptide nucleic acid (PNA) is made up of N-(2-aminoethyl) glycine monomers connected by peptide bonds. According to a study, PNA molecules used to mute miR-122 performed better than a 2′-OMe oligonucleotide. [45]

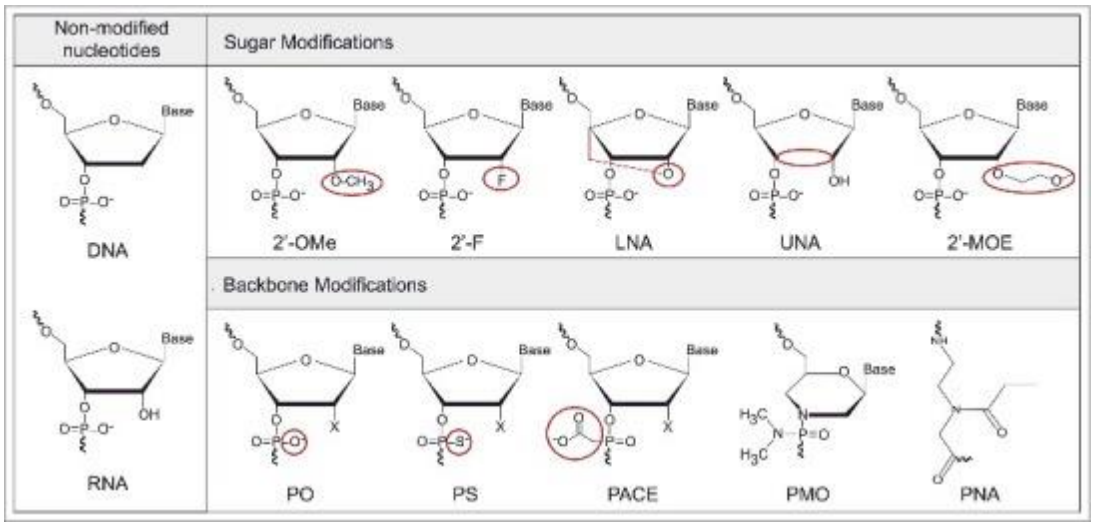


Figure 10 Chemical modifications applied in the AMOs design. [31]

It is crucial to create new technologies to investigate how miRNAs work and how they are regulated in human disorders.

In order to mute undesired miRNA function and gene activity, the use of AMOs in clinical practice for the treatment of cancer appears to be a potential alternative.

No complete therapeutic approach to altering miRNA function has yet been created. Attempting to selectively target miR-122, experimental AMO-based medicines have already entered clinical trials (Miravirsen developed by Santaris Pharma and RG-101 developed by Regulus). Despite the encouraging outcomes, further research is still needed to confirm the safety, specificity, and stability of the hybridization between the anti-miRNA oligonucleotide sequence and the target miRNA.

Recent research has demonstrated that genetically engineered miRNA from mesenchymal stem cell exosomes has a tremendous potential to support angiogenesis and tissue repair in diabetic feet. Its primary mechanisms include encouraging angiogenesis by taking part in transcriptional regulation of gene expression, speeding up wound re-epithelialization, reducing inflammation and apoptosis, controlling cell regeneration, and protecting cells and regulating insulin resistance. These functions all contribute to the healing of diabetic foot wounds.

By preventing cell apoptosis and raising the sensitivity of insulin target tissues, mesenchymal stem cell exosomes gene-modified miRNA can help with pancreatic tissue repair, obesity and insulin resistance, and delay the onset of diabetes and its comorbidities. Exosomes miRNA also exhibits significant promise as a biomarker for the early detection, efficacy assessment, and prognosis diagnosis of diabetic foot problems. Seeking the best mesenchymal stem cell exosomes gene-modified miRNA therapy is expected to become a new technology for diabetic foot treatment.

1.1.5 MicroRNA in liver disease

As they control the expression of genes that can be crucial for the physiological status of the organism, miRNAs have a role in the pathological progression of many disorders.
Numerous human diseases, such as cancer, immune system abnormalities, liver dysfunction, and neurodegenerative diseases, have been linked to variations in the expression levels of these short non-coding RNAs. In reality, experimental data demonstrates that these diseases may be a direct result of changes in cellular pathways brought on by mutations in the genes encoding miRNAs, in the binding sites on their targets, or in the molecular processes that control their production.
The functions of the liver, both healthy and unhealthy, are regulated by miRNAs. MiRNAs are appealing therapeutic options for the detection and management of liver illnesses since altered miRNA expression is linked to altered liver metabolism, liver damage, liver fibrosis, and tumor growth.

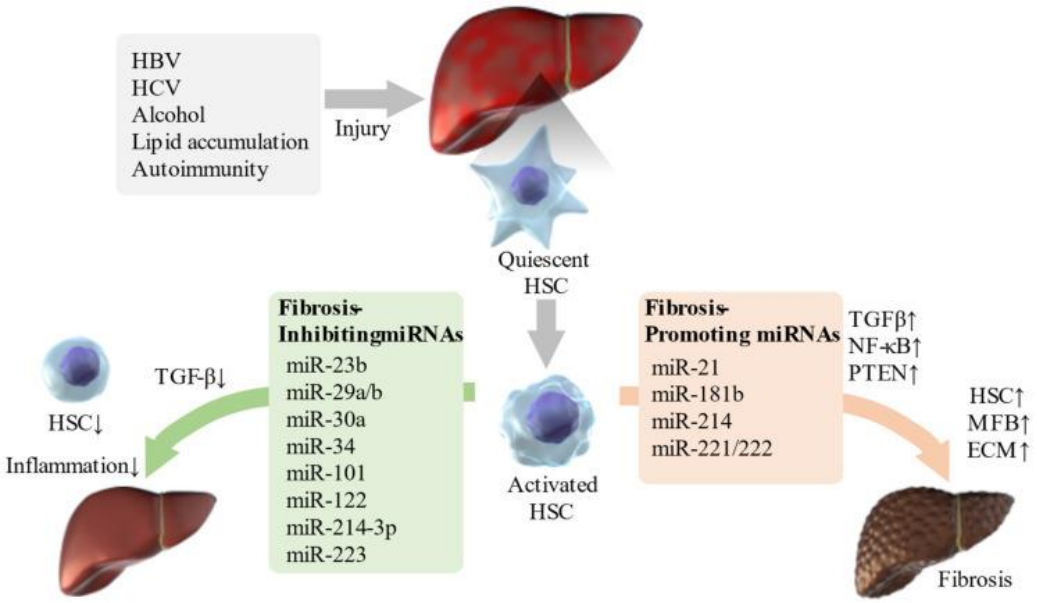


Figure 11 schematic diagram of the relationship between miRNAs and liver fibrosis[46]

In livers that have been harmed by viruses, alcohol, cholesterol buildup, and autoimmune, HSCs become active.
After liver injury, the hepatic stellate cells (HSCs) undergo a response called "activation", transforming the cells into proliferative, fibrogenic and contractile myofibroblasts, representing the main collagen-producing cells in the injured tissue. In addition to releasing cytokines and a number of hepatomatogens, activated HSCs are thought to be pro-inflammatory cells that are also involved in the recruitment of Kupffer cells, circulating monocytes, and macrophages through the production of chemokines. When the liver is wounded, hepatocytes and HSCs interact in a complex way that leads to mitochondrial

inefficiency, which in turn causes a deposit of fats in the hepatocytes that leads to liver lipotoxicity.
These mechanisms for how HSC are activated imply that they play a significant role in the development of metabolic diseases. [47]
This process involves a number of miRNAs, which have an impact on multiple pathways-involved molecules as TGF-, NF-B, and PTEN. This may lessen inflammation or, on the other hand, lead to ECM deposit and cirrhosis.
MiR-122 is thought to be a hepatocyte-specific miRNA since systematic study of the distribution of miRNAs has revealed that it is a highly abundant, liver-specific miRNA that makes up roughly 70% and 52%, respectively, of the entire hepatic miRNome in adult mouse and human. High levels of miR-122 expression can be found in mature liver, however they are noticeably downregulated in HCC. Patients with non-alcoholic steatohepatitis (NASH) had lower levels of the liver miR-122 than healthy controls. Alcohol-associated liver disease (ALD) is also accompanied by decreased hepatic levels of miR-122.
One of the exosomal miRNAs linked to obesity that contribute to the pathophysiology of dyslipidemia and glucose intolerance is miR-192.
According to a recent study, miR-192 may control liver inflammation in people with NAFLD by encouraging macrophage activation and proinflammatory activities. The miRNA that is most noticeably downregulated in CSC+HCC cells is miR-192.
Through its targeting of numerous genes, MiR-194 also contributes to the growth and metastasis of HCC.
As a neutrophil-specific miRNA with the highest level of expression in neutrophils, X-chromosome linked miR-223 is essential for reducing neutrophil maturation and activation. It is reasonable to assume that this neutrophil-specific miR-223 plays a significant role in the pathophysiology of ALD and NAFLD as hepatic neutrophil infiltration is a characteristic of these two disorders. In NAFLD model, miR-223 directly inhibits several inflammatory genes and oncogenes including (C-X-C motif) chemokine 10 (*Cxcl10*) and transcriptional coactivator with PDZ-binding motif (*Taz*) in hepatocytes, thus ameliorating NAFLD.
miR-223 is commonly repressed in human HCC.
One of the most prevalent miRNAs found in the bloodstream, miR-21, is highly expressed in a variety of human tissues, including the liver.
Mice and people with NAFLD/NASH had considerably higher levels of hepatic miR-21 expression. By blocking several transcription factors that encourage the release of very low-density lipoprotein (VLDL) and prevent lipogenesis, miR-21 encourages steatosis. PPAR alpha (hepatic PPAR) expression is likewise decreased by miR-21, which accelerates the development of NASH.
Furthermore, miR-21 is regarded as an onco-miR in liver cancer and encourages HCC development. One of the most overexpressed miRNAs in HCC is miR-21, and patients with HCC have significantly higher serum levels of this miRNA.
When it comes to regulating the actions of immune cells, such as macrophages, miR-155 is strongly expressed in these cells. Despite being found in the liver at low levels, miR-155 has been shown to control liver damage, inflammation, fibrosis, and carcinogenesis.
By decreasing hepatic Ppara in hepatocytes and by targeting inhibitors of the LPS/TLR4 pathway such SHIP1, IL-1 receptor-associated kinase-M, and suppressor of cytokine

signaling 1 in macrophages, miR-155 encourages alcohol-induced steatohepatitis and liver fibrosis in ALD.

MiR-155 is regarded as an oncogenic miRNA that links tumorigenesis with inflammation. Finally, miR-155 is highly expressed in epithelial cell adhesion molecule+ HCC cells, which promotes malignant features like colony formation, cell migration, and invasion. Hepatic miR-155 is up-regulated in human HCC samples, activated NF-B signaling appears to upregulate miR-155 expression in hepatocytes and liver cancer. Three extensively expressed miRs, miR-29a, miR-29b, and miR-29c, make up the miR-29 family. 6 HSCs in the liver exhibit the greatest levels of miR-29b expression. In patients with advanced fibrosis, the expression of miR-29a, b, and c in the liver is downregulated. The metabolism of lipids and glucose in the liver also involves miR-29. In HCC, MiR-29 also has a tumor-suppressive impact. In human HCC tissues, miR-29a/b/c are downregulated, and individuals with HCC who show low levels of miR-29a have poorer prognoses. [4]

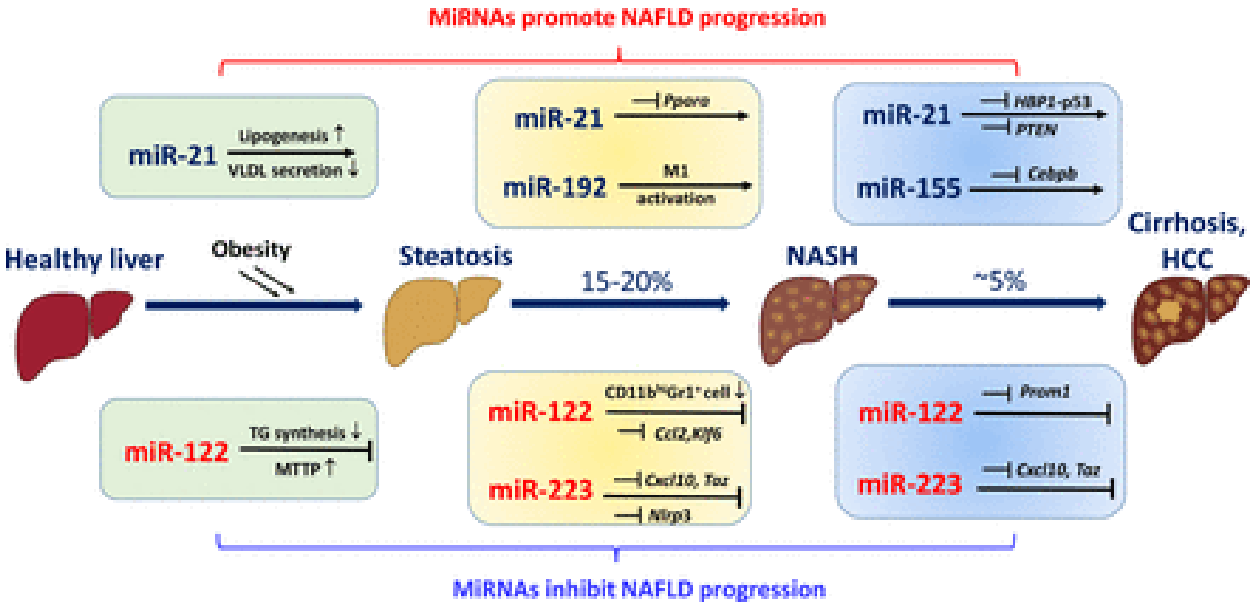


Figure 12 Roles of microRNAs (miRNAs) in the progression of non-alcoholic fatty liver disease (NAFLD)[4]

1.1.6 MiR-21

MiR-21, one of the many microRNAs, is of special relevance due to its connection to chronic liver disease. It has been discovered that this is extensively expressed in a variety of human organs, including the liver and activated stellate cells (HSCs), which are in charge of hepatic fibrosis. MiR-21 is also one of the most overexpressed miRNAs in HCC, with both tissue and serum levels markedly increased.
MiR-21 is a protein that is increased in the liver throughout a variety of biological processes, such as fibrosis, cancer, and inflammation.

