Human Liver and microRNA profiling: effects of donor and recipient age-mismatch in transplant

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1.INTRODUCTION

1.1 Liver Physiology

The body's largest organ, the liver weighs 1400-1600 grams in men and 1200-1400 grams in women. The fold of peritoneum known as the falciform ligament separates the right and left lobes anteriorly, while the fissure for the ligamentum teres separates them inferiorly, and the ligamentum venosum fissure separates them posteriorly.

The liver is the human body's largest glandular organ and is located strategically to collect all the food supply flowing through the portal vein from the pancreas, spleen and intestine.

The hepatic parenchyma comprises, at the microscopic level, various hexagonal or pyramidal classical lobules. In turn, the lobules are mostly composed of thin layers of hepatocytes (the most common kind of liver cell), radiating from the central vein to the edge of the lobule. Small blood vessels, called sinusoids, are placed between the radiating rows of hepatocytes. Though the portal vein, the sinusoids obtain blood that is rich in oxygen from the hepatic artery and nutrients from the intestines. The oxygen and nutrients spread into the liver cells through the capillary walls.

Specialized macrophages called Kupffer cells are found within the sinusoids and these cells play a significant role in the process of recycling old red blood cells. The portal area, which is the complex at the corners of each lobule, is made up of parts of the hepatic portal vein, the hepatic artery, the bile duct, and the nerve. The bile is able to drain from the hepatocytes via the many minute bile ducts that together form the hepatic duct, which is the main bile duct of the liver. This goes on to join the cystic duct, which leading from the gallbladder subsequently comprises the common bile duct and then drains into the duodenum. At the center of each lobule there is a central canal which is a blood vessel receiving blood from the hepatic portal vein and the hepatic artery through the sinusoids, and goes on to drain the blood into the hepatic vein. (Fig 1.1)

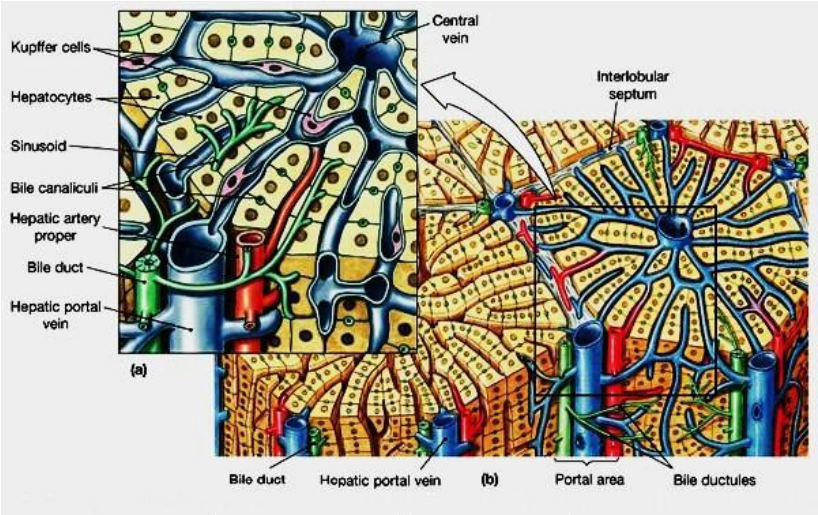


Figure 1.1 Parenchymal liver structure: a)the cords of hepatocytes and blood-containing sinusoids radiate to the peripheral portal from the central vein; b) pyramidal lobules (http://3.bp.blogspot.com/-)

1.2 Liver Functions

In terms of functionality, the liver can be considered as an assortment of various compartments that exert control over metabolic (metabolism of glucose and lipids), catabolic, bio-transformatory (breaking down of serum proteins, hormones and alteration of contaminants), synthetic (coagulation factors and serum proteins), removal (biliary components) and storage functions

(glycogen, triglycerides, metals and vitamins) (Pilkis et al, 1992; Strange ;1984).

Blood circulation in liver occurs in such a way as to bring very significant volumes of blood into contact with the lobules' cells. Therefore these cells can very easily take in materials from the blood as well as secrete materials into it. This occurs extremely frequently, because the liver's function consists of maintaining the proper concentrations of many components in the blood. The majority of liver functions are carried out by the hepatocytes; the phagocytic Kupffer cells lining the sinusoids, however, play the role of cleansing the blood.

Liver Functions:

- The metabolism of carbohydrates
    - Glycogenesis
    - Glycogenolysis
    - Gluconeogenesis
- The synthesis and secretion of the bile that is necessary to emulsify fats. Some of the bile is stored

in the gallbladder while some of it empties directly into the duodenum.

- The breakdown of hormones such as insulin
- The metabolism of proteins
- The metabolism of lipids:
    - the synthesis of cholesterol
    - triglyceride production
- The breaking down of hemoglobin to create metabolites that are added as pigment (bilirubin and biliverdin) to bile.
- the production of protein C, protein S and antithrombin as well as blood coagulation factors I (fibrinogen), II (prothrombin), V, VII, IX, X and XI,
- The conversion of ammonia into urea.
- The breaking down of toxic substances and the majority of medicinal products in the process of drug metabolism.
- The storage of multiple substances such as, vitamin B12, iron, copper and glucose in the form of glycogen.

- Various Immunological effects: thanks to the many immunologically active cells contained in the liver's reticuloendothelial system, it functions as a 'sieve' for the antigens it receives through the portal system.
- The production of red blood cells in first trimester fetuses. Bone marrow nearly wholly assumes this role by the 32nd week of gestation.