1.1.4.1. Molecular role in the progression of liver disease

It has been demonstrated that the host miRNAs of the hepatitis B virus and hepatitis C virus (HBV, HCV) can affect the viral genome as well as different host cellular factors. The virus can also affect the levels of cellular miRNA expression to its benefit, thereby fostering an environment favorable for the development of infection and liver damage. HBV encodes protein X (HBx), which is crucial for viral replication and the development of hepatocellular carcinoma associated with HBV. By causing the overexpression of miR-21 in hepatocytes and hepatocellular carcinoma, HBx speeds up the progression of liver damage by suppressing tumor suppressor genes such phosphatase and tensin homolog and programmed cell death protein 4 (PDCD4) (PTEN) and by inhibiting apoptosis by targeting interleukin-12 (IL-12). [48] [49] [50]
A study has shown that up-regulation of miR-21 occurs in HCV infection, which in turn suppresses the production of type I Interferon (IFN) normally induced by HCV, thus promoting viral replication. [51]
Patients with NAFLD or NASH also have miR-21 deregulation, which encourages the storage of liver lipids and cell proliferation by interfering with the Hbp1-p53-Srebp1c251 pathway and suppressing PTEN.
The FOXO1 protein, which is involved in lipid metabolism and is crucial in the emergence of lipid-related disorders, can also be dysregulated by mir-21, which further contributes to the development of fatty liver disease.
FoxO protein is also connected to the VLDL biogenesis. There are a variety of FoxO transcription factor binding partners, including proliferators-activated receptor (PPAR), PPAR, RAR, Smad, thyroid hormone receptor (TR), and PPAR gamma coactivator-1 (PGC1). Insulin strictly controls the process through which VLDL are secreted from the liver after being synthesized in hepatocytes. Increased insulin action inhibits the formation of VLDL in the liver. The postprandial plasma triglyceride excursion is constrained by this effect. In contrast, decreased insulin action increases the synthesis of VLDL, and more VLDL is circulated, which helps to transfer lipids to peripheral tissues for energy. For the preservation of lipid homeostasis, these reciprocal pathways are essential. An significant new understanding of the mechanism behind the insulin-dependent regulation of VLDL production comes from recent research identifying FoxO1 as a major regulator of hepatic MTP. FoxO1 sits in the nucleus and binds to IRE as a trans-activator to increase promoter activity in the absence of insulin action. When FoxO1 is phosphorylated in response to insulin, it is excluded from the nucleus and the expression of the target gene is inhibited.

This theory contributes to the understanding of the substantial correlation between hypertriglyceridemia and hepatic VLDL overproduction and insulin resistance. As a result, FoxO1's decreased capacity to undergo insulin-dependent protein trafficking can cause MTP to remain nuclear localized and express itself even in the presence of hepatic insulin resistance, which can contribute to the emergence of hypertriglyceridemia in people with metabolic syndrome. [52]

Through the suppression of the peroxisome proliferator-activated receptor alpha (PPAR) signaling pathway, MiR-21 also appears to have a role in the induction or facilitation of cell damage, inflammation, and fibrosis. [53]

The liver fibrosis is also caused by the activation of hepatic stellate cells (HSC), which is characterized by an excessive production of collagen. Additionally, a microRNA-21/programmed cell death protein 4/activation protein-1 (miR-21/PDCD4/AP-1) feedback loop was used to maintain miR-21 at consistently high levels. MiR-21 or AP-1 inhibitors disrupting this cycle greatly reduced the pro-fibrotic activity of HSCs. On the other hand, fibrogenesis was increased by enhancing this cycle with a small interfering RNA (siRNA) against PDCD4. The transforming growth factor (TGF-) signaling pathway that underlies the activation of HSC is supported by the up-regulated miR-21. The course of liver fibrosis appears to be heavily influenced by the miR-21/PDCD4/AP-1 self-regulation cycle. [54]

MiR-21 can be classified as an onco-miRNA: it promotes cell proliferation, migration, and invasion in many cancer cells when it is overexpressed. PTEN is a tumor suppressor gene, and miR-21's association with PTEN is one of its important functions.

The expression of PTEN was downregulated in human hepatocytes transfected with precursors of miR-21, and FAK, a protein tyrosine kinase that is typically dephosphorylated by PTEN itself, was phosphorylated. This resulted in enhanced cell motility.

Therefore, it may be assumed that mir-21 and the metastatic process are related. [49]

Another study identified a role for miR-21 in the resistance of HCC to sorafenib therapy, which is likely the result of the down-regulation of PTEN and the subsequent activation of AKT, which suppresses autophagy. A role for miR-21 in this pathway was then confirmed by the subsequent antagonism of miR-21 by certain oligonucleotides, which made the cells susceptible to sorafenib once more and markedly boosted autophagy. [55]

In example, in one study, miR-21 overexpression in DU145 cells boosted the production of HIF-1 and VEGF and caused tumor angiogenesis. MiR-21 appears to play a role in angiogenic processes in many patient malignancies. By partially inactivating AKT and ERK and reducing the production of HIF-1 and VEGF, overexpression of the miR-21 target PTEN also prevented tumor angiogenesis. The HIF-1 and VEGF expression as well as angiogenesis were reduced by the AKT and ERK inhibitors LY294002 and U0126. Furthermore, miR-21-upregulated tumor angiogenesis was reversed when HIF-1 expression was inhibited on its own, showing that HIF-1 is necessary for this process. This research demonstrates that miR-21 promotes tumor angiogenesis by targeting PTEN, which activates the AKT and ERK1/2 signaling pathways and increases the production of HIF-1 and VEGF. HIF-1 is a crucial downstream target of miR-21 in controlling tumor angiogenesis. [56]

1.1.4.2. Mir-21 as biomarker

Because they are extremely stable and simple to find in circulation, miRNAs have the potential to develop into innovative, non-invasive biomarkers.
It can be argued that some miRNAs are preferable than conventional biomarkers for the early diagnosis, prognosis, and assessment of chronic liver disease.
Through the activation or enhancement of molecular signaling pathways with pro-fibrotic effects, as well as through the deregulation of signaling pathways related to processes of migration, cell invasion, apoptosis, and angiogenesis with carcinogenic effects, miR-21 contributes to the progression of liver damage. Therefore, it is not surprising that in the research, the levels of miR-21, an onco-miR, are higher in HCC tissues than in liver tissue absent of tumors.
The determination of miR-21 as a reliable diagnostic tool is suggested by the fact that serum levels are also greater in HCC patients than in healthy controls.
In patients with HCV-related chronic hepatitis (CHC) or NAFLD, a study examined the relationship between miR-21 levels and the degree of hepatic damage to histology (measured by fibrosis). The results showed a small rise in miR-21 in the advanced stages (F4) but not in the earlier stages. [57]
There are conflicting findings in the literature regarding NAFLD/NASH: elevated tissue levels of miR-21 were discovered; regarding serum levels, one study showed lower levels of miR-21 compared to healthy controls; while another study found higher serum levels of miR-21 in NAFLD patients compared to healthy controls. [58] [59]

In one study, the levels of miR-21 were compared in HCC patients, CHC patients, and healthy people. There was no difference between CHC and HCC patients, while individuals with CHC had high levels compared to healthy controls. Only CHC patients with elevated ALT levels had elevated miR-21 serum levels, whereas sera from CHC patients with normal ALT levels had normal miR-21 serum concentrations. The Histology Activity Index (HAI), a score based on the degree of liver necrosis, inflammation, and fibrosis, and serum levels of miR-21 showed a substantial correlation. This suggests that the serum level of miR-21 may be associated to necroinflammatory activity liver in patients with CHC.
The serum miR-21 level is acceptable for detecting necroinflammatory activity in the liver and not the presence of HCC, according to the study authors, who also claim that miR-21 serum levels are more closely associated to liver damage activity than hepatic fibrosis in CHC patients.[60]
In one study, the levels of the miR-21 gene were compared in patients with HCC, those with chronic hepatitis and cirrhosis but no HCC, those with chronic hepatitis but no cirrhosis but no HCC, and healthy controls. The findings indicate that individuals with cirrhosis and HCC had levels that were considerably higher than those with cirrhosis alone and that those without cirrhosis and HCC had levels that were in between the two. Furthermore, a positive association between the levels of miR-21 and the HBV viral load was discovered.
MiR-21 appears to be suitable for differentiating HCC with an effective diagnostic power of sensitivity, specificity, and level of expression, according to the results of this study, which showed a sensitivity of 74.1% and a specificity of 75.0% in differentiating HCC

patients from patients without HCC but an inability to differentiate HCC from chronic hepatitis. [61]

Most likely, determining many miRNAs in serum has greater diagnostic utility than determining only one miRNA. MiR-21, miR-199, and miR-122, which are widely expressed miRNAs, may be more accurate for the diagnosis of HCC.

Although the majority of studies show a strong link between miR-21 levels and the prognosis of HCC or chronic liver disease, other investigations have produced contradictory results. Furthermore, the trend of miR-21 in the various stages of chronic liver disease and its potential link with variables like the degree of fibrosis are not well studied.

Finally, it seems difficult to compare these studies with one another due to a variety of factors, including the use of control groups, RNA extraction techniques, different sample sizes and selection, and differences in the samples used to measure the level of miR-21 (tissue, serum, plasma, and whole blood) (healthy volunteers, hepatitis and cirrhosis). A conclusion regarding the potential eligibility and dependability of miRNAs as diagnostic and prognostic biomarkers of HCC, but also of chronic liver diseases, representing the stages of natural history prior to the onset of HCC, in order to enable early diagnosis, accurate staging and appropriate treatment requires urgent further research into these studies and, more importantly, the standardization of the methods by which these are conducted.

1.2 Chronic liver disease

The liver is one of the most important organs and performs many essential functions for the body: metabolic, at a carbohydrate, lipid and protein level, but also catabolic, as it stores and removes toxic substances. When the liver is affected by a pathological process, these numerous functions it performs are compromised, in a variable and generally progressive manner depending on the degree of involvement of the hepatic parenchyma. The mechanisms underlying liver damage are extremely complex and not yet fully understood. The damage can be direct, by the causative agent (viral, ischemic or chemical / toxic) at the level of the hepatic parenchyma, or, as in most cases, indirect, i.e. caused by the inflammation resulting from the interaction between the injurious agent and immune system. The liver responds to this damage with a variable pattern of hepatocyte repair and regeneration, fibrosis or cellular necrosis, also based on the extent of the damage and above all on the permanence and persistence of the cause. Chronic liver disease is nowadays one of the most frequent chronic diseases in the world, significantly affecting in terms of morbidity and mortality, but also in terms of direct and indirect costs related to the diagnosis, treatment and management of the numerous complications. Liver disease can be caused by many etiological agents: the main ones are viral (hepatitis B [HBV], hepatitis C [HCV]), metabolic (Non-Alcoholic Fatty Liver Disease or NAFLD and Non-Alcoholic Steato-Hepatitis or NASH) and toxic (Alcoholic Liver Disease or ALD and Alcoholic Steato-Hepatitis or ASH). Despite having a different pathogenetic mechanism from each other, all chronic liver diseases converge to the development of fibrosis, as an attempt to repair in response to the damaging insult to the hepatic parenchyma, and to the consequent evolution into liver cirrhosis, which with its complications and the increased risk of onset of hepatocellular carcinoma, represents the terminal stage of chronic liver disease. [62]

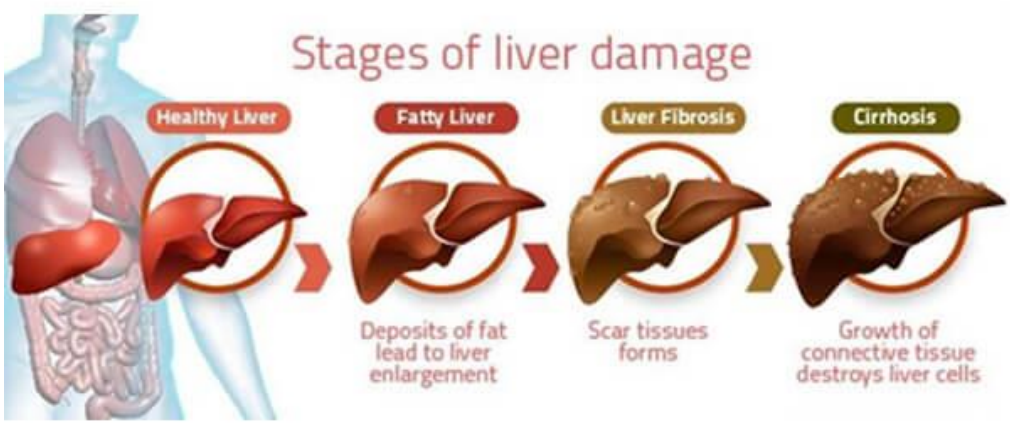


Figure 13 stages of liver damage[63]

Liver disease can begin with an acute phase, or acute hepatitis, defined as an inflammatory and necrotic process that resolves within six months. Usually, this phase is characterized by a significant increase in transaminases and by the presence of associated clinical manifestations that largely depend on the severity of the necroinflammatory involvement of the hepatic parenchyma, such as jaundice and, even if rarely, acute liver failure. Chronic hepatitis represents the possible evolution of acute forms, which, in most cases, go unnoticed as they are asymptomatic or paucisymptomatic. Chronic hepatitis is defined as a clinical-pathological entity characterized by the persistence of hepatocyte necrosis phenomena for over six months, caused by widespread inflammation of the liver and associated with a variable degree of fibrosis.
Chronic hepatitis is often diagnosed randomly as a result of other tests.
The time between the onset of the disease and the onset of cirrhosis, the terminal stage of chronic liver disease, is variable and depends on numerous factors such as the aggressiveness of the disease, the timeliness and effectiveness of the therapy, the elimination of any risk factors and the body's ability to respond. This inflammation and fibrosis process evolves subclinically over many years, without affecting liver function.
The presence of cirrhosis can lead to the development of portal hypertension, a condition characterized by an increase in the pressure gradient between the portal vein and hepatic veins, which can determine the onset of complications typical of advanced stages of liver disease such as the onset of esophageal varices and gastric disorders, ascites, encephalopathy, hepatorenal syndrome, and discoagulopathy. [64]
Cirrhosis of the liver is also the major cause of the onset of hepatocellular carcinoma (HCC), one of the most common cancers worldwide today and a cause of high mortality in the population.About 90% of HCC cases arise in fact in cirrhotic patients, with the exception of countries where the predominant viral etiology of chronic hepatitis is HBV, such as China, Japan, South Korea, Philippines and Vietnam: in these cases, HCC can arise,

in a high percentage of cases, even in non-cirrhotic livers.

1.2.1 Hepatitis B

The hepatitis B virus (HBV) is a DNA virus belonging to the Hepadnaviridae family. Characteristic of HBV is that of having a circular, partially double-stranded DNA that is converted into a template DNA, called cccDNA, highly stable and responsible for chronic infection.
In developed countries, including Italy, HBV-related mortality and the incidence of HBV-related HCC has undergone a significant reduction in recent years thanks to information campaigns, the improvement of social and health conditions and above all the introduction of vaccination. mandatory since 1991, for the screening of pregnant women and the possible administration of anti-HBV prophylaxis to those born to positive mothers. A further contribution was made by raising the awareness of the population about the HIV virus, which shares the same mode of transmission with HBV.
HBV infection is transmitted parenterally, through exposure to infected blood and body secretions, but can also be transmitted vertically (from mother to newborn). Inappropriate sterilization of medical instruments, the use of contaminated blood products and dialysis instruments and the use of infected needles for the administration of intravenous drugs remain the main routes of transmission.[65] [66]
HBV causes damage to the liver parenchyma following a complex interaction that the virus establishes with the immune system.
Generally, acute infection is asymptomatic or has mild and not very specific symptoms, Failure to heal from the acute form predisposes the transition to the chronic form of hepatitis. Fulminant hepatitis is rare, but is highly lethal in the absence of a liver transplant. The natural history of the disease is divided into five phases, through the use of serological markers (HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc IgM and IgG), virological

(HBV-DNA), biochemical (alanine amino -transferase [ALT] and aspartate amino-transferase [AST]) and histological.

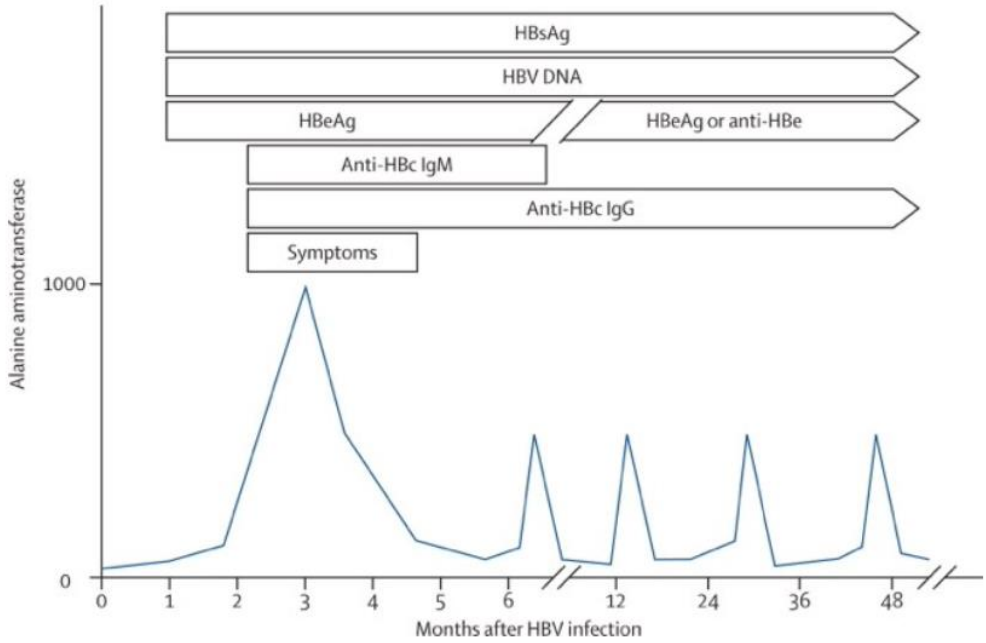


Figure 14 serological markers in the transition phase from acute hepatitis B to chronic hepatitis B.

HBsAg is detectable in serum after about 2-10 weeks of exposure to HBV, even before the onset of symptoms and the increase in hepatocytolysis indices, and remains positive throughout the acute phase of the disease. In case of healing, anti-HBs antibodies will appear, associated with the presence of anti-HBc antibodies; the mere presence of anti-HBs antibodies is indicative of successful vaccination. In the case of non-healing and transition into the chronic phase, HBsAg will continue to be detectable. The presence of HBV-DNA in the liver and the absence of HBsAg in the serum, on the other hand, indicate a condition called occult infection. [67]

When HBV infection is acquired in childhood, the early stage of chronic infection (first stage, immunotolerance) is characterized by high levels of HBsAg, presence of HBeAg, very high HBV-DNA (> 20,000 IU / mL) , normal ALT concentrations and minimal inflammation and fibrosis on histology. This phase can last as long as 20-40 years with minimal disease progression and minimal cure rate. The second phase (chronic immunoreactive hepatitis, HBeAg-positive) 33 is characterized by high levels of HBV-DNA, a significant increase in ALT and a necroinflammatory or cirrhotic picture on histology. These patients have a high risk of progression to HCC; however, seroconversion from HBeAg to anti-HBe with disease remission (asymptomatic carrier state, third phase), or progression to chronic anti-HBe positive hepatitis (fourth phase) may occur. The third

phase (inactive carrier, HBeAg negative) is characterized by a lowering of HBV-DNA and a return to the normal limits of the ALT concentration. HBsAg is present but in low concentrations. Liver histology is normal or with a minimal degree of fibrosis.[68] [69]

A patient in this phase may remain so or may present a reactivation of viral replication with resumption of liver disease. This is the reason why inactive carrier patients must be monitored over time, are not treated with antiviral therapy (unless the use of immunosuppressive drugs) and must avoid exposure to hepatotoxic risk factors such as alcohol and drugs. HBeAg seroconversion before age 30 is associated with a lower risk of developing hepatocellular carcinoma and better survival than seroconversion in later life. [70] [71]

Inactive carrier patients who instead will be subject to a recurrence of the disease will find themselves in the phase of chronic HBeAg-negative hepatitis (fourth phase), characterized by a continuous or fluctuating necroinflammatory process, with periods of remission alternating with elevation of HBV-DNA and ALT , which will go hand in hand with liver damage. It is the most common form of chronic hepatitis B (85% of cases). Moreover, sometimes HBV can also manifest itself with extrahepatic symptoms, with rheumatological (e.g. panarteritis nodosa and vasculitis) or renal (e.g. membranous and membranoproliferative glomerulonephritis), mainly due to the blood circulation of immune complexes.
About 25% of patients with chronic HBV hepatitis do not survive due to complications of cirrhosis or HCC. [72]
A radical change in the natural history of HBV-related liver disease has been achieved with the introduction of new nucleoside (Lamivudine, Entecavir) and nucleotide (Tenofovir) antiviral treatments. These drugs have a remarkable rate of efficacy in the suppression of viral replication, higher than interferon therapy alone, and greater tolerability, given the lower incidence of adverse effects (asthenia, anorexia, flu-like syndrome, myalgia in mild cases, significant neutropenias. and thrombocytopenias in severe cases) which sometimes required a lower dose until discontinuation of interferon therapy. Furthermore, even if these drugs are not able to eradicate the infection, as is the case for the new drugs for hepatitis C, it is possible to obtain a suppression of viral replication, thus allowing to stop or slow down the progress of the disease and therefore the onset of fibrosis (sometimes they can also induce a regression) and subsequent cirrhosis, thus guaranteeing patients a state of inactive carrier, asymptomatic and without risk of progression. [73] [74]

1.2.2 Hepatitis C

The hepatitis C virus (HCV) is an RNA virus that belongs to the Flavivirus family. The natural targets of HCV are hepatocytes but a probable target is represented by B lymphocytes, which could explain the increased incidence of lymphoproliferative diseases in patients with HCV-related liver disease. Replication occurs through an RNA-dependent RNA polymerase, lacking a proof-reading system. [75]

HCV is represented by six different genotypes, each with a characteristic geographic distribution: in developed regions, such as the United States, Western Europe and Australia, genotypes 1a and 1b prevail, followed by genotypes 3 and 2; in developing countries, on the other hand, genotypes 3 (especially India and Pakistan), 4 (especially in Egypt), 5 in South Africa and 6 in the regions of South-East Asia prevail. Determining the genotype in the patient with hepatitis C is of extreme importance as the genotype influences the response to therapy. [76]

As in the case of the hepatitis B virus, HCV is also transmitted parenterally.

Poor socioeconomic conditions, unprotected sexual intercourse, and the use of unsterilized equipment in healthcare settings are linked to an increased risk of infection. Maternal-fetal transmission occurs but is rare and often associated with HIV-1 co-infection in the mother. [77]

Like HBV infection, HCV infection is rarely diagnosed during the acute phase of the infection, which usually has a totally asymptomatic course or with mild flu-like symptoms. [78]

HCV is a disease with a high prevalence in the population and which presents numerous extrahepatic manifestations, capable of determining a lowering of the quality of life even in the absence of severe liver disease and cirrhosis. [79]

The natural history of chronic hepatitis C is influenced by numerous intrinsic factors of the affected individual, viral and environmental. [80]

Most of these are immutable: advanced age, male sex, genetic predisposition, presence of diabetes and insulin resistance, burden viral and viral genotype. Modifiable factors are obesity, sedentary lifestyle and voluptuous habits such as alcohol abuse, smoking and cannabis use. [81]

Alcohol in particular, which represents a hepatologic agent, accelerates the progression of liver damage caused by other etiological agents, especially when consumed in high quantities by individuals with hepatitis C. Several studies have shown that males are more susceptible than females to developing fibrosis and cirrhosis in case of HCV infection, possibly due to an effect inhibitor of estrogens on fibrosis. [82]

Co-infection with the hepatitis B virus determines an important acceleration of the progression of the disease, a problem that has been largely reduced thanks to the introduction of vaccination.

In addition to liver disease, there are important extrahepatic manifestations of HCV infection. Most of these syndromes are associated with autoimmune or lymphoproliferative states and are related to the possible interaction between HCV and lymphoid cells. [83]

These manifestations range from vasculitis associated with weakness and arthralgia,[84] o more severe cases characterized by the presence of membranoproliferative glomerulonephritis, peripheral polyneuropathies and type II cryoglobulinemia, of which HCV is the main cause. A higher incidence of non-Hodgkin's lymphoma in HCV infection was also observed. [85]

Similarly to what happened for hepatitis B, even in the case of hepatitis C the introduction of new drugs in recent years has allowed a radical change in the natural history of the disease. These drugs, defined DAA (Direct Acting Antiviral) are characterized by an excellent tolerance profile and a low incidence of adverse effects compared to the old therapies based on interferon and ribavirin and are extremely easy to take and better

compliance by the patient, being administered orally. The very high therapeutic efficacy allows a complete and definitive elimination (eradication) of the disease in 95% of patients, thus allowing both a resolution of secondary complications and the arrest of the progression of liver damage, as the etiological agent is eliminated responsible for all necroinflammatory processes affecting the hepatic parenchyma. In addition, the complete eradication of the virus allows a considerable advantage also in the perspective of a possible liver transplant, allowing the patient to arrive at the transplant itself in the absence of disease and thus avoid post-surgery relapses, unfortunately very common in the pre- DAA. [86]

1.2.3 Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH)

NAFLD is a general term used to describe a continuum of liver conditions ranging from simple steatosis ("fatty liver"), to nonalcoholic steatohepatitis (NASH), characterized by hepatic steatosis and hepatocyte necroinflammation, with or without fibrosis. To diagnose NAFLD it is necessary to exclude other causes of liver damage such as drugs, viruses, autoimmune processes and, above all, significant alcohol consumption (> 20g / day in women and> 30g / day in men corresponding respectively to 2 or 3 drinks / day). [87] A meta-analysis estimated that the global prevalence of NAFLD is around 25%, with peaks of around 30% in regions such as the Middle East and South America. However, forecasts indicate that this disease will experience a significant increase in future years. The same meta-analysis highlights how NAFLD / NASH are pathologies closely linked to dysmetabolic conditions such as diabetes, obesity, hypertriglyceridemia and dyslipedemia, with a prevalence reaching peaks of 70% in patients with type two diabetes. [88]

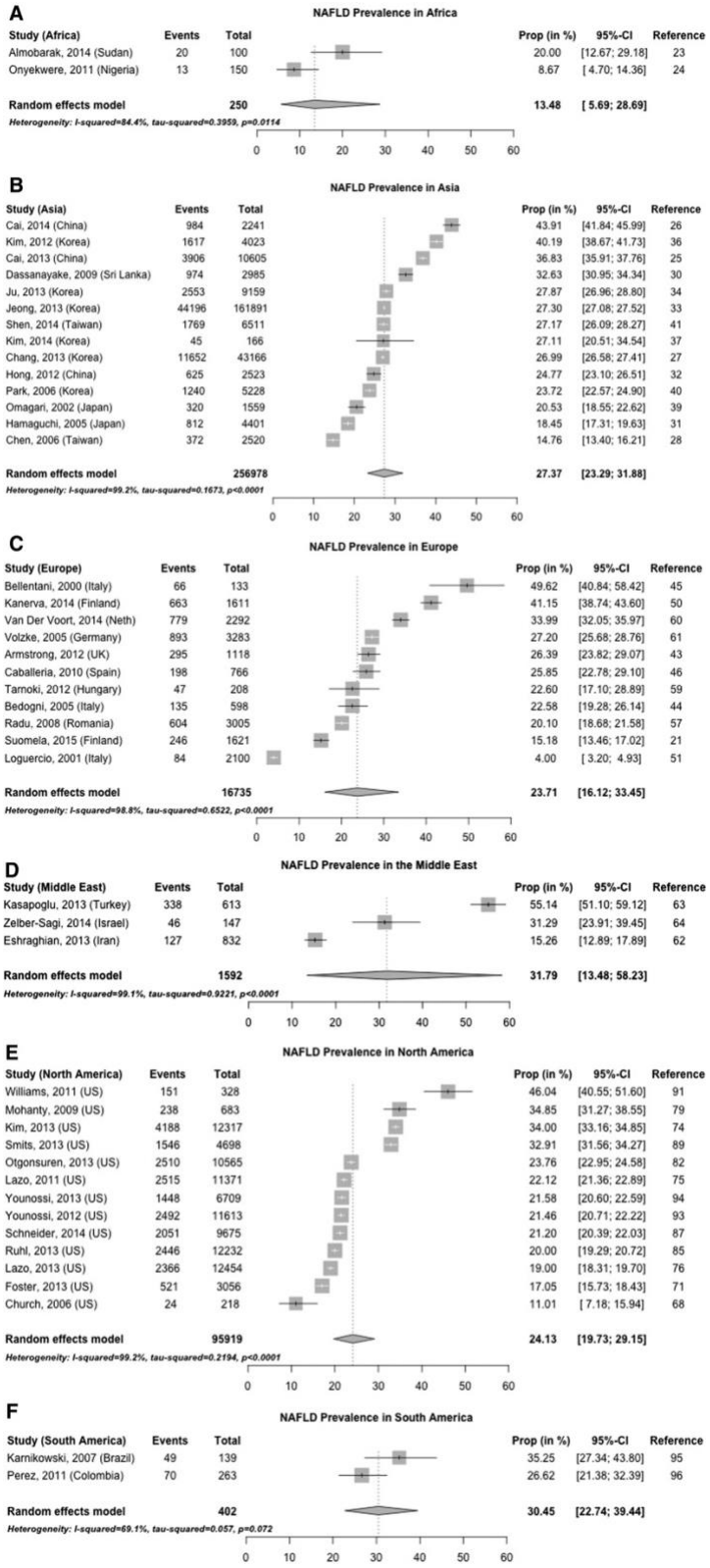


Figure 15 Meta-analysis of the global prevalence of NAFLD[88]

This is all relevant as NAFLD is often a progressive disease associated with significant complications such as cirrhosis, hepatocellular carcinoma (HCC) and overall mortality.
NAFLD is a complex and multifactorial disease, influenced by numerous genetic and environmental factors. Most studies agree that NAFLD is a hepatic manifestation related to the metabolic syndrome, that is a clinical condition characterized by the presence of visceral obesity and high BMI, LDL hypercholesterolemia and low HDL levels, hypertriglyceridemia, arterial hypertension and insulin resistance. In fact, the prevalence of NAFLD increases with increasing body mass index (BMI), from 25% in subjects with weight normal, 67% in overweight subjects and 91% in obese subjects. Furthermore, the metabolic syndrome causes cardiovascular events and mortality in patients with NAFLD. [89]

Although fatty liver disease is asymptomatic, has a negligible risk of developing into cirrhosis, and patients have a similar life expectancy to the general population, a small percentage of individuals with NAFLD may develop into NASH, which predisposes the liver to possible evolution into fibrosis and cirrhosis, with related complications.[90]
It has also been shown that NASH appears to be more prevalent and significantly accelerated in the presence of insulin resistance and diabetes mellitus. The explanation is given by the fact that with the presence of insulin resistance there is an increase in the production of glycerol and non-esterified fatty acids (Free Fatty Acids [FFA]) due to the lack of ability of insulin to effectively block lipolysis and due to increased dietary intake, typical of patients with metabolic syndrome. FFAs are absorbed by the liver, which produces triglycerides and very low-density lipoproteins (VLDL). In addition, excess carbohydrates resulting from a high-calorie diet stimulate de novo lipogenesis, which is another important contribution to the increase in triglycerides in the liver. FFA metabolites cause hepatocellular damage in the form of endoplasmic reticulum stress, mitochondrial dysfunction with ROS production and oxidative stress, apoptosis, activation of pro-inflammatory cytokines, inflammation, necrosis and dysmorphic features such as swelling and formation of Mallory's bodies -Denk. [91]
The accumulation of lipids at the level of hepatocytes favors, in genetically predisposed subjects, the onset of chronic liver damage, with activation of Kupffer cells and hepatic stellate cells, responsible for the formation of fibrosis.[92]
A meta-analysis that included 86 studies showed that patients with uncomplicated fatty liver disease had an annual HCC incidence of 0.44 per 1000 person-years, while patients with NASH had an annual HCC incidence rate of 5.29 per 1000 person-years.
Another meta-analysis of 16 studies showed that NAFLD patients have a higher risk of fatal and non-fatal cardiovascular events than patients without NAFLD, and that the severity of the disease is related to the severity and frequency of these events. [93]

1.2.4 Alcoholic Liver Disease (ALD) and Alcoholic Steato-Hepatitis (ASH)

Alcohol is one of the most common causes of chronic liver disease in most industrialized countries, as well as being a global health problem with enormous social and economic consequences. Overall, alcohol abuse causes about 20-25% of liver cirrhosis cases. High

alcohol consumption is closely correlated with the onset of fatty liver disease, but only a minority (about 10%) of patients progresses into cirrhosis. [94]

The estimate of daily alcohol consumption can be made using an arbitrary unit called alcohol unit (AU) or drink. The AU corresponds to 12-13 g of pure ethanol, and is contained in a 125 ml glass of wine, or in 330 ml of beer or in 40 ml of spirits. The acceptable daily intake limit is a maximum of 20 g / day for women and 30 g / day for men. Female sex, obesity, genetic predisposition and cigarette smoking can promote the onset of alcohol-related liver disease (ALD) and contribute to the overall risk of developing alcohol-related steatohepatitis (ASH). [95]

The most important risk factors for the development of alcohol-dependent disease remain the time of exposure and the amount of alcohol consumed, although there is no cutoff that can predict its occurrence.