Parenchymal liver cells (hepatocytes) are able to detoxify and re-process ingredients absorbed from food thanks to the unique protein and gene expression patterns that they possess. Nutrients received by the liver are transformed and the organ secretes multiple proteins including albumin, various plasma carrier proteins, and the majority of coagulation factors. Lipids are delivered to other tissues in the form of lipoproteins and carbohydrates are stored as glycogen in the liver itself, which enables it to maintain the stability of blood glucose levels. Another function of the liver is synthesizing bile, which plays a fundamental role in the digestion of fats. The liver is also responsible for producing almost 80% of the cholesterol contained in the

human body. The liver is capable of capturing, storing, and releasing minerals, vitamins A, D, K, B12, potent growth factors such as HGF, hormones, and other substances that are biologically active. The liver thus plays a crucial role in homeostasis and maintaining the 'quality' of blood. The result of acute or chronic liver function deficiency is 'hepatic encephalopathy', coma, and death (Pacheco et al, 2009). The major function of the liver, that of filtering toxic substances from the blood, explains liver cells' astonishing capacity for proliferation. A percentage of hepatocytes are routinely killed off by continuous toxic stress, which causes surviving cells to initiative the required proliferative response. Hepatocytes in the majority of mammals take the form of polyploid cells displaying unique and highly articulated functions and gene expression signatures that are the foundation of an extremely specific phenotype. Hepatocytes go on working to maintain bodily homeostasis even in cases where 90% of the organ is absent and the liver cells that remain are all carrying out substantial proliferation, which illustrates how committed they are to their function. Studies involving the multiple sequential

transplantation of hepatocytes and partial hepatectomies reveal the virtually unlimited regenerative capacity of hepatocytes (Rhim et al, 1994; Overturf et al, 1997).

Liver function covers a great deal of ground, given that this organ contributes to a wide variety of biochemical and physiological processes, ranging from metabolic and synthetic function to processes of detoxification. Nonetheless, it is the hepatocyte itself whose function is so essential in maintaining healthy life and dealing with states of disease. In severely ill patients, the beginning of liver failure is a sure forecast of a terminal outcome. However, whereas the glomerular filtration rate acts as a clear quantifier of key renal function, no quantitative test for liver function is clinically available at most practice sites. Although indirect assessments of this function are commonly conducted (for instance, through the measurement of levels of its end products and by-products in blood) and acute injury can be detected by measuring an increase in transaminases, there is no test widely available that would allow the direct measurement of hepatocellular function (Bennink et al, 2012).

In a regenerating liver, a partial reversion to a fetal phenotype is indicated by actively proliferating hepatocytes that express alpha-fetoprotein (AFP) and other fetal markers (Meier et al, 2006). While hepatocytes do perform extremely specialized and essential functions throughout the body, there is a general consensus that they are not entirely terminally differentiated. Indeed, this phenotypic plasticity characteristic of hepatocytes enables them to trans-differentiate, becoming bile duct epithelial cells and oval cells that can serve as facultative progenitor cells when required (Nishikawa et al, 2005). Hepatocytes are often considered to be functional stem cells on the basis of their phenotypic plasticity as well as their extensive capacity to regenerate themselves when mature. Similar to cancer cells or hematopoietic stem cells, these cells are able to reproduce *in vivo* as well as in culture. Cultured hepatocytes are able to reactivate telomerase activity, which partly accounts for their extraordinary ability to proliferate (Nozawa et al, 1999). Genetic changes are recognized as governing hepatic cells proliferation; in addition, it has been recently found that epigenetic

modulations represent important mechanisms in genomic imprinting in embryos; even more significantly, they contribute to regulating the expression of multiple tumor suppressors. The balance in the expression of the genes controlling xenobiotic metabolism versus those involved in proliferation is found to be significantly modified by DNA methyltransferases. DNA methylation is considered to be crucial in the control of hepatocyte proliferation and differentiation during the development and regeneration of the liver (Anderson et al, 2009; Waterland et al, 2009).

The proliferation of liver cells is dependent on paracrine-signaling that involves extrahepatic humoral factors and a coordinated cross-talk among the various populations of liver cells. The scientific community has recently begun to focus on a new cell-to-cell communication concept. This communication is facilitated by exosomes, which are membrane-bound vesicles the size of a nanometer involved in the modulation of proliferation for multiple types of cells; they are commonly considered to represent a prospective therapeutic instrument in the promotion of cancer immunity (Li et al, 2005; Aharon and Brenner

2009). Exosomes can also be produced by liver cells, according to the evidence (Conde-Vancells *et al*, 2008).

1.3. Liver regeneration

In contrast to most other mammalian organs, hepatic regeneration is not solely dependent on stem cells. Rather, functional parenchymal cells divide in order to re-establish the initial mass during regeneration of the liver (Fausto, 2005). The liver is the only mammalian organ capable of swiftly regaining its former dimension, structure, and function even when as little as 10% of the original tissue remains (Myronovych et al, 2008). Liver regeneration occurs through an highly complex and orchestrated process characterized by very tightly coordinated cascades of signaling pathways, both intra and extracellular. Hepatic regeneration is in many respects quite similar to the development of the liver during embryogenesis; furthermore, it demonstrates its distinctive embryonic memory when faced with serious injury. A better understanding of the mechanisms that govern hepatic development and regeneration can also help us to

comprehend the etiology of chronic and acute diseases of the liver as well as hepatocarcinogenesis and developmental defects (Fausto et al, 2006).

1.4 Pathology

Jaundice, which is a yellowing of the eyes and skin, is a common indicator of a damaged liver. Jaundice occurs whenever there is a buildup of bilirubin, a yellow breakdown product of red blood cells, in the blood. Hepatic diseases include:

- **Hepatitis**, an inflammation of the liver that primarily results from one of several viruses but can also be caused by certain poisons, hereditary conditions, or autoimmunity.
- **Cirrhosis**, which is the development of fibrous tissue in the liver in the place of dead hepatic cells. Liver cells death can be caused by factors such as alcoholism, viral hepatitis, or contact with other chemicals that are toxic to the liver.
- **Cancer of the liver** (primarily manifesting as hepatocellular carcinoma or cholangiocarcinoma as

well as metastatic cancers, usually originating in other parts of the gastrointestinal tract).