Alcohol abuse can cause liver damage ranging from mild conditions such as simple steatosis to more serious conditions such as hepatitis and fibrosis, up to overt cirrhosis and the consequent high risk of developing hepatocellular cancer. Although steatosis is reversible, the persistence of alcohol intake and abuse predisposes the patient to disease progression. Cirrhosis occurs in about 10% of patients and is initially micronodular but later evolves into a mixed or macronodular pattern. Hepatocarcinoma typically develops in this context. [96]

The liver damage caused by alcohol is due to the fact that the liver is the main organ responsible for detoxifying numerous substances, including alcohol. The alcohol metabolites (acetaldehyde, acetate, ethanol and fatty acid esters, ethanol- protein adducts) are able to create damage directly on site, acting as hepatotoxins. Alcohol is oxidized by enzymes such as alcohol-dehydrogenase (ADH) which transforms alcohol into acetaldehyde and acetaldehyde dehydrogenase (ALDH) which transforms the latter into acetate and NADH.

The increased NADH / NAD + ratio also promotes the synthesis of fatty acids that contribute to the development of steatosis. The oxidation of excess NADH requires high quantities of oxygen which will be absorbed by arterial blood, effectively creating an insufficient supply of oxygenated blood to hepatocytes: this explains the reason why the first area to show hypoxic damage is that around the centrilobular vein (perivenous spaces) up to the periportal spaces. [97]

Acetaldehyde has been shown to be a toxic compound capable of promoting pro-fibrotic and inflammatory processes in the liver, for example by activating transforming growth factor (TGF) -β in hepatic stellate cells (HSC) and creating immunogenic adducts (malondialdehyde) by covalent bonding with proteins, lipids and DNA. [98]

Another factor that contributes to the development of inflammation in alcoholic liver disease is intestinal lipopolysaccharide (LPS): alcohol is able to damage the intestinal barrier leading to an increase in circulating LPS levels which leads to the activation of NF -κB and tumor necrosis factor (TNF) -α and induce an increase in circulating pro-inflammatory cytokines. TNF-α, in turn, further worsens intestinal permeability. Furthermore, ethanol activates genes that regulate lipogenic enzymes such as the sterol regulatory element-binding protein-1c (SREBP-1c), which promotes the synthesis of triglycerides and the accumulation in the cytoplasm of hepatocytes, favoring steatosis. [99]

1.2.5 Cirrhosis

Cirrhosis is a slow-progressing disease, widely spread throughout the world, and represents the common and terminal stage of chronic liver disease, regardless of the causative agent that causes it. It typically develops through inflammatory and pro-fibrotic processes at the level of the hepatic parenchyma, in which the altered deposition and accumulation of connective tissue progressively prevents proper functioning. Histologically, cirrhotic liver is characterized by the presence of fibrotic connective tissue and regenerative nodules, which develop in response to the chronic damage to which the liver is subjected. Based on the size of the nodules it can be classified as micronodular or macronodular (minor or major nodules of 3mm). Micronodular cirrhosis typically originates in the context of alcoholic liver disease, while macronodular cirrhosis typically has viral origin.[100]
The main causes of cirrhosis are chronic liver disease, mostly represented by chronic hepatitis B (CHB), chronic hepatitis C (CHC), steatosis and non-alcohol-related steatohepatitis (NAFLD / NASH) and alcohol-related (ALD) / ASH). Other less frequent causes are autoimmune hepatitis, metabolic disorders, iatrogenic and vascular causes. The risk of alcohol-related cirrhosis is closely associated with drinking habits. Metabolic syndrome often overlaps with high alcohol consumption and, likewise, alcohol abuse often overlaps with hepatitis in many patients. chronic virus-related creating a substantial increase in the risk of disease progression.[101]
The pathophysiological mechanisms underlying liver cirrhosis are hepatocyte necrosis and the deposition of disorganized fibrotic tissue within the parenchyma, as a response mechanism to liver damage. This causes an alteration of the hepatic microcirculation which leads to the progressive reduction of the functioning hepatic mass. Furthermore, these alterations are the basis for the development of portal hypertension, a key event in the development of all complications related to liver cirrhosis.
In chronic liver diseases, the perpetuation of damage to hepatocytes activates the proliferation of inflammatory cells, Kupffer cells and platelets which, in turn, release cytokines: Interleukin (IL) -2, IL-6, IL-1, TNFα and factors of growth such as platelet derived growth Factor-ß (PDGF-B) and TGF-ß. These inflammatory cytokines cause the activation of hepatic stellate cells (HSC) which, from cells used to store vitamin A, acquire a phenotype similar to fibroblasts and become cells capable of synthesizing collagen. Initially the deposition of collagen is limited to the subendothelial areas, progressively this process will cause extensive fibrosis, incorporating the hepatocytes in disordered nodular structures, the regeneration nodules.[102]
The disease is typically asymptomatic in the early stage and is termed compensated cirrhosis. Sometimes this stage can be randomly diagnosed through other tests. With the onset of complications mainly due to the subversion of hepatic microcirculation and portal hypertension (ascites and spontaneous bacterial peritonitis, esophageal varices, encephalopathy, hepatorenal syndrome and discoagulopathy) and liver dysfunction, the condition called decompensated cirrhosis is established. These complications often lead to hospitalization, reduced quality of life and high mortality.
The average survival of a cirrhotic patient depends on the precocity of the diagnosis: with an early diagnosis, before the onset of complications, survival can be much higher than ten years, while it drops to about two years in case of decompensated cirrhosis. [103]
Nowadays, the treatment of cirrhosis and its complications allows for an improvement in

the quality of life and a slowdown in the progression of the disease, but the only curative treatment remains liver transplantation.

1.2.6 portal hypertension

The portal system is a venous system, used for the drainage of blood from the abdominal organs towards the liver, characterized by a high flow rate and low resistance. Blood from the liver reaches the inferior vena cava via the hepatic veins and subsequently enters the systemic circulation.
The main trigger of portal hypertension is the subversion of the normal structure of the liver parenchyma caused by cirrhosis: the apposition of fibrotic tissue and the formation of regenerative nodules, the capillarization of sinusoids and the contraction of vascular smooth muscle cells (VMSC) and of HSC, favored by the presence of vasoconstrictors (eg endothelin) causes an increase in intrahepatic vascular resistance.
Clinically, portal hypertension is defined as a sustained increase in the pressure gradient between the portal and systemic circulation. The most common parameter used to measure portal pressure is the hepatic venous pressure gradient (HVPG), which represents the pressure difference between the portal vein and the inferior vena cava. In healthy individuals, normal HVPG is 1-5 mmHg. In compensated cirrhosis, an HVPG between 6-10 mmHg is a sign of mild portal hypertension that has no symptoms or complications, while portal hypertension becomes clinically significant when the HVPG exceeds 10 mmHg. Decompensated cirrhosis is characterized by an HVPG greater than 12 mmHg, and is typically characterized by the development of porto-systemic shunts, ruptured esophageal varices, and bleeding. [104]
Most of the complications that occur in chronic liver disease are due to portal hypertension and define the patient's transition from the compensated cirrhotic phase to decompensated cirrhosis. These complications are the formation and subsequent bleeding from esophageal varices, congestive gastropathy, the formation of ascites and encephalopathy. Less frequent but equally fearful complications are spontaneous bacterial peritonitis, hepatorenal syndrome, coagulopathy and portal thrombosis.
These complications lead to a deterioration of the patient's clinical condition with a drastic decrease in median survival from about 10-12 years to about 2 years.[105]

1.2.7 Hepatocellular Carcinoma

Hepatocellular carcinoma accounts for 80% of liver cancers. hepatocellular carcinoma is classified as the fifth most common malignancy and the fourth cause of cancer death, but its prevalence is estimated to increase in the future. [106]
Men are more at risk of developing HCC than women, especially in high-incidence regions

with a 3.7: 1 ratio possibly due to a protective effect of estrogen.[107] Cirrhosis increases the risk of HCC, especially if due to hepatitis C (HCV), hepatitis B (HBV), non-alcoholic fatty liver disease (NAFLD). However, the predominant causes are chronic hepatitis caused by HBV and HCV, which together cause about 80% of HCC in the world. Aggravating risk factors are advanced age, male sex, HBV or HIV coinfection, alcohol consumption, diabetes mellitus, while a reduction in risk is achievable by eradicating the infection by drug therapy. eradication must occur before the onset of cirrhosis, otherwise the risk of progression to HCC persists. Chronic HBV hepatitis presents a risk of the onset of HCC even in the absence of cirrhosis, although 70-90% of HCCs are diagnosed in cirrhosis.[108]
As for HBV, HCC associated with NAFLD also occurs frequently in the absence of cirrhosis.
Other rarer causes of HCC are chronic biliary diseases, genetic and metabolic diseases of the liver. [109]

Carcinogenesis

The pathogenesis of HCC is complex and is characterized by numerous molecular events that affect cell cycle regulation, chromosomal instability, DNA methylation, epithelio-mesenchymal transition (EMT), stem cell deregulation and numerous alterations borne by microRNAs (miRNAs). Although the mechanisms of liver damage that progressively lead to the onset of HCC are different depending on the etiological agent, the classic sequence of progression is liver inflammation following the injurious insult, fibrosis which subsequently evolves into cirrhosis and tumor development. Damaged or necrotic cells release molecular mediators such as Damage-Associated Molecular Patterns (DAMPS) into the microenvironment while for example viral cells can release Pathogen-Associated Molecular Patterns (PAMPS): these molecules are able to activate an innate immune response by the activation of Pattern Recognition Receptors (PRR) including the Toll-Like receptor (TLR). Primarily there will be an acute inflammatory response, which is reversible once the cause of the damage has been eliminated, but if this does not happen, the inflammation becomes chronic and is the cause of the progression into fibrosis and cirrhosis.
Numerous genetic mutations and epigenetic modifications have been found in hepatocellular carcinoma. The most frequent somatic mutation concerns the TERT promoter, a gene that regulates the expression and reactivation of telomerase, other frequent mutations in HCC concern the CTNNB1 genes, coding for β-catenin (important for the stabilization of the cytoskeleton, for the stability of intercellular junctions, and also plays the role of transcription factor in the signaling pathway called wingless / Wnt.), inactivating TP53 mutations (cell cycle regulator). CTNNB1 mutations have been associated with tumors caused by HCV and HBV, with a tendency to invasion and metastasis.
In addition to CTNNB1, other genes involved in the WNT pathway, such as AXIN1, AXIN2 and APC can be mutated in HCC. ARID1A and ARID2 mutations have recently been discovered, implicated in chromatin remodeling and tendentially associated with

HCCs of non-viral etiology.[110; 111]

The mutation of the CUL7 gene is also involved in liver carcinogenesis associated with metabolic syndrome, the amplification of which might influence cell proliferation. Despite the progress of research in this field, none of the molecular classifications of HCC are able to predict the prognosis or the risk of cancer recurrence. The only effective strategy is primary prevention, vaccination against HBV, minimizing the chance of HCV infection, maintaining a healthy lifestyle and avoiding risk factors such as alcohol, smoking, obesity, and minimizing exposure food with aflatoxins.[112]

1.3 The gut-liver axis

The term "gut-liver axis" refers to the two-way communication between the gut and its microbiota and the liver via the biliary tract, which results from interactions between signals produced by environmental, genetic, and nutritional variables. By releasing bile acids and numerous other bioactive mediators into the biliary tract and the systemic circulation, the liver communicates with the intestine.

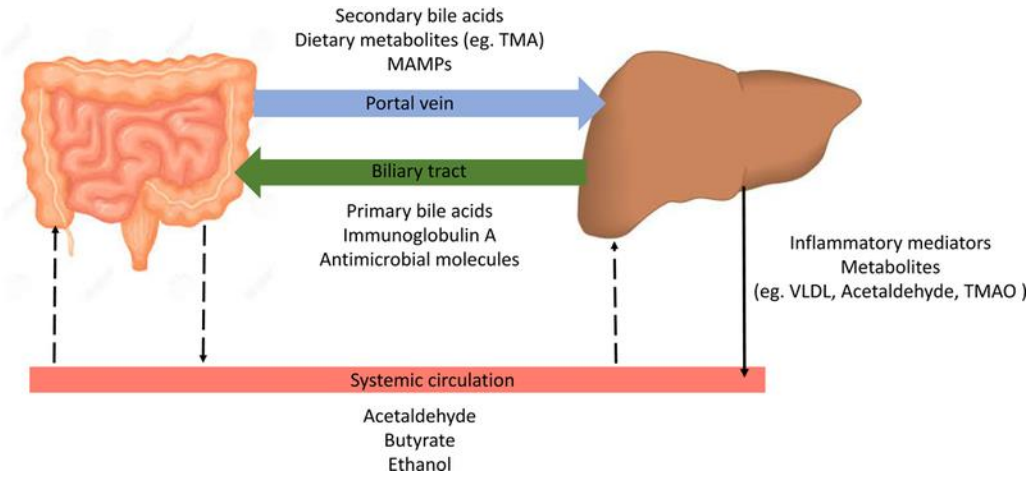


Figure 16 bidirectional communication between gut and liver[2]

Because the gut and liver are interdependent, it is understandable why problems with the intestinal barrier produce an increase in the portal inflow of bacteria or their products into the liver, where they can lead to or increase a variety of hepatic disorders. It has long been understood that gut microbes play a role in liver illnesses such alcohol-related liver disease (ALD) or bacterial infection in advanced liver disease.[2]

1.3.1 The mucus barrier, the epithelial barrier and the gut-vascular barrier

The portal vein, which transports products from the gut to the liver, and the feedback of bile and antibodies from the liver to the intestine establish the reciprocal relationship between the microbiome and the liver. In reality, bile acids play a crucial role in controlling the gut microbiota in addition to interacting with nuclear receptors to govern metabolic processes. The gut mucosal barrier, which is made up of intestinal epithelial cells that maintain gut homeostasis by separating the gut microbiota and host immune cells, serves as the interface between the liver and the microbiome.
The mucus barrier serves as a physical barrier between the microbiota and the epithelial lining, preventing an excessive inflammatory response.
Where the microbiota is found, such as in the terminal ileum and colon, the mucus barrier is thicker and varies in thickness across the various gut segments.

The gut barrier is made up of a monolayer of enterocytes, goblet cells, tuft cells, and enterochromaffin cells that lies just beneath the mucus layer.
These cells work together to defend the gut from harm caused by infectious diseases and the microbiota. This barrier combines three different types of defenses: physical (tight connections between adjacent epithelial cells), electrical (the brush border's negative charge opposes the microbiota's negative charge), and chemical (epithelial cells release a sequence of antimicrobial peptides). Additionally, the epithelium is guarded by a number of mucosal immune cells, which are discussed later.
But only a small portion of them will be able to spread systemically. Some will go to the lymph nodes in the mesentery. The gut vascular barrier (GVB), another barrier, keeps bacteria from entering the portal circulation and getting to the liver. However, some pathogenic bacteria and probably some pathobionts have evolved strategies to elude this barrier. [113]
Interesting, certain pathologic diseases like celiac disease, non-alcoholic steatohepatitis (NASH), and non-alcoholic fatty liver impair this barrier (NAFLD).
Early steps in the development of NASH include disruption of the gut vascular barrier (GVB) and intestinal epithelial barrier. According to a study, mice fed HFD for just one week experience a dysbiosis brought on by the diet that results in GVB damage and bacterial translocation into the liver. When HFD-fed mice's feces are transplanted into certain pathogen-free recipients, it damages the GVB and enlarges the epididymal adipose tissue. Genetic modification that causes -catenin activation solely in endothelial cells, avoiding GVB disruption and NASH development, demonstrates that GVB disruption requires on interfering with the WNT/-catenin signaling system. The GVB leakage marker was also found to be upregulated in the colons of NASH patients, according to the study. We can therefore conclude that NASH development can be stopped by medications that guard against breaking the barrier. [114]

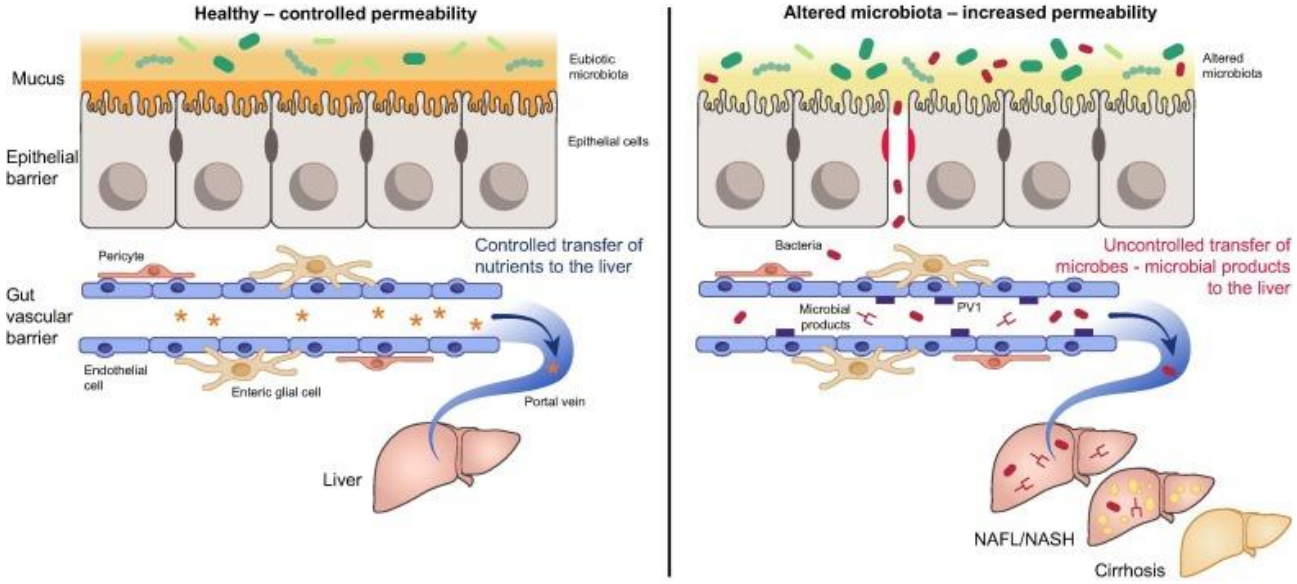


Figure 17 the intestinal barrier with a healthy-controlled permeability versus with an altered microbiota-increasead permeability[115]

1.3.2 Microbiota in liver inflammation and in liver disease

Inflammatory bowel disease has long been thought to be primarily influenced by the microbiome.
There are three main ways that intestinal microbiota might encourage metabolic illness.
When transplanted into germ-free mice, the microbiota from obese humans exhibits changed Bacteroidetes/Firmicutes ratios, which increase energy harvest and obesity. This is the first way that microbiota can affect how effectively energy is obtained from diet. The production of hazardous metabolites from food is another way that microbial metabolism can have a bad effect on the host. For instance, the conversion of choline to the heart disease risky phosphatidylcholine by the microbiota.
Additionally, TLR/NLR agonists from the microbiota that activate pro-inflammatory signaling in the organs that regulate central metabolism may have a role in this metabolic disorder, at least in part.
Due to the size of the gut microbiota and the portal vein link, it is possible that some gut bacteria and their byproducts are entering the liver. It makes sense to think that in rare occasions, a very small but possibly not negligible minority of bacteria could get past the gut epithelium and fast reach the liver.
Numerous studies show that Kupffer cells respond to very low concentrations of LPS by activating NF-B and producing pro-inflammatory cytokines, similar to most populations of macrophages. This suggests that these cells would respond to physiologically relevant levels of microbial products that reach the liver.
Additionally, activation of liver NLRs may occur more frequently in settings of greater uncommon danger, such as an infection, whereas activation of liver TLRs on Kupffer may play a role in liver homeostasis. It has been demonstrated that the gut microbiota's higher levels of TLR/NLR activation contribute to chronic liver inflammatory illness.

- Alcoholic liver disease

  There is growing evidence that alcohol increases gut permeability and liver exposure to endotoxin from the gut, which activates TLR4 and causes alcohol-induced tissue damage.
  Alcohol appears to promote intestinal permeability through a mechanism that is driven by expression of the tight junction protein is altered by drinking.
  A central role for gut-derived microbial products in alcohol-induced liver dysfunction is suggested by the increased intestinal permeability, which increases the load of a range of microbial products that can lead to excessive activation of both TLR and NLR driven pathways. Alcohol can also stimulate liver TLR/NLR activation by changing the microbiota's constitution.
  Alcohol not only increases TLR/NLR activation directly, but it also causes an increase in Proteobacteria and Gram negative bacteria in general. This increase in acetaldehyde accumulation causes an increase in tyrosine phosphorylation of tight junction and adherent junction proteins, which can increase intestinal permeability to bacterial products.

These findings support the idea that endotoxins, in particular, are major contributors to alcohol-induced liver illness and raise the possibility that inhibiting endotoxin's ability to be recognized by TLR4 could be used to cure or prevent alcohol-induced liver damage.

- Non-alcoholic fatty liver disease

According to a study, mice on a high-fat/simple carbohydrate diet have increased intestinal permeability, elevated blood endotoxin levels, and mildly elevated levels of pro-inflammatory cytokines, all of which are associated with NAFLD and other aspects of the metabolic syndrome.
A change in the composition of the gut microbiota caused by the loss of genes involved in innate immune recognition of the microbiota has been observed in studies in mice, and this enhanced activation of compensatory innate immune signaling pathways. These phenomena are linked to the emergence of numerous metabolic syndrome symptoms, which, when combined with a western diet, lead to NAFLD. For instance, an altered microbiome with various bacterial species that were either over or underrepresented was seen in the TLR5-deficient animals. Studies on mice conducted recently by Flavell and colleagues provide some of the strongest evidence yet that changed microbiota can induce NAFLD. They discovered that mice lacking either NLRP3 or NLRP6, two NLRs that trigger the inflammasome in response to the detection of foreign protein and/or host-derived danger signals, are significantly more susceptible to both NAFLD and metabolic syndrome caused by methionine/choline deficiency diets.
Diet may influence the composition of the gut microbiota, which may then affect how the host-microbiota dynamic functions in NAFLD. In fact, high-fat diets change the phyla ratios and promote the proliferation of Proteobacteria in the gut microbiota, which can both boost the microbiome's pro-inflammatory potential. Furthermore, studies in mice demonstrate that high-fructose diets cause the expression of tight junction proteins to drop off quickly, changing the way the gut barrier functions. When the products of the leaky gut microbiota are detected, the increased expression of liver TLRs correlates with the reduction in gut tight junction protein expression, which is likely to cause inflammation.
Yet, the molecular factors (eukaryotic) or metafactors (prokaryotic) connecting gut microbiota to NAFLD are poorly understood. Hepatic miR expression changes as a result of NAFLD, although it is yet unclear whether gut microbiota also affects hepatic miR expression to cause NAFLD. Since little is known about the molecular processes connecting the gut microbiota to the host liver pathology, this knowledge gap represents a significant research challenge that needs to be overcome. [116]

2. AIM OF THE PROJECT

The pandemic prevalence and economic cost of obesity and its metabolic complications such as type 2 diabetes, jointly named diabesity, represent key health concerns. Diabesity is characterised by triglycerides (TG) storage in the liver. Even if is not yet known how the gut microbiota induces NAFLD through a change in liver miRNAs, the researchers collaborating with our team (Matteo Serino from INSERM in Toulouse) assume that miRNAs mechanistically link gut microbiota dysbiosis to hepatic TG storage.
To gain insights into the pathophysiological relevance of gut microbiota-liver miRs axis, our team and the team from Toulouse propose to develop the following work-packages and releted aims:

- Impact of miRNA-21 modulation on hepatic tg storage during diabesity, with the aim of evaluating the function of miRNA-21 in hepatic TG storage during diabesity.
- Fine identification of hepatic messengers RNAs (mRNAs) targeted by miRNA-21, with the aim of identifying the target mRNAs of miRNA-21 during diabesity.
- Identification of the metabolic network between gut metatranscriptome (microbial active pathways), gut microbiota-derived metabolites, miRNA-21 and hepatic tg storage. With the aim of identifying microbial active pathways and gut microbiota-derived metabolites in portal circulation which are associated with miRNA-21 and hepatic TG storage during diabesity.
- Other hepatic miRNAs controlled by gut microbiota during diabesity in mice and humans.

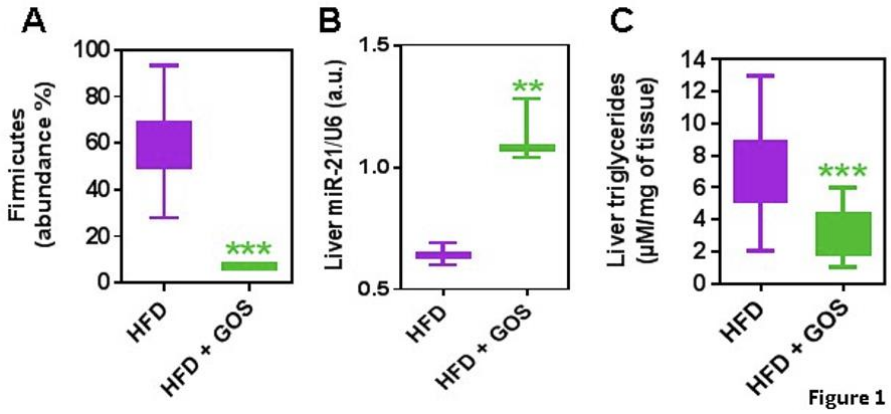


Figure 18 impact of GOS prebiotics on gut microbiota, hepatic mir-21 expression and liver TG in a murine model of metabolic adaptation to an HFD.

This data obtained by the team from Toulouese showed that hepatic expression of miR-21 is strongly and significantly associated with both hepatic TG and gut microbiota in a murine model of metabolic adaptation to a diabetogenic high-fat diet (HFD). In particular this data is demonstrating that modulating gut microbiota by GOS (glucooligosaccharides) reduces Firmicutes bacteria (Fig.1A), increases hepatic miR-21 expression (Fig.1B) and reduces hepatic TG (Fig.1C).

The team also analysed hepatic miR-21 in mice responders (diabetic-sensitive, **DS**) vs. non-responders (diabetic-resistant, **DR**) fed with the HFD. MiR-21 had a significantly higher hepatic expression in DS vs. DR mice (**Fig.14A**), associated with a higher DS hepatic TG (**Fig.14B**), and lower *Firmicutes* bacteria in DS than in DR mice gut microbiota (**Fig.14C**). Therefore, in these phenotypes (DS vs. DR mice), hepatic miR-21 expression is positively correlated to hepatic TG storage.

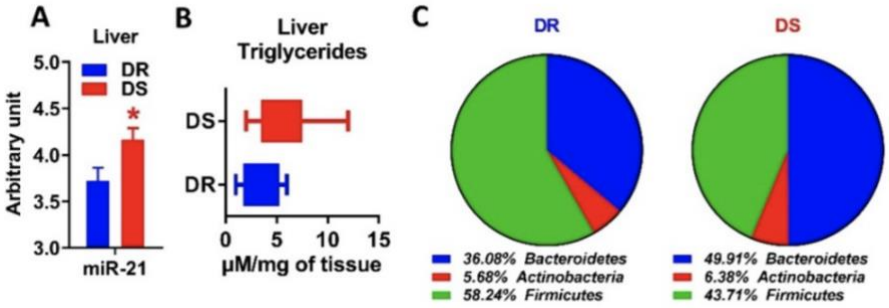
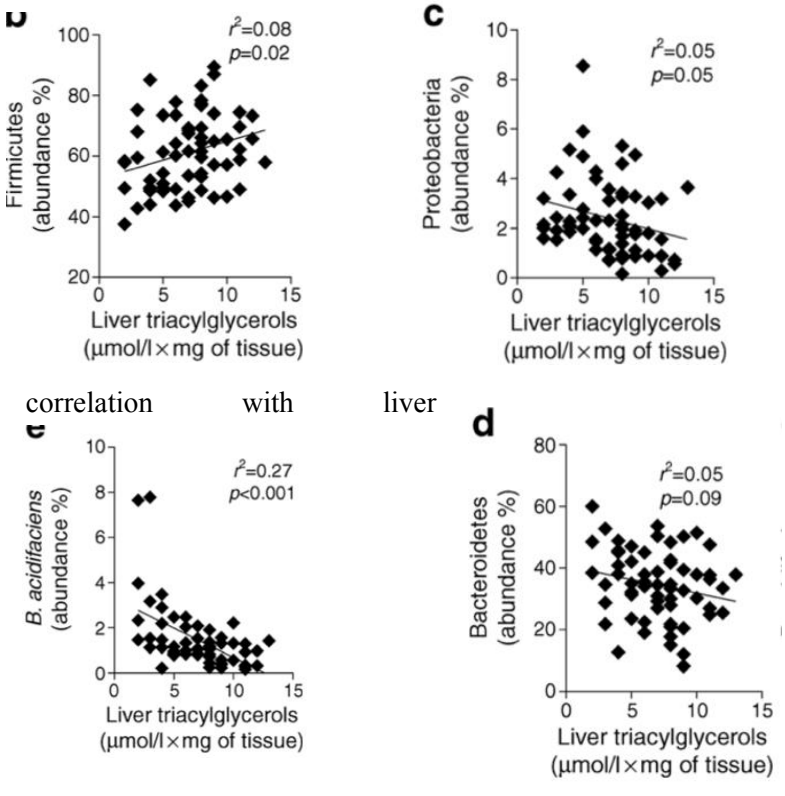


Figure 19 Analysis of hepatic miR-21 espression, hepatic TG and gut microbiota.