- **Wilson's disease**, an hereditary disease that causes a retention of copper in the body.
- **Hemochromatosis**, an hereditary disease that produces an accumulation of iron in the body, which gradually results in damage to the liver.
- **Primary biliary cirrhosis**, an autoimmune disease that affects the small bile ducts
- **Gilbert's syndrome**, a genetic disorder in the metabolism of bilirubin that affects approximately 5% of the population.
- **Primary sclerosing cholangitis**, an autoimmune condition that produces inflammation of the bile duct.
- **Budd-Chiari syndrome**, a hepatic vein obstruction.

1.5 Liver grafts

In 1963 a team of surgeons with Dr. Thomas Starzl at its head performed the first human liver transplant in history (Starzl et al.; 1963). In the following years, Starzl went on to carry out further transplants and the first operation displaying short-term success was conducted in 1967, as indicated by a post translation survival period of one year. Over the 1970s several feasible surgical techniques were developed, but the transplantation of the liver continued to represent an experimental practice with the one year survival rate of patients hovering around 25%. When Sir Roy Calne eventually introduced ciclosporin, patient outcomes were significantly improved and over the course of the 1980s liver transplantation came to be recognized as a standard clinical treatment for all patients exhibiting the proper indications, adult as well as pediatric subjects (Starzl et al.; 1981). Generally speaking, as long as the recipient does not exhibit any other conditions that would make a successful transplant impossible, liver transplantation can be considered a potential treatment for any acute or chronic condition that results in permanent

dysfunction of the liver. Unqualified contraindications include active septic infections, the active abuse of drugs or alcohol and metastatic cancer located externally to the liver. At one time HIV infection was included among these absolute contraindications, but in recent years this assessment has come under reconsideration. Relative counterindications, on the other hand, include serious heart disease as well as pulmonary or other disease and advanced age, which may prevent or interfere with the success of transplantation. Liver transplantation is positively indicated in cases of chronic liver diseases that will result in irreparable liver scarring or liver cirrhosis.

The important role of elderly donors

In relation to patients with end-stage liver disease, transplantation of the liver (liver transplantation LT) represents the sole opportunity for a cure and, in most centers, generally results in a 70% to 85% survival rate 5-year out. Demand for this operation has increased over time, but the rate of organ donation by deceased donors has not increased at the same rate. The result is a

significant shortfall in the number of organs that may be used for transplantation (United Network for Organ Sharing). The acceptance of progressively elderly donors has represented one answer to this shortfall. While only 28% of the total number of donors in 1988 were aged 50 years or over and only 0.05% were more than 65 y.o., these percentages had grown by 1995 (42% over 50 and 5% over 65). They continued to increase, reaching 65% and 10%, respectively by 2007 (United Network for Organ Sharing). In Italy, liver donation by older donors has become a common practice, with a donor age that is elevated in relation to the United States and other European countries (Avolio et al., 2011). It is necessary to approach the age of donors conservatively to ensure a high level of quality among donated organs, but at the same time if an excessively strict restriction were to be applied, some organs that might otherwise be appropriate for transplantation would end up being unintentionally excluded. Given the ongoing shortage of available organs and the fact that the geriatric population is rapidly expanding, a realistic strategy for enlarging the donor pool

would be by easing restrictions on donor age. The principal issue remains, however, whether or not more mature organs grant patients the same survival advantage that younger donated organs do. Fortunately, there is evidence that the liver holds a privileged place among organs in that there are minimal pathophysiological changes displayed as a result of aging and a healthy aging process does not significantly reduce the overall function of the liver (Kampmann et al.,1975; Popper, 1986). Several initial studies do show an increased risk of initial poor function, primary nonfunction occult tumor transmission in cases involving older donors (Wall et al.,1990; Healey et al., 1998); however, more than one recent study demonstrates that, in relation to specific types of patients, satisfactory results can be achieved using the livers of donors aged up to 80 y.o. (Wall et al., 1993; Emre et al., 1996; Jiménez Romero et al., 1999; Grazi et al., 2001; Cuende et al., 2002; Mazziotti et al., 1999; Nardo et al., 2004). Nonetheless, the lack of consensus among transplant centers in relation to an upper age limit for accepting donors can be read as the lack of a clear definition for this issue. Furthermore, it has

not been conclusively demonstrated whether using these so-called “extreme” donors might result in post-transplant adjunctive risks beyond the acceptable parameters (Tector et al.,2006). There is still an ongoing and heated debate over whether or not it is safe to use grafts for LT that are designated as elderly (> 50 years) or even senior (>70 years). When Detre et al (Detre et al., 1995) analyzed an LT cohort of 7,988 recipients in 1995, they found a higher rate of re-transplant and graft failure when livers had been obtained from donors aged 50 or older. Similar results have been reported in relation to large cohorts analyzed more recently (Feng et al., 2006; Hoofnagle et al., 1996). After analyzing an LT cohort of more than 20,000, Feng et al reported finding a direct correlation between an increase in the relative risk of graft failure and an increase in the age of donors. When combined with additional risk factors such as African-American race, height, or cerebrovascular cause of death, a donor age of > 70 years was found by the same study to result in the highest risk of graft failure. Yet the results reported by other studies are dissimilar. For instance, when analyzing 741 cases, Anderson et al

(Anderson et al., 2008) found no statistically relevant difference in the survival rate of patients receiving livers from >60 year old donors versus those receiving livers from <60 year old donors. In their study of 55 donors aged > 70 years, Gastaca et al found an excellent rate of patient survival, equal to 91% at 3 years (Gastaca et al.,2005). Another study by Cescon et al analyzed a series of 17 liver transplantations using octogenarian donors and reported a 3-year patient survival rate of 75%, notwithstanding an evident increase in the risk of viral recurrence in the case of HCV recipients (Cescon et al., 2003 a). Cescon et al, in an additional study, (Cescon et al., 2003 b), used direct experience to observe LTs using >80-year donors and had two main findings: first, that early results are comparable to the results achieved using livers from donors who are younger, and secondly, that the policy of using older donors could offer transplant recipients a substantial chance of prolonged survival. Even transplants using donors older than 80 years have reported successful results (Emre et al., 1996, Jimenez Romero et al., 1999; Wall et al., 1993) as have those with donors older than 90 years (Filipponi et al.,

2003), this suggests that the liver fares much better in relation to aging-related damage than other organs do (Cuende et al.,2002).