Hence, since the gut–liver axis manages our capacity to sense gut microbes, the team from Toulouse investigated whether bacterial groups from the gut microbiota may be associated with the diversity of hepatic triacylglycerol content in HFD-fed mice. They sequenced the gut microbiota from all mice and performed a non a prioribased analysis to identify putative correlations between bacterial phyla and metabolic variables of interest. Out of the total 12 phyla identified, three were present in all mice: Firmicutes, Proteobacteria and Bacteroidetes.



correlation with liver

Firmicutes showed a significant positive correlation with liver triacylglycerol content and Proteobacteria and Bacteroides Acidifaciens showed a significant negative triacylglycerols.

In contrast, single linear regression analysis revealed that Bacteroidetes showed a

non-significant correlation with liver triacylglycerol content.

This as regards the work of the team that collaborates with us.

Instead our team's aim is understanding molecular mechanisms underlying the pathogenic role of microRNA-21 in hepatocytes of obese patients.
In order to do this we have 3 different steps:
1) Identify the direct target RNAs of mir21 by using the iCLIP2-seq technique and RNA-seq.
2) Validate in cells the most promising, and those releted to liver metabolism, dysregulation target RNAs of miR21 by several biochemical and molecular techniques, such as:

- western Blot
- qPCR
- Gene Reporter Assay

3) Investigate in cells the physiological and pathological role of miR-21 targets by performing a global bioinformatic analysis, such as IPA analysis.

For now we are at the point 1).

3. MATERIALS AND METHODS

3.1 Cell Culture

MATERIALS
All cell lines used in this thesis were purchased from Sigma Aldrich

cell lines:

HepG2
Hep G2 [HEPG2] is a cell line exhibiting epithelial-like morphology that was isolated from a hepatocellular carcinoma of a 15-year-old, White, male youth with liver cancer. HepG2 cells are considered as a consolidated cellular model of HCC. Due to their high degree of morphological and functional differentiation in vitro, Hep G2 cells are a suitable model for studying lipid metabolism and dynamics in human hepatocytes in vitro. [117]

AML-12
AML12 (alpha mouse liver 12) cells are derived from liver of transgenic mice overexpressing transforming growth factor (TGF) α and has mainly been used for studies on lipid metabolism extending to steatosis/non-alcoholic fatty liver disease. [118]

HEK293-T
Human embryonic kidney 293 cells (HEK 293) are a specific immortalised cell line derived from an aborted fetus or human embryonic kidney cells grown in tissue culture taken from a female fetus in 1973.
HEK 293 cells are simple to culture and transfect. They were used as hosts for gene expression. Typically, these experiments involve transfection into a gene of interest and then analysis of the expressed protein. The widespread use of this cell line is due to its transfectability with various techniques.

Colture media:

- Minimum Essential Medium (Eagle) (ATCC): HepG2
- Dulbecco's Modified Eagle's Medium (DMEM): 293T
-DMEM F12 Medium: AML-12

Fetal bovine serum (FBS)
PBS (1X)
Tripsina

Penicillina-Streptomicina (100X)
L-Glutamina (100x)

METHOD

The cells were cultured and expanded at 37 ° C, in a 5% CO2 atmosphere in a medium containing 10% FBS, 2mM L-Glutamine and 1X PEN-STREP.

Trypsinization:

Remove the growth medium from each dish.
Do a cell wash with 1ml of trypsin.
Add 1 ml of trypsin.
Incubate the cells for 4 'at 37 ° C and 5% CO2.
Add 9 mL of culture medium to deactivate trypsin. Collect cells in 15 mL tubes.
Centrifuge for 5 'at 1300 rpm.
Discard the supernatant.
Resuspend the cell pellet in 10 mL of medium.
Add in new dish X ml according to the desired dilution

3.2 RNA extraction

MATERIALS:

Trizol (Invitrogen)
Chloroform
Isopropanol
Di-ethyl-pyro-carbonate (DEPC) (Sigma)
Ethanol

METHOD:

Remove media
Waqh with PBS RNase/Dnase free (X2)
Add 1ml Trizol /10cm dish
Collect cells with scraper and in eppendorf tube

Add 200ul chloroform
Mix rigorously for 15sec (by inverting tubes in paper)
Wait 2-3min at RT

Centrifuge 15min at 14 000g 4°C

Transfer aqueous phasis (500 uL) in 1.5 ml tube

Precipitate RNA with 500ul isopropanol (and add 1 ul of glycolblue if there's little RNA)
Mix by inverting 5-6 times
Wait 10min at RT

Centrifuge 10min 14 000g 4°C
Aspire the supernatant with the vacuum (leave some drops on the bottom)

Wash pellet with 1ml EtOH 70% (no invert, no vortex, do not pipette)

Centrifuge 10min at 14 000g 4°C
Aspire the supernatant with the vacuum (leave some drops on the bottom)

Spin briefly and remove all EtOH with pipette

Dry pellet at RT 10min

Resuspend pellet in 50ul H2O DEPC / or 200-300 if it's highly visible

Warm sample 10min at 55-60°C, then gently pipette up and down for about 20 times (DO NOT TOUCH THE PELLET)
Put on ice

3.3 Northern Blot

DAY 1

Sample preparation
-15-20 ug of RNA
-Max volume per lane: 25 ul

Add 15-20 ug of RNA to the same volume of TBE-urea 2X Buffer and leave the sample shaking at 70° for 10 minutes
Load the samples on a pre-cast gel (10% TBU)
Running buffer is TBE 1X (Vf= 500 ul)
Run: 10 min at 90V, then increase at 160V

Probe Preparation
(20 ul of probe for each membrane)
1ul of DNA oligonucleotide (5 pmol/ul)
1 ul of 10X Buffer for T4 PNK
13 ul of H2O
1 ul of T4 PNK
2 ul (γ-32P) ATP (in radioactive room)

Transfer
For the sandwich, put in order from below:

- sponge
- paper
- turned gel
- nylon membrane
- paper
- sponge (to fill the support)

transfer buffer is TBE 0.5X
(same buffer of the run dilueted to 0.5X)
activate the nylon membrane for 1 minute in H2O, then keep it in transfer buffet
perform the transfer at 32V for 1h30 in cold room
Once the transfer is finished, crosslink the membrane at 1200J (UV linker)

Pre-hybridization
Put the membrane in a glass tube and add the pre-hybridization solution (enough volume to cover the membrane with a thin line of solution and remove bubbles)
Leave in rotation at 37° C for at least 30 minutes or until the evening before to hybridize (in the radioactive room)

Hybridization
Add 20 ul of the probe in the glass tube and leave it in rotation over night at 37°C
DAY2

All passages must be done in the radioactive room

Washes
(25 ml/wash/membrane)

Perform 2 washes with 2X SCC 0.05% SDS using 25 ml for each membrane, for 20 minutes at room temperature (25°C), max speed
Perform 2 washes with 0.1 SCC 0.1% SDS for 20 minutes at room temperature, max speed

Develop
Expose the film and if it needs over night leave the cassette at -80°C

3.4 Reverse Transcription

First-strand cDNA Synthesis

The following procedure can be used to convert up to 5µg of total RNA or up to 500ng of poly(A) RNA into first-strand cDNA.

1. Mix and briefly centrifuge each component before use. Combine the following:

Component:
-Experimental RNA (up to 5µg/reaction)
-Primer [Oligo(dT)15 (0.5µg/reaction) and/or
Random Primer (0.5µg/reaction) or
gene-specific primer (10–20pmol/reaction)]
-Nuclease-Free Water
For a Final volume of 5µl

2. Heat in a 70°C heat block for 5 minutes. Immediately chill in ice water for at least 5 minutes. Centrifuge 10 seconds in a microcentrifuge. Store on ice until reverse transcription mix is added.

3. Prepare the reverse transcription reaction mix, 15µl for each cDNA reaction. Combine on ice, in the order listed.

Component:
GoScriptTM 5X Reaction Buffer
MgCl2 (final concentration 1.5–5.0mM)
PCR Nucleotide Mix (final concentration 0.5mM each dNTP)
Recombinant RNasin® Ribonuclease Inhibitor (optional)
GoScriptTM Reverse Transcriptase
Nuclease-Free Water (to a final volume of 15µl) X
For a Final volume of 15µl

4. Combine 15µl of reverse transcription mix with 5µl of RNA and primer mix.

5. Anneal in a heat block at 25°C for 5 minutes.

6. Extend in a heat block at 42°C for up to one hour.
Reactions can be stopped at this point for analysis of the cDNA or may be frozen for long-term storage.

7. Inactivate Reverse Transcriptase: Before proceeding with qPCR, inactivate the reverse transcriptase in a heat block at 70°C for 15 minutes.

3.5 qPCR

cDNA quantification using qPCR

prepare the plate mixing in each well 2 ul of cDNA + 8 ul master mix.

SYBR Green qPCR Master Mix:
Fast: 5 ul
Primers (forward + Reverse) 1 ul
H2O 2 ul
For a total volume of 8 ul/well

3.6 Cell transfection

There are various methods of transfection, generally they can be grouped into three categories: physical, chemical and biological. The one that we used is the lipofection, which is a chemical method.

Lipofectamine 2000 is a common transfection reagent used to increase the transfection efficiency of RNA (including mRNA and siRNA) or plasmid DNA in vitro cell cultures by lipofection. Lipofectamine contains lipid subunits that can form liposomes in an aqueous environment, which entrap the transfection payload. Liposomes can easily merge with the cell membrane since they are both made of a phospholipid bilayer.

MATHERIALS

Lipofectamine2000 (Invitrogen)
Opti-MEM
Anti-miR miRNA-21 inhibitor
OMeUCAOMeACAUOMeCAGUOMeCUOMeGAUAOMeAGCUOMeA

Anti-miR Negative Control

METHOD

For 10 cm cell dish

1) for each transfection sample prepare oligomer-lipofectamine 2000 complexes as follows:

a) Diluite 500 pm oligo in 500 ul of opti-MEM
(Final concentration= 100 nM)
Mix gently

b) Mix lipofectamine 2000 gently before use, then diluite 10 ul in 500 ul of opti-MEM
Mix gently
Incubate for 5' at room temperature

c) After 5 minutes incubation, combine the diluted oligomer with the diluted lipofectamine 2000
Mix gently
Incubate for 20 minutes at room temperature

2) Add the oligomer-lipofectamine2000 complex to each dish conteining 5 ml medium

Mix gently by rocking the plate back and forth
Incubate the cells at 37°C in a CO2 incubator for 24h
The next day add other 5 ml of Medium

3.7 RNA immunoprecipitation

Immunoprecipitation (**IP**) is the technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein.

The immunoprecipitation can be:

- individual protein immunoprecipitation (IP)
- protein complex immunoprecipitation (Co-IP)
- Chromatin immunoprecipitation (ChIP)
- RNA immunoprecipitation (RIP)

The one that we performed for our project was RIP.
RNA immunoprecipitation targets ribonucleoproteins (RNPs). Live cells are first lysed and then the target protein and associated RNA are immunoprecipitated using an antibody targeting the protein of interest. The purified RNA-protein complexes can be separated by performing an RNA extraction.

METHOD:

Seeding cells
Prepare a 10 cm dish at confluence by taking 3 days before 3 ml of cells + 7 ml of medium complete

Immunoprecipitation
10 cm dish at 80% confluence
Wash the cells with 5 ml of PBS
Add 1 ml PBS and collect cells by scraping
Centrifugate for 3' at 9000 rpm at 4°C, then put on ice
Remove PBS
Add 1 ml of lysis buffer (LB) + complete 25X
Incubate for 10' in ice
Centrifugate at max speed at 4°C for 10'
Take the supernant (where there are the proteins)
Take 1% as input

Dynobeads - protein G
Wash the beads with LB for three times
After the last wash resuspend in the volume needed
Incubate 30' at 4°C in rotation

Antibodies – dynobeads
Ab 2A8 [0,5 mg/ml]: 2 ul
Ab 11A9 [0,25 mg/ml]: 4 ul
IgG [0,2 mg/ml]: 5ul
Incubate over night at 4°C

The day after

Washing
Wash with 1 ml HSB twice
At the end add 500 ul of Trizol for the RNA extraction

Buffers:

Lysis Buffer (LB) 250 ml

- Tris HCl pH 7.5 50 mM
- NaCl 100 mM
- Triton 1%
- Sodium Deoxycholate powder 0,5%
- SDS 0,1%
- H20

+
Complete 25X

High Salt Buffer (HSB) 500 ml

- Tris HCl pH 7,5 50 mM
- NaCl 200 mM
- EDTA pH 8 1 mM
- Triton 1%
- Sodium Deocycholate powder 0,5%
- SDS 0,1%
- H2O

Antibodies:

2A8
Purified mouse monoclonal IgG1 in a buffer containing 0.1 M tris-Glycine (ph 7,4), 150 mM NaCl with 0.05% sodium azide

11A9
Purified rat monoclonal IgG in a buffer containing 0.1M Tris-Glycine (ph 7.4), 150 mM NaCl with 0.05% sodium azide.

3.8 Individual-nucleotide resolution Crosslinking Immunoprecipitation sequencing (iCLIP2-seq)

To identify the direct target of miR21 we are using the iCLIP-seq, a technique to map the RNA interaction sites of an RNA-binding protein across the transcriptome.
This new protocol comprises separate adapter ligations, two cDNA amplification steps and bead-based size selection.
We start by UV crosslinking the cells, to generate covalent bounds between RBP and target RNAs
And after lysis and partial RNA digestion we perform the RNA immunoprecipitation.
iCLIP allows for a very stringent purification of the linked protein-RNA complexes, using immunoprecipitation followed by sds-page and transfer to nitrocellulose.
The radiolabelled protein-RNA complexes are then excised from nitrocellulose, and treated with proteinase K to release the RNA, leaving one or two amino acids at the crosslink site of the RNA.
The RNA is then reverse transcribed, causing cDNAs to often truncate at the crosslink site, which is the key insight and unique feature in the development of iCLIP, as it allows identification of the site of RNA-protein interaction at high resolution.
The small amount of resulting cDNAs is then PCR amplified and sequenced using a next-generation sequencing platform.
And for the purification we use ProNex size selection system.