It is widely thought that this is the reason why early post-implantation function is similar to that seen in the case of much younger donors. In theory, there is no upper age limit for using cadaveric liver donors and the selection of organs is carried out solely on the basis of available pre-LT pathological factors (such as fibrosis and steatosis). Throughout the world, an expanded donor criteria (marginal) grafts can be used to augment the available supply of organs for transplantation. Furthermore, multiple studies have compared the outcome resulting from marginal versus non-marginal graft transplantation in 103 cases in which liver transplantation was required by chronic hepatic failure. According to Bacchella (Bacchella et al., 2008) overall marginal graft outcome and the survival of recipient was most decisively defined by the first month post-transplantation. Additionally, recipients of marginal grafts who were designated high- MELD (Model For End-Stage Liver Disease) displayed a high rate of mortality in

the first week following transplantation. Recipients of marginal and non-marginal grafts were found to achieve analogous outcomes following the first month after transplantation. A significant issue in liver transplantation is making the best match between the degree of graft injury and the recipient's clinical status; the researchers thus conclude that using marginal graft results in increased early mortality in liver transplantation, especially in the case of high-MELD recipients. For the other groups, the survival rate remained unchanged between marginal and non-marginal grafts one week, one month and one year after transplantation. It can therefore be concluded that the survival rate after the first month following transplantation remained the same. In the case of HCV + patients, a study by Cescon et al (Cescon et al., 2003 c) provides the first evidence that long-term survival is possible, in particular for non-HCV+ patients. A very recent Spanish study of more than 300 livers obtained from donors aged over 70 years demonstrates similar results in those who received grafts from donors aged over 80 years: there was a 15.8% incidence of primary graft failure, a 72% 1-year graft

survival rate and a 51% 5-year survival rate (Cuende et al., 2002). To conclude, the data they report confirm the outcomes previously found in a larger series, which supports the safe use of >80 year old donors for LT as a result of the fact that these grafts are able to achieve normal functional recovery. This policy does impose a strict process of selecting among available organs in order to diminish the adjunctive risk factors associated with poor outcome. While it has yet to be determined what life expectancy applies to patients who receive these organs, there is evidence that, particularly in the case of non-HCV+ subjects, it is possible to achieve long-term patient and graft survival. When considering the distribution of grafts from elderly donors to HCV+ patients, the high rate and rapidity of HCV recurrence represents a significant concern. The most common indication for liver transplant is chronic liver disease related to the hepatitis C virus. With the exception of a few publications (Doyle et al., 2008), all the studies which evaluated the survival of grafts and patients with livers from older donors regularly report that hepatitis C virus-seropositive recipients showed worse

outcomes than recipients whose cirrhosis had other causes (Nardo et al., 2004, Lake et al., 2005; Borchert et al., 2005; Kim et al., 2005). Furthermore, a study by Melendez and Heaton, (Melendez et al., 1999) suggests that marginal grafts should only be used with patients who are clinically stable and are able to tolerate potential re-transplantations. It is important to weigh carefully the possible risks and benefits connected to using livers from older donors. Even in the case of donors ≥ 80 years, acceptable early functional recovery is possible by carefully selecting donors, avoiding additional risk factors in donors (i.e. decreasing cold ischemia time and avoiding grafts that show moderate or severe steatosis), and through a vigilant process of allocation (i.e. hepatitis C seronegative recipients with moderate MELD scores). It is particularly important whenever possible to avoid matching an old graft with an older recipient who is infected with the hepatitis C virus. To conclude, in LT it is advisable to avoid other risk factors for poor graft function (and especially a long ischemia time and recipient MELD score >15) in order to improve the outcomes of older donors (Grazi et al, 2005). However, in

order to definitively assess this option, we must first clearly comprehend the cellular and molecular mechanisms that hamper hepatic regeneration in older patients.

The importance of donor/recipient age in transplantation

The currently available literature does not clearly indicate whether or not the process of liver ageing is connected to a simultaneous decline in function (Verzaro et al., 2008) and, in particular, which estimated age limit can be associated with functional decline. Nearly all the cell types that contribute to the outcomes of liver transplantation are affected by aging: donor-derived hepatocytes (Iakova et al., 2003; Brouwer et al.,1985; Okaya et al., 2005; Sastre et al., 1996) as well as recipient cells (inflammatory cells and the cells which gradually populate the graft and which are derived from the bone marrow of the recipient) (De la Fuente et al., 2004; Harris et al.,1998). The liver's capacity to respond to stress is significantly affected by the aging

process (De la Fuente et al., 2004; Selzner et al., 2007; Sanz et al., 1999; Berenguer et al., 2005; Rifai et al., 2004). Whether it be inflammation (Sanz et al., 1999), ischemia-reperfusion injury,(Okaya et al., 2005; Harris et al., 1998) or hepatitis C virus (HCV) recurrence,(Berenguer et al., 2005; Rifai et al., 2004) this stress response triggers a complex cascade that involves donor-derived hepatocytes as well as endothelial cells and recipient-derived nonparenchymal cells (i.e. T-cells, macrophages and platelets). Nonetheless, the overall function of the liver does not show significant decline, thanks to the effective counterbalance provided by its large, functional reserve, its capacity for regeneration and its dual blood supply. Furthermore, for unknown reasons atherosclerosis less frequently affects the visceral vessels of the abdomen (Berenguer et al., 2002). Cells from both donors and recipients are involved in these processes; as a result, the effects that the age of both donors and recipients has on short and long-term outcomes should be considered in tandem rather than independently. Recent studies indicate that the recurrence of more severe HCV is associated with

increased donor age (Rayhill et al., 2007; Khapra et al., 2006; Berenguer et al.,2002). On the other hand, it is not yet clear what effect donor age has on non–HCV-related long-term outcomes and reperfusion injury. The findings of some series suggest that recipients who receive older grafts are at increased risk for primary non-function and display an elevated incidence of biliary complications (Shah et al., 2007; Ploeg et al., 1993); other studies, however, do not demonstrate any significant difference for any of the outcome parameters under examination (Emre et al., 1996). From a pathophysiological standpoint, the liver shows minimal age-related alterations in terms of morphology, ultrastructure and function than do other tissues and organs of the body such as the heart, kidneys and lungs (Popper, 1986). Such alterations include a variation in the size of hepatocytes and their mitochondria, a reduction in overall mass, reduced phagocytosis among Kupffer cells, a propensity for nuclear polyploidy, endothelial cell endocytosis, increased lipofuscin pigment and reduced blood flow as well as alterations in a range of hepatic functions such as drug metabolism, protein

synthesis and biliary secretion (Popper , 1986; Kampmann et al., 1975). Taken together, these observations constitute the biological rationale for expanding donor eligibility to older individuals.

Even under the dissimilar conditions provided by a liver transplantation recipient, a grafted donor liver should develop and survive. However, it is not yet clear if a grafted liver's age can be affected by factors presented by the recipient. The extent to which the liver is influenced by its microenvironment remains a matter of investigation; it has not yet been determined whether an aging liver might be able to "rejuvenate" when transplanted into a younger recipient. Indeed, liver rejuvenation has not been fully investigated in the liver transplantation field. There remains a relative scarcity of literature that addresses this issue and the few studies that have been conducted so far do not appear to corroborate this hypothesis. To address this issue, Eguchi et al. (2010) investigated the re-expression of SMP-30 in a biopsied adult liver (n = 6) that was transplanted into a pediatric recipient, given that SMP-30 can be considered to be a marker of senescence. The

immunohistochemical staining showed that no liver of a pediatric patient who had been the recipient of an adult donor showed SMP-30 re-expression or an increase in SMP-30. On the basis of these findings, the researchers concluded that adult grafted livers do not appear to rejuvenate in pediatric recipients.

1.6. Liver aging

As developed countries population continues to grow older, an understanding of the ageing process that could allow healthy ageing has significant medical implications. Not only elderly individuals are more vulnerable to most acquired disorders of the liver, they are also more susceptible to the consequences of liver disease (Hoare et al., 2010). Along with age, also the occurrence of liver disease increases whereas the capacity to sustain a hepatic insult decreases as people grow older. Changes at cellular and sub-cellular levels underlying this predisposition are the focus of a great deal of scrutiny. There has been increased interest in the role that ageing plays within the sphere of hepatology, in particular in response to the recent recognition of the decisive importance of age in determining the clinical outcome in cases of infection of chronic hepatitis C virus (Poynard et al., 1997) and the relevance of the age of donors on post –LT graft survival (Keswani et al., 2004). In addition, death related to liver in older individuals increases substantially when compared to younger people who display the same condition (Regev

et al., 2001). Unlike the majority of other organs, the liver does not display marked or well-documented changes in both structure and function during the aging process (Schmucker, 1985, O'Mahony and Schmucker, 1994; Schmucker, 1998; Schmucker, 2004). The few existing comprehensive studies on liver morphology during aging were qualitative in nature and performed using rodent models. A weakness of the studies that have employed tissue from human liver has been their dependence on postmortem samples or samples from patients who had been diagnosed with liver disease.

Irrefutable evidence exists suggesting that the ageing process take place at the cellular level and that inflammation can 'prematurely' induce such changes. Lipofuscin, that is cytoplasmic accumulation of highly oxidized insoluble proteins related to age, is the most widespread change on the specimens obtained though diagnostic liver biopsy (Schmucker, 2002; Jung et al., 2007). These accumulations of protein that is highly cross-linked are believed to be related to a failure to degrade damaged and denatured proteins and chronic oxidative stress (Jung

et al., 2007). There is growing availability of evidence suggesting that lipofuscin creates interference with cellular pathways because of its capacity to capture metallic cations and facilitate further formation of free radicals (Jolly et al., 1995). There are fewer good quality descriptions about sub-cellular hepatocyte changes related to age and the majority of these data derives from animal studies. The clear decline in the surface area of smooth endoplasmic reticulum due to age (Schmucker et al., 2002; Schmucker et al., 1980) correlates with a decreased concentrations of hepatic microsomal proteins as well as enzymatic activity such as glucose-6-phosphatase (Schmucker et al., 1980). There are other changes in the structure of hepatic cells which include, (a) a decrease in smooth surfaced endoplasmic reticulum, (b) and loss of volume of the dense body compartment, for instance, secondary lysosomes and increased hepatocyte polyploidy (Schmucker, 1990). Quantitative evidence is available describing how hepatocytes in males of one inbred rat strain (Fischer 344) increase in volume through maturity and then successively become smaller in such a way that cells in immature and

senescent animals present an equivalent size (Schmucker, 1978). The occurrence of a decline related to age in chaperone-mediated autophagy, the cellular pathway leading to depletion of molecules and sub-cellular organelles, is suggested by recent data. A mouse model associated with a decrease in liver function related to age suggested that the restoration of autophagy was associated with preservation of the function of liver (Zhang et al., 2008). Additional data point out a change in hepatocyte nuclear morphology connected to age increase. A more substantial variation in nuclear size (Watanabe et al., 1982), associated with higher incidence of polyploidy of hepatocytes, is also known to exist (Schmucker et al., 1990) Approximately 27% of human hepatocytes show polyploidy in individuals aged over 85, compared to approximately 6% for subjects in their twenties (Kudryavtsev et al., 1993)