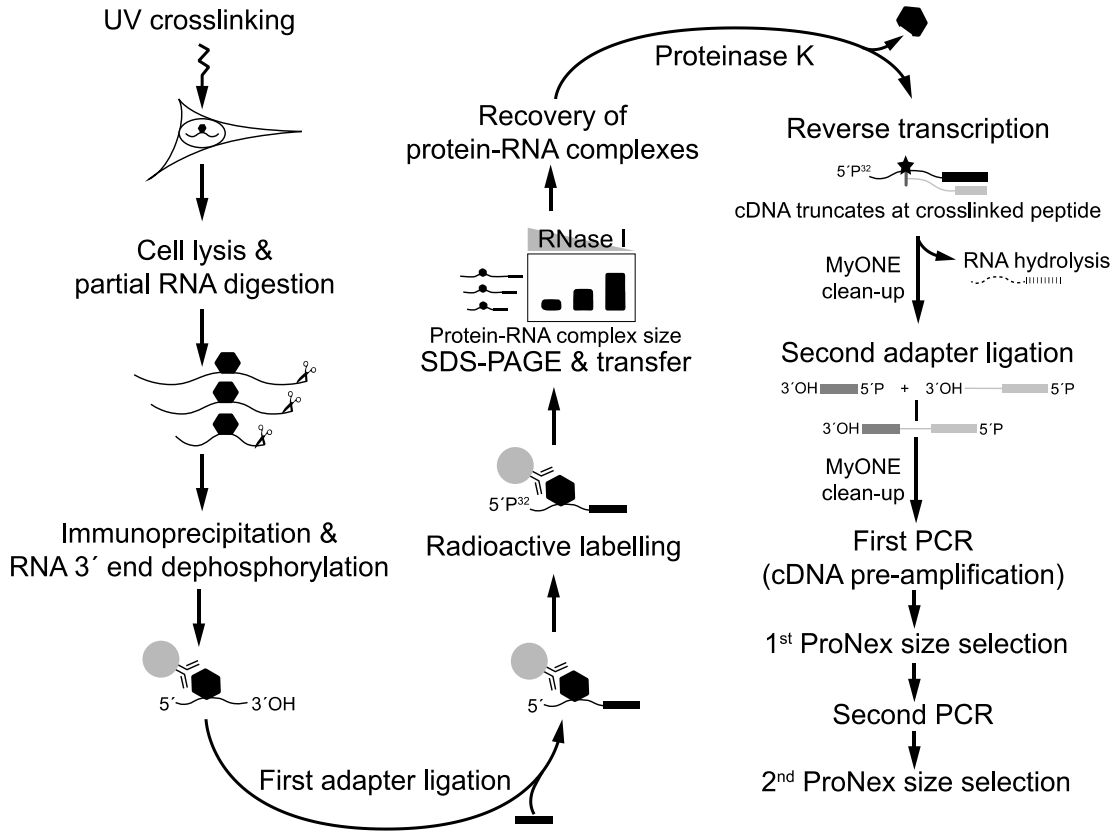


Figure 20 iCLIP2 steps

Protocol:
DAY 1

1. UV-C crosslinking
- Remove the medium, wash once with 5 ml PBS and 6 ml ice-cold PBS to cells in 10 cm plate (80% is sufficient). Remove the PBS and place the dish on ice-plate covered with a thin layer of water
- Remove the lid from cells and irradiate once with 150 mJ/cm3 in a UV-C crosslinker at 254 nm at 5 cm of distance from UV bulbs (more or less 30 sec and put 2 ml of PBS into the dishes and keep them at 4°C in the meantime)
- Harvest cells by adding 2 mL of PBS and scraping them
- Spin at 1000 g (3200 rpm) for 1 min at 4°C to pellet cells, then remove the PBS supernatant
- Snap freeze pellets (liquid nitrogen) on dry ice and store at -80°C until use

2. Immunoprecipitation – protocol for 1 IP
a. Bead preparation
- Use 70 ul of protein G Dynabeads per IP (50 ul IP and 20 ul for preclearing)
- Wash beads 2 x with lysis buffer (LB) + proteinase inhibitor
- Resuspend beads in 70 ul LB

- Take 50 ul in a new tube and add 10 ug of Anti-Ago2 (WAKO for AML12 mouse cell line)
- Rotate tubes at RT for 30 – 60 min (until lysat is ready) and it will be ready to be added into the lysate

b. Lysis and partial RNA digestion
- Resuspend cell pellet in 1 ml lysis buffer (with protease inhibitors)
- Spin at 4°C at top speed for 10 min and transfer the supernatant to a new tube
- Dose sample protein concentration and normalize them to the lowest sample
- Bradford solution (from 5X to 1X in H2O)
- Add 1 ul of sample to 1 ml of Bradford solution
- Analyze on photometer the absorbance (595 nm)
- To dose the samples, you can pool all the same samples together and then divide them equally in a 1.5 ml tube
- Prepare the optimized RNase I dilution in lysis buffer (high 1:100 and low 1:500) and add 10 ul to the lysate together with 2 ul Turbo DNase (referred to as lowest RNase sample)
- Digest RNA for exactly 3 min shaking at 37°C and 1100 rpm.
- After incubation transfer to ice for 3 min
- Load 500 ul of lysate onto a Proteus mini clarification spin column
- Spin at 4°C at 16,000 g (12,900 rpm) for 1 min
- Transfer flow-through to a new tube
- Repeat with second half of the lysate and combine both

c. Immunoprecipitation
- Preclear the lysate with 20 ul of beads previously washed
- Add the cell extract to the beads
- Rotate beads-lysate mix O.N. at 4°C

DAY 2
- Discard the supernatant and wash with 1 mL of HSB x2
- Incubate the second wash for at least 2 min on ice
- Wash with 1 mL of PNK buffer x2 and then resuspended in 1 ml PNK buffer

3. RNA 3’ end dephosphorylation
- Discard supernatant
- Resuspend the beads in 20 ul of the following mixture:

15 ul Water
4 ul 5X PNK buffer, pH 6.5
0.5 ul RNase inhibitor
0.5 ul T4 PNK enzyme (with 3’ phosphatase activity)

- Incubate for 20 min at 37°C in a thermomixer at 1100 rpm
- Wash with PNK buffer x 1
- Wash with HSB x 2 (incubate the second wash for at least 2 min on ice)
- Wash with PNK buffer x 2

4. First adapter ligation to the 3’ end of the RNA

- Carefully remove the supernatant and resuspended the beads in 20 ul of the following mixture:
8µl Water
5µl 4× ligation buffer
0.5 µl RNase inhibitor
1.5 µl Pre-adenylated L3-App (20 µM)
4µl PEG400
1µl T4 RNA ligase
- Incubate O.N. at 16 °C in a thermomixer at 1100 rpm

DAY 3

- Add 500 µl PNK buffer
Wash 2× with 900 µl high-salt wash buffer (incubate the second wash for at least 2 min on ice)
- Wash 2× with 900 µl PNK buffer and leave in 1 ml of the second wash. Transfer to new tubes after the first wash
Radioactive Room
- Collect 200 µl (20%) of beads from the previous step and remove the supernatant
- Add 4µl of hot PNK mix:
3 ul Water
0.4 ul 10× PNK buffer (NEB)
0.4 ul 32P-γ-ATP
0.2 ul T4 PNK enzyme (with 3′ phosphatase activity)
- Incubate for 5 min at 37 °C in a thermomixer at 1100 rpm
- Remove the hot PNK mix and wash beads with 1× PNK buffer
- Discard the supernatant in solid radioactive waste (in a closed tube)
- After removing the supernatant, add 20 µl of 1× NuPAGE loading buffer to the beads and 1% of beta-mercaptoethanol
- Remove the supernatant from remaining cold beads (from step 4) more or less 800 ul. Then add the radioactively labelled beads to the cold beads and incubate at 70 °C for 5 min.
- Place on magnet to precipitate the beads and transfer supernatant to new tubes.
- Place the new tubes again on the magnet and load the eluate on the gel (step 5)

5. SDS-PAGE and nitrocellulose transfer
- Load the samples on a 4–12% NuPAGE Bis-Tris gel according to the manufacturer's instructions (Life Technologies)
- Use 0.5 L of 1× MOPS running buffer. Also, load 5 µl of a pre-stained protein size marker (Loading Dye Page ruler)
- Run the gel at 100V for 1h30 in the Xcell Blot Module
- Remove the ATP-containing dye front and discard as solid radio-active waste
- Use 0.9 L of MOPS 20X / 10% EtOH or NuPAGE 20X Transfer /10% EtOH
- Transfer the protein-RNA complexes from the gel to a nitrocellulose membrane, reconstituted in H2O for 1 minute, using the Bio-Rad Blot Module according to the manufacturer's instructions at 30 V O.N.

DAY 4

- After the transfer, rinse the membrane in cold PBS buffer, then wrap it in saran wrap
- Place radioactive and visible dots next to the membrane to later align the picture of the autoradiograph with the membrane
- Expose the screen for 1h at -80°C if there is a strong radioactive signal (or over day if very low), then develop the autoradiograph film

6. RNA isolation

- Isolate the protein-RNA complexes from the low-RNase experiment using your autoradiograph as a mask for cutting the respective region out of the nitrocellulose membrane. Use the radioactive, visible dots to align mask and membrane. The region can be taken either in a single piece or further divided.
- Place the membrane fragments into 1.5 ml tubes. Since the membrane is too large to fit down to the bottom of the tube, cut it into several pieces by using a clean scalpel blade before placing it into the tube
- Add 10 µl proteinase K in 200 µl PK buffer to the nitrocellulose pieces (all should be submerged)
- Incubate in a thermomixer for 20 min at 37 °C and 1100 rpm
- Add 200 µl of PK + urea buffer by pipetting up and down and incubate in a thermomixer for further 20 min at 37°C and 1100 rpm
- Collect the solution and add it together with 400µl phenol/ chloroform
- Incubate in a thermomixer for 5 min at 30 °C shaking at 1100 rpm (do not vortex)
- Separate the phases by spinning for 5min at 16,000g (12,900 rpm) at RT
- Transfer the aqueous layer into a new tube (be careful not to touch the gel matrix with the pipette).
- Spin the supernatant again for 1 min and transfer into a new tube
- Precipitate by addition of 1 µl GlycoBlue and 50 µl 3 M sodium acetate pH 5.5.
- Mix and add 1 ml 100% ethanol, mix by inverting several times and place O.N.

DAY 5

- Put the tubes on ice to thaw
- Spin for 20 min at 21,100g (14,800 rpm) at 4 °C. Remove the supernatant; wash the pellet with 900 µl 80% ethanol, and spin again for 5 min. Air-dry for 3 min. Resuspend the pellet in 5 µl water and transfer to a PCR tube

7. Reverse transcription (RT)

- Add the following reagents to the resuspended pellet

1 µl primer RToligo (0.5 pmol/µl)
1 µl dNTP mix (10 mM)

- Rt thermal programme

70°C 5 min
25°C hold

Until the RT mix is added, mix by pipetting:
RT mix
7µl Water
4µl 5X RT buffer
1µl 0.1 M DTT
0.5 µl RNase inhibitor
0.5 µl Superscript III

- RT thermal programme continued:
25°C 5 min
42°C 20 min
50°C 40 min
80°C 5 min
4°C hold

- Add 1.65 µl 1 M NaOH and incubate at 98 °C in the thermocycler for 20 min
- Then add 20µl 1M HEPES-NaOH pH 7.3 (this will eliminate radioactivity from strongly labelled samples after the next step and prevent RNA from interfering with subsequent reactions)

8. Second adapter ligation to the 3′ end of the cDNA

a. MyONE clean-up
- From a thoroughly mixed MyONE Silane bead solution, use 10 µl MyONE Silane beads per sample. Attract the beads magnetically and discard the supernatant
- Wash the beads with 500 µl RLT buffer and resuspend them in 125 µl RLT buffer
- Add 125µl washed beads to each sample and mix
- Then add 150 µl 100% ethanol and carefully mix by pipetting. After 5 min at room temperature, mix the sample once more by pipetting and repeat the 5 min incubation step a second time.
- Magnetically attract the beads and discard the supernatant
- Resuspend the beads in 900 µl 80% ethanol and transfer the mix to a new tube
- Magnetically attract the beads, discard the supernatant and wash with another 900 µl 80% ethanol
- Let the ethanol incubate on the sample for 30s at room temperature and repeat the wash with 900 µl 80% ethanol for a third time
- Briefly spin the mix in a microcentrifuge, magnetically attract the beads and discard the supernatant
- Air-dry the beads for 5 min at room temperature and resuspend them in 5 µl water
- Incubate the water on the sample for 5 min at room temperature and proceed without removal of beads

b. Second adapter ligation

- Add the second adapter and DMSO to the cDNA-bead solution
- Heat the mix for 2 min at 75 °C and immediately keep it on ice for < 1 min

- Prepare:
2 µl L#Clip2.0oligo (10 µM) (mix of the 25th oligos)
1 µl 100% DMSO
- Prepare the following ligation master-mix on ice:
0.3 µl Water
2µl 10× NEB RNA ligase buffer (with DTT)
0.2 µl ATP, 100 mM
9µl 50% PEG 8000
0.5 µl High conc. RNA ligase

- To ensure homogeneity, mix the ligation master-mix by vigorous stirring, pipetting and flicking
- Briefly centrifuge the mix in a microcentrifuge
- Then add 12 µl of ligation master-mix to 8 µl sample-adapter mix and mix it thoroughly
- Add another 1 µl RNA ligase to each sample (final volume: 21 µl), mix by stirring and agitate it O.N. at room temperature and 1100 rpm.

DAY 6 (and DAY 7)
c. MyONE clean-up
- In this step, fresh MyONE Silane beads will be added to the cDNA-bead mix. Therefore, magnetically attract 5 µl of fresh MyONE Silane beads, discard the supernatant and wash them with 500 µl RLT buffer.
- Repeat magnetic attraction and buffer removal in order to resuspend the beads in 60 µl RLT buffer per sample
- Add 60 µl beads to the cDNA-bead slurry and mix
- Then add 60 µl 100% ethanol and mix by pipetting the solution carefully
- After incubating the sample for 5 min at room temperature, mix it once more by pipetting and repeat the 5 min incubation step a second time.
- Magnetically attract the beads and discard the supernatant.
- Resuspend the beads in 900 µl 80% ethanol and transfer the mix to a new tube.
- Magnetically attract the beads, discard the supernatant and wash with another 900 µl 80% ethanol.
- Let the ethanol incubate on the sample for 30s at room temperature and repeat the wash with 900 µl 80% ethanol for a third time.
- Briefly spin the mix in a microcentrifuge, magnetically attract the beads and discard the supernatant.
- Air-dry the beads for 5 min at room temperature and resuspend them in 23 µl water. Incubate the water on the sample for 5 min at room temperature.
- Magnetically attract the beads and add the eluate to the PCR mix of the next step

9. First PCR (cDNA pre-amplification)
- Prepare the following PCR mix:
22.5 µl cDNA
2.5 µl Primer mix of P5Solexa_s and P3Solexa_s, 10 µM each

25 µl 2× Phusion HF PCR MasterMix
Note – We use shorter primers for the cDNA pre-amplification to obtain smaller PCR products, which allow for efficient size selection with ProNex Chemistry.
- Run the following PCR:
98 30 s
98 10 s 6 cycle
65 30 s 6 cycle
72 30 s 6 cycle
72 3 min
16 hold

10. First ProNex size selection to remove primer-dimers
Notes - To remove excess primer dimers, size-select your samples with ProNex Chemistry. In
order to estimate the efficiency of the size se- lection, we recommend to include two samples
with GeneRuler Ultra Low Range Ladder (ULR Ladder; Life Technologies). While one sample is
used for size selection, the second serves as a reference in the TapeStation run and can be stored at room temperature.
The following scheme will help to prepare the size selection control:
ULR Ladder reference:
1 µl ULR Ladder
49 µl Water or ProNex Elution Buffer
ULR Ladder for size selection:
1 µl ULR Ladder
24 µl Water
25 µl 2× Phusion PCR MasterMix
- Equilibrate the ProNex Chemistry to room temperature for 30 min and resuspend the beads by vigorous vortexing
- For 50µl of sample (either PCR product or ULR Ladder for size selection), add 147.5 µl ProNex Chemistry (beads). This is a 1:2.95 v/v ratio of sample to beads.
- Mix by pipetting 10× up and down.
- Incubate the ProNex Chemistry on the samples at room temperature for 10 min.
- Place the samples on a magnetic stand for 2 min. Discard the supernatant.
- Leave the beads on the magnetic stand and add 300 µl ProNex Wash Buffer to the samples. If necessary, scale up the volume of ProNex Wash Buffer to cover all beads on the magnet.
- While the beads are magnetically attracted, incubate the ProNex Wash Buffer for 30–60 s before removal.
- Repeat the last wash of the magnetically attracted beads with an- other 300 µl ProNex Wash Buffer for 40–60 s, subsequently discard the supernatant and allow the samples to air-dry for ca. 8–10 min (< 60 min) until cracking starts.
- Remove the beads from the magnetic stand and start eluting the samples.
- Be careful to elute the beads of the samples in 23 µl water (or ProNex Elution Buffer), whereas the ULR Ladder sample (for size selection) requires to be eluted in 50 µl.