None of these changes related to age results in significant declines of the functions of the liver. There are data, however, showing specific age-related changes that include decrease of hepatic volume and decline in hepatic perfusion. Both these changes can affect specific functions

of the liver, for example first pass pharmacokinetics (Wynne et al., 1989, Marchesini et al., 1988). Data coming from clinical tests on liver function are, however, inconclusive and do not identify considerable deficits related to age in liver functions (Thompson et al., 1977; Tietz et al., 1992; O'Mahony and Schmucker, 1994). Various studies show moderate changes connected to age in biliary function, such as diminished bile flow and bile acid secretion. Whether or not hepatic functions are compromised in elderly people or senescent animals is still unclear. Hepatologist Hans Popper declared that, "aging exerts a limited effect on the constitutive liver functions and more on its response to extrahepatic factors. . ." (Butler et al., 2008). A more clinically significant change related to age may be manifest decline in the hepatic regeneration rate that follows chemically induced injury or partial resection (hepatectomy). There is general consensus that fewer hepatocytes enter the S-phase following partial hepatectomy in older individuals and senescent animals than in younger subjects and those that do so less rapidly; this delay associated to age threatens the rate of hepatic

regeneration. This can be explained by the significant increase in mortality due to hepatic diseases in older individuals compared to younger subjects. Regev and Schiff found a 3–5-fold increase in deaths caused by hepatic diseases in population over 65 y.o. when compared to under 45 people (Regev et al., 2001). The augmented demand for donor livers for transplantation is another element of concern: evidence exists showing that livers from older donors might be less viable than those obtained from young donors (Washburn et al., 1996, Selzner, 2009). Another element that should be considered is the age of recipient; as a matter of fact, Fortner and Lincer report a 15% increase in post transplant mortality in people between 55 and 75 y.o. (Fortner et al., 1990). Aging, thus, hampers hepatic regeneration in terms of the rates of post-resection hepatocyte proliferation.

Schmucker et al (2011) clearly outline the highlights of liver aging:

- **Increase in Reactive Oxygen Species Related to Age**: Reactive oxygen species (ROS) are considered

to represent a causative factor that is responsible for various pathophysiological changes throughout the aging process. According to a recent study by Haga et al., an increased expression or phosphorylation of p66Shc adapter protein is implicated in the enhanced generation of ROS and the initiation of apoptosis in hepatocytes following a partial hepatectomy in elderly mice; the same however is not true in young animals' livers (Haga et al., 2010). The study shows a similar hepatocyte proliferation in young and old cohorts, but an impaired cell growth only in old mice. In addition, ablation of p66Shc reduces post-hepatectomy oxidative stress and apoptosis in elderly mice, and this suggested that this protein associated to age might be critical for the inhibition of liver regenerative capacity in old animals.

- **Loss of Telomere Length Related to Age**: An hypothesis suggested that the reduction related to age in hepatocyte telomere length leads to a decrease in cell mitosis and apoptosis, therefore

producing a decline in the proliferation of cell. Takubo et al. showed, for example, a significant age-related loss in the length of hepatocyte telomere in humans, and Aikata et al. independently confirmed these data (Takubo et al., 2000; Aikata et al., 2000).Takubo et al. additionally reported a significantly higher rate of telomere shortening in hepatocytes than in the majority of other epithelial cell types that display high turnover rates such as, for example, enterocytes as well as esophageal epithelium (Takubo et al., 2000). A recent study demonstrates that the hepatocyte's telomere length in humans displays a yearly reduction rate between 55 and 120 base pairs (Takubo et al., 2010). Visible variations in the structure of cells are not consistently reflected in concomitant alterations in function. Denchi et al. showed that, through the use of a telomere restriction fragment deficient mouse model, the loss of telomere integrity did not have the effect of hampering the regeneration of liver after a partial hepatectomy (Denchi et al., 2006). The

hepatocytes did enter S-phase, however, mitosis, anaphase, and telophase were not found to subsequently occur. Further research into liver regeneration using this model may be granted credence by the recent finding that rejuvenating telomerase activity in a telomerase-deficient mouse model did act to reverse some heavily documented deficits associated with aging (Jaskelioff et al., 2011). There is one caveat, however, which is that the aging process displayed by this particular telomerase-deficient mouse model may not effectively replicate normal aging in humans.

- **Aging Effects on the Hepatocellular Response to Factors of Growth**: twenty years ago, Sawada was able to demonstrate that there was a noticeably higher hepatocyte proliferative response to EGF in young rats than in elderly animals , suggesting that the factor of age in old rats impaired cell responsiveness to growth factors (Sawada et al., 1989). Several studies showed that the activation of

a hepatocyte extracellular receptor kinase (ERK) was lower in old rodents than in young animals after a partial hepatectomy (Palmer et al., 1999; Hutter et al., 2000).

Kamat and others, subsequently focused on the molecular pathways regulating the proliferation of hepatocyte (Kamat et al., 2008). These researchers have reported remarkable decline associated with age in the expression of hepatocyte EGF receptor mRNA and protein, and in EGF receptor phosphorylation as well as the successive ERK activation. A number of studies by Wang and associates outlined the centrality of transcription factors in processes of hepatic regeneration and the critical role that FOXM1B gene plays in hepatocyte proliferation (Wang et al., 2001; Wang, et al., 2002). Though the use of a mouse model FOXM1B-deficient in, these researchers demonstrated that adenovirus transfection with FOXM1B was able to restore the regenerative capacity of the liver in mature animals bringing it to a higher level than that recorded in

young adult mice. Non-transfected mice deficient in FOXM1B showed no sign of increased hepatocyte proliferation.

- **Other Causes of Diminished Regeneration:** Several studies by Le Couteur et al. reported significant -changes related to age in the structure of the liver sinusoidal endothelium; changes include, a loss of fenestrae and a thickening of the endothelial cells, which is a process also known as pseudo-capillarization (Le Couteur et al., 2001;. Le Couteur et al., 2002;Le Couteur et al., 2007)

  A great deal of evidence supports the idea that the age of liver donors or recipients affects only partially the survival rate of post transplantation patients. These studies imply that a pre-transplantation regimen of growth factors in liver recipients who are elderly deserves to be investigated further.

1.7 The miRNAs

MicroRNAs or miRNAs are endogenous, highly conserved, expressed non-coding RNAs that have a length of 20–24 nucleotides; they have been found to regulate the expression of genes and the differentiation of cells, metabolism, cell proliferation, apoptosis, and immunity development and disease (Friedman et al., 2009). Although some miRNAs are expressed in a broad array of tissues and cell types, most miRNAs are expressed only in very specific tissues and organs (Lagos-Quintana et al.,2002, Sempere et al.; 2004). It was in *Caenorhabditis elegans* that MiRNAs were first described (Lee et al; 1993), along with various eukaryotic cells with the exception of algae, fungi and marine plants (Boutz et al., 2011) More than one thousand specific miRNA have been described thus far in humans (Davis-Dusenbery , et al.2010; Deiters et al., 2010; Li et al.; 2009; Steitz et al., 2009; Eulalio, 2008). MiRNAs are responsible for regulating several different physiological processes, including the translation efficiency or stability of particular mRNAs. Given that individual miRNAs are

capable of regulating many different mRNAs (encoded by target genes numbering from 250 to 500), it follows that approximately 20–80% of transcribed human genes are quite likely to be regulated by miRNAs (Deiters et al.; 2010 , Eulalio, 2008). The effectiveness of miRNAs in binding and neutralizing their targets is dependent on multiple parameters, for instance the primary sequence of the miRNA and target mRNA, the miRNA's three-dimensional structure, co-factors, and so on). Ever since 1993 when the first miRNA *lin-4* was discovered in *C.* elegans (Lee et al.; 1993), there has been a dramatic increase in our understanding of how miRNAs function. Besides their important role in controlling physiological processes, miRNAs have also been found to play an important part in various pathologies; these pathologies include liver diseases such as hepatitis, fibrosis and hepatocellular carcinoma (HCC) (Boutz et al.; 2011, Li et al.; 2009, Lagos-Quintana et al., 2002).

Researchers have become extremely interested in understanding the role of miRNAs in tumorigenesis following recent findings that demonstrate the differential

expression of both miRNAs and their target mRNAs in cancer (Calin et al.; 2006 , Calin et al.; 2007). Generally speaking, miRNAs use multiple mechanisms, including mRNA cleavage, a complimentary base pairing with the 3' UnTranslated Region (UTR) of their target mRNAs resulting in translational repression, and mRNA decay that is initiated by miRNA-guided rapid deadenylation in order to negatively to regulate the expression of genes in vertebrates.

1.8 miRNA biogenesis

MiRNAs are single-stranded RNA sequences, 16- to 29-nucleotides in length, that operate at the post-transcriptional level to negatively regulate the expression of genes (Bartel 2004; Guo et al. 2010a; Zhang et al. 2009). A multistep process is responsible for producing these mature miRNAs (Fig. 1) (Kutanzi et al.; 2011).

In short, the transcription of miRNA is accomplished by RNA polymerase II, long primary transcripts (pri-miRNAs) (Lee et al. 2002, 2004) that are polyadenylated (AAAAA) and capped (7MGpppG). (Fig.1) With the excision of a 65–

75 nucleotide, the nuclear RNase III Drosha and its cofactor Pasha (alternatively known in mammals as DGCR8) act to crop these pri-miRNA transcripts into pre-miRNAs having a hairpin shape (Lee et al, 2003; Kim, 2004). This cleavage event holds great importance in that it predetermines the sequence of mature miRNA and generates an ideal substrate for subsequent events (Lee et al, 2003; Lund et al, 2004). Pre-miRNA, the processing intermediate, is then exported out of the nucleus by exportin-5 (Exp5), which is a member of the family of Ran-dependent nuclear transport receptors (Yi et al, 2003; Bohnsack et al, 2004; Lund et al, 2004).

Successively, pre-miRNA is cleaved by cytoplasmic RNase III Dicer (Bernstein et al, 2001; Grishok et al, 2001; Hutvagner et al, 2001; Ketting et al, 2001; Knight and Bass, 2001). This acts to process the pre-miRNA in order to produce a transient 22-nucleotide miRNA:miRNA*duplex, which is subsequently loaded into the miRNA-associated, multiprotein RNA-induced silencing complex (miRISC). This complex includes the Argonaute proteins and the now-mature single-stranded miRNA is preferentially retained in

it. The mature miRNA goes on to bind to complementary sites within the mRNA target in order to negatively regulate gene expression in one of two ways; these ways depend on the degree of complementarity that exists between the miRNA and its target. Specifically, miRNAs which bind to mRNA targets that present imperfect complementarity function to block target gene expression at the protein translation level. However, there is recent evidence to suggest that miRNAs might also have an effect on the stability of mRNA. In general, the complementary sites for miRNAs that use this mechanism are to be found in the 3' untranslated regions (3' UTRs) of target mRNA genes. The miRNA that bind to their mRNA targets with perfect or virtually perfect complementarity, on the other hand, function to induce target-mRNA cleavage. The miRNAs that use this mechanism bind to miRNA complementary sites which are normally to be found in the mRNA target's coding sequence or open reading frame (ORF).

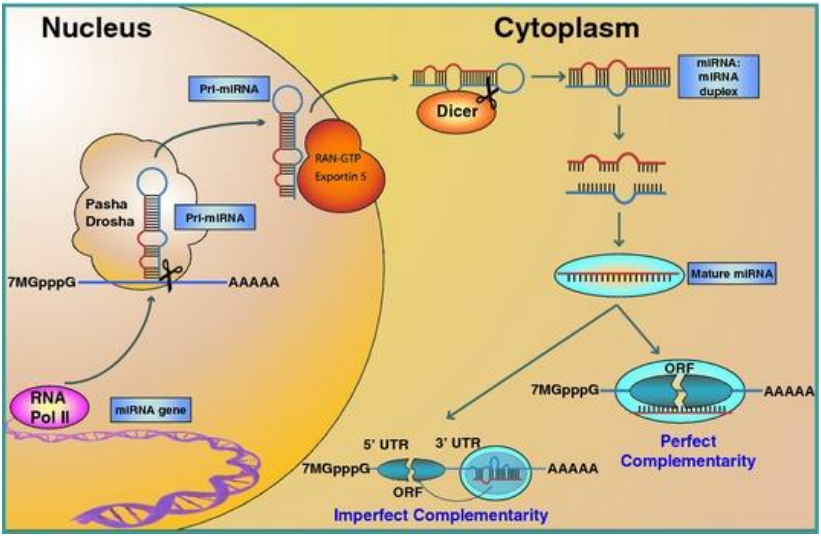


Fig. 1 the biogenesis and function of miRNA. Mature miRNAs are produced through a multistep process. (by: MicroRNA-mediated drug resistance in breast cancer. Kutanzi , Yurchenko, Beland , Checkhun , Pogribny. Clin Epigenetics. 2011 Aug;2(2):171-185. Epub 2011 Jun 27)