- Resuspend all samples by pipetting, and let them stand for 5 min at room temperature.
- Return the samples to the magnetic stand for 1 min, then carefully transfer the eluted cDNA to a clean tube.
- To check the selection efficiency of your samples, compare the ULR Ladder with and without ProNex size selection on a High Sensitivity D1000 TapeStation Kit or Bioanalyzer
Notes - The efficiency of this size selection step is estimated from the ratio of intensities of the 75 nt and 50 nt fragments of the ULR Ladder. This ratio should be around 2.5.

11. Second PCR amplification (PCR cycle optimization)
- Prepare the following PCR mix:
3.5 ul Water
1 ul cDNA (from step 3.11)
0.5 ul Primer mix P5Solexa/P3Solexa, 10 µM each
5 ul 2× Phusion HF PCR MasterMix
- Run the following PCR:
98°C 30 s
98°C 10 s (6 – 11 cycles)
65°C 30 s (6 – 11 cycles)
72°C 30 s (6 - 11 cycle)
72°C 3 min
16°C hold

- Run 2µl of the amplified library on capillary gel electrophoresis using the High Sensitivity D1000 Kit in a TapeStation system.

Preparative PCR
- Prepare the following PCR mix:
8 µl Water
10 µl cDNA
2 µl Primer mix P5Solexa/P3Solexa, 10 µM each
20 µl 2× Phusion HF PCR MasterMix

- Test 2 µl of the amplified library with capillary gel electrophoresis using the High Sensitivity D1000 Kit in a TapeStation system or Bioanalyzer.
- If everything looks fine, amplify the second half of the library and combine with the first half.

12. Second ProNex size selection to remove residual primers
- Equilibrate the ProNex Chemistry (beads) to room temperature for
- 30 min and resuspend the beads by vigorous vortexing.
Based on the optimal sample-to-ProNex (v/v) ratio of 1:2.4, calculate and add the needed volume of beads to your samples (e.g. for 50µl sample, add 120µl beads).
- Mix by 10× pipetting up and down.

- Incubate the ProNex Chemistry on the samples at room temperature for 10 min.
- Place the samples on a magnetic stand for 2 min. Discard the supernatant.
- Leave the beads on the magnetic stand and add 300 µl ProNex Wash Buffer to the sample
- While the beads are magnetically attracted, incubate the ProNex Wash Buffer for 30–60s before removal. If necessary, scale up the volume of ProNex Wash Buffer to cover all beads on the magnet
- Repeat the last wash of the magnetically attracted beads with another 300 µl ProNex Wash Buffer for 40–60 s, subsequently discard the supernatant and allow the samples to air-dry for ca. 8–10 min (< 60 min) until cracking starts.
- Repeat the washing and allow the sample to air-dry for ca. 8–10 min < 60 min until cracking start
- Remove the beads from the magnetic stand and start eluting the samples.
- Be careful to elute the beads of the samples in 20 µl water (or ProNex Elution Buffer), whereas the ULR Ladder sample (for size selection) requires to be eluted in 50 µl.
- Resuspend all samples by pipetting and let them stand for 5 min at RT
- Return the samples to the magnetic stand for 1 min, then carefully transfer the eluted cDNA to a clean tube or well.
- To check for successful primer removal test 2 µl of the purified library with capillary gel electrophoresis using the High Sensitivity D1000 Kit in a TapeStation system or Bioanalyzer. To check the selection efficiency, compare the ULR Ladder with and without ProNex size selection on a High Sensitivity D1000 TapeStation Kit or Bioanalyzer.

4. Results

4.1 Mir21 expression in AML-12 and HepG2 cell lines

First, we analyzed Mir21 expression in both AML-12 and HepG2 cell lines with northern blot first and qPCR after, and as we can see the mir21 expression is comparable to the let7-c expression, that we used as control since let7-c is one of the most ancient and highly conserved microRNAs

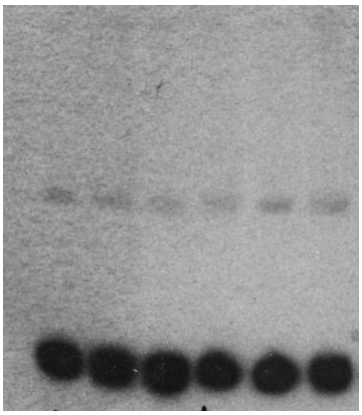


Figure 21 Northern Blot result about mir21 expression in AML-12 and HepG2

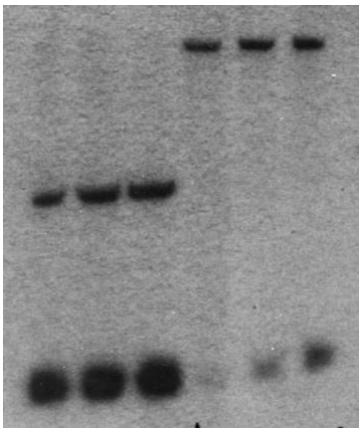


Figure 22 Northern Blot result about let7-c expression in AML-12 and HepG2

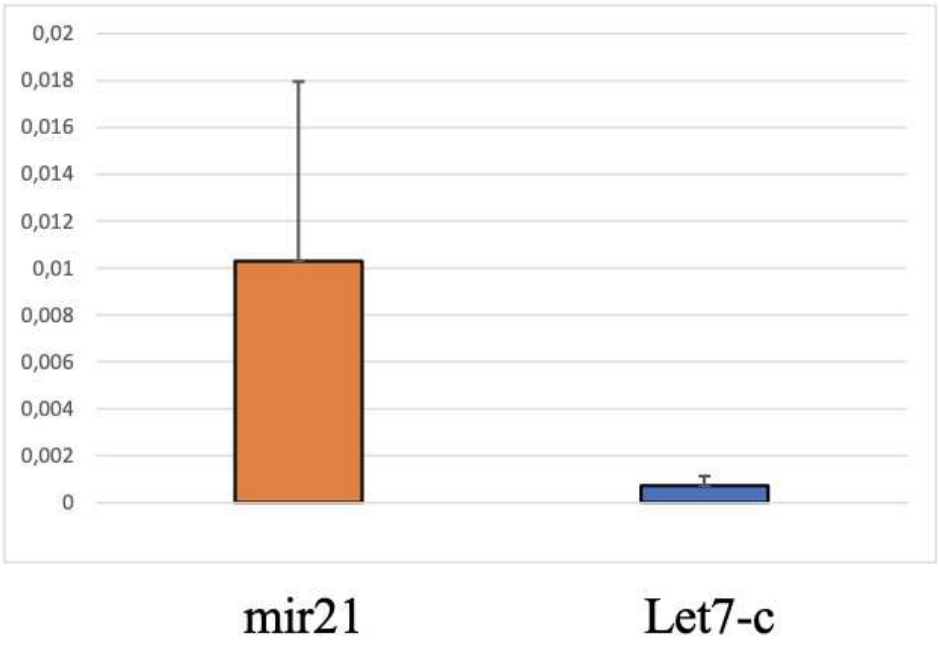
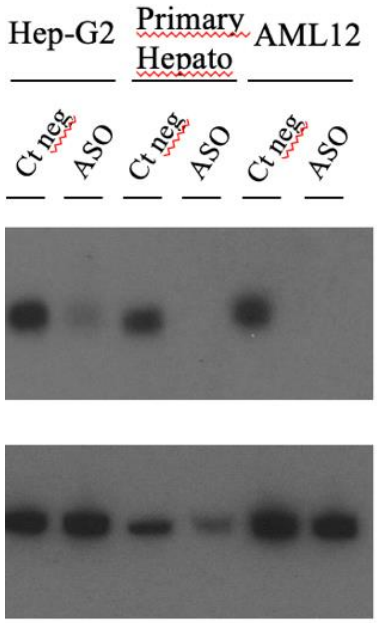


Figure 23 semiquantitative qPCR in AML-12

4.2 miR21 knockdown in vitro and primary liver cell lines

So once confirmed the high expression of micro-RNA 21 we did a knock-down in the AML-12, HepG2 cell lines and in the primary cell lines thanks to another equipe.



From this results we can clearly see that the knock down of mir21 worked well in all the cell lines compered with the negative control and also with U6 expression.

Figure 24Norther Blot result about the knock down of miR-21 (first pic) compered with U6 (second pic) in different cell lines

4.3 RIP in AML-12 and HepG2

An Importanat part of the iCLIP is the RNA immunoprecipitation, so we performed the RIP on the AML12 and HepG2 to see if it will work out in the iCLIP.

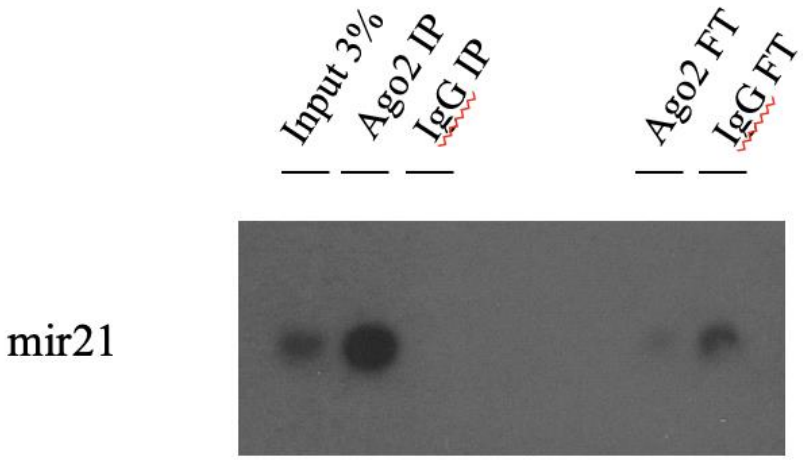


Figure 25 Northern Blot of the RIP in AML-12

Here there is the result of the Northern Blot of the RIP in the AML-12 cell lines, where we can clearly see the immunoprecipitation worked.

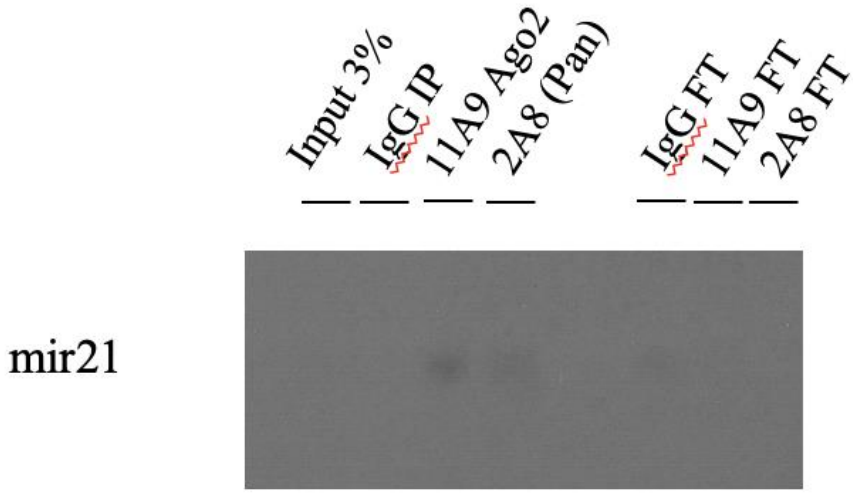


Figure 26 Northern Blot of the RIP in HepG2

From the second picture we can still see that the immunoprecipitation of Ago, and consequentially of the RNA, worked.

4.4 First iCLIP test

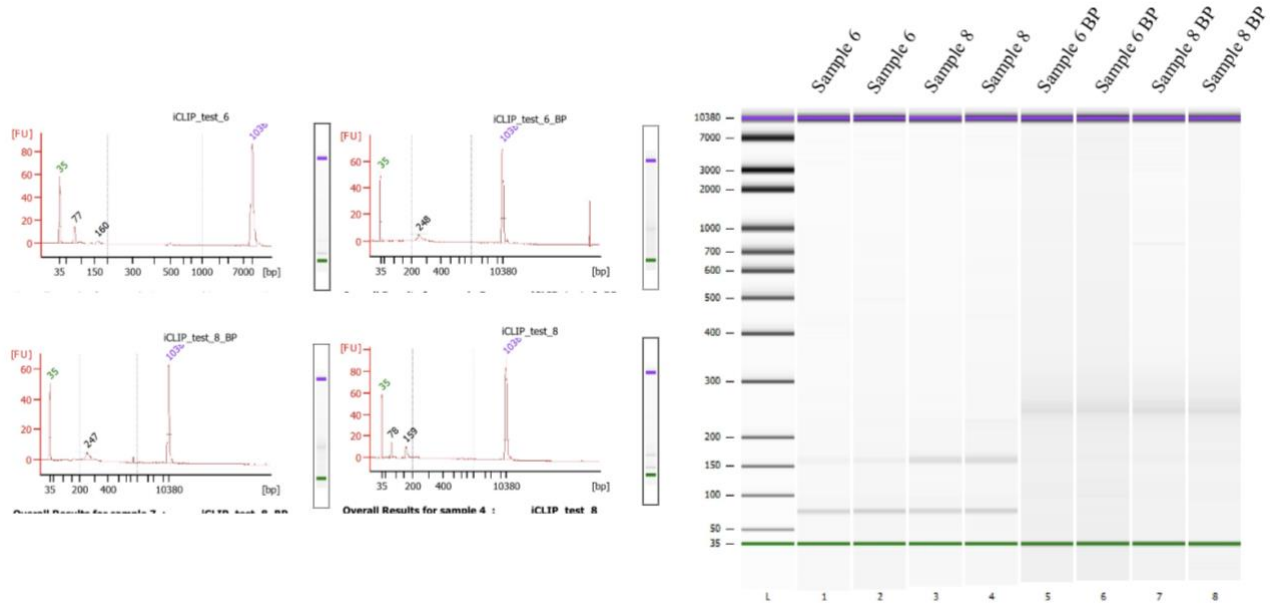


Figure 27 first iCLIP2 result

We performed the first iCLIP test with the AML12
We tried with both purification systems: blue pippin size selection and the ProNex size selection.
With the ProNex size selection (sample 6 and 8) there is an interference band coming out, that there is not with the blue pippin' size selection system (sample 6 BP and 8 BP). In both system we can see the mRNA and targets comin out.

For this first test there were some problems:
first of all we didn't get enough proteins from the cells, we should have had 2,2 ug of proteins and we had half of it.

So for sure we will run another test.
but this time probably we will change the purification system, and we will do the blue pippin size selection instead of ProNex size selection.

5. Future prospectives

For the next iCLIP test with the blue pippin we will start with more starting material and 10 micrograms pf anti-Ago proteins antibodies per IP. Furthermore we want to do the iCLIP-sequencing for AML12 and HepG2 upon miR21 k.d. and then the Rna-seq analisys for AML12 and HepG2 upon miR21 k.d. to check on miR-21 mediated repression of direct targets. the bionformatic analisys will be performed by the bioinformatic of the team.
Moreover our team wants to do Funcional and biochemical validation of the found targets.

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