1.9 Role of miRNAs

It seems that MiRNAs and their targets constitute regulatory networks that are remarkably complex, as a single miRNA is able bind to and regulate multiple different mRNA targets and, conversely, multiple different miRNAs are able to bind to and cooperatively exert control over a single mRNA target (Lewis et al, 2003). As of today,

researchers have identified more than 1,200 mammalian miRNAs, which are potentially able to target as much as one third of the protein-coding genes (The miRBase Sequence Database—Release 16.0) that are involved in cell differentiation, development, signal transduction, metabolic pathways, proliferation and apoptosis (Bartel 2004; Selbach et al. 2008). Given that miRNAs mainly function by repressing the expression of their targets, there is a minimum threshold amount that must be reached in order for miRNAs to effectively exert their function (Brown et al., 2007; Sarasin-Filipowicz et al., 2009). Abundantly expressed miRNAs therefore appear to be more important than miRNAs that are expressed at relatively low levels.

While there is increasing recognition of the critical role played by miRNAs, researchers have yet to completely understand the mechanism of their action. It is widely known that miRNAs regulate genes, and this is thought to occur through either the induction of mRNA cleavage or the specific inhibition of translation (Chekulaeva et al., 2009). The crucial role that miRNAs play cellular physiology was sophisticatedly demonstrated in mice that lack the Dicer

enzyme which is necessary for processing the miRNA precursor into mature miRNA (Kanellopoulou et al., 2005). The majority of research into miRNA so far has focused mainly on the role they play in the cytoplasm. Recent studies, however, suggest a range of roles for miRNAs, such as regulating DNA methylation (Kim et al.,2008; Sinkkonen Let al., 2008), regulating target genes by acting at the 5' UTR(Orom et al.,2008), and importing mature microRNA into the nucleus; these suggest additional functional modes (Hwang et al., 2007. There is increasing evidence to suggest that the role of miRNA is not limited to the regulation of gene expression at the post-transcriptional level, but that they are also able to modify chromatin (Figure 2). As demonstrated by Hwang et al, 2007, human miR-29b (unlike the miRNA of other studied animals) is mainly localized to the nucleus. MiR-29b presents a distinctive hexanucleotide terminal motif that can act as an element of transferable nuclear localization, directing the nuclear enrichment of the micrRNAs or small interfering RNAs that is connected to (Hawang et al.; 2007).

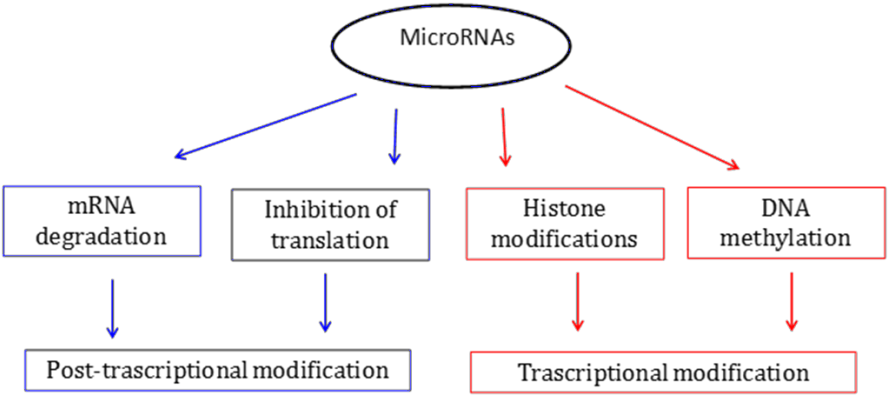


Figure 2: The various gene regulation modes carried out by miRNAs (Bala S.; et al. 2009)

1.10 miRNAs and the liver

The adult liver is rendered unique by its intrinsic capability of regenerating through the proliferation of fully differentiated cells (Michalopoulos, 2007). Under normal circumstances, adult hepatocytes are quiescent and in mice will divide only once or twice a year while the frequency of division in humans is even lower (Lee, 2001). In responding to the injury or loss of liver tissue, however, adult hepatocytes are capable of dividing numerous times

(Overturf et al., 1997; Azuma et al, 2007). The hepatocyte cell cycle is regulated by a multifaceted network of cytokine and growth factor signaling that occurs between hepatocytes and other types of liver cell in order to ensure that the regeneration of the liver is both rapid and vigorous (Fausto et al., 2006). It has been shown that miRNAs work post transcriptionally to regulate the genes that orchestrate proliferation in both development and cancer, but their role in processes of organ regeneration is not yet well known. A study by Song et al (2010) analyzed changes occurring in miRNA expression during the regeneration of mouse livers, which lead to the finding that miR-21 and miR-378 act to regulate organ regeneration. Specifically, this issue was investigated by generating mice who had a hepatocyte-specific inactivation of DGCR8, which is a vital constituent of the microprocessor complex. As DGCR8 functions to anchor the primary miRNA transcript for its cleavage by Drosha, it acts upstream of Dicer and, when it is deficient, there is a disruption in the processing of miRNAs but not other small RNAs (Michalopoulos, 2007). These findings therefore suggest that miRNAs are responsible for

regulating hepatocyte G1 to S phase progression in the course of liver regeneration.

A recent study by the Chinese group Hou et al (2011) identified the miRNome in both the normal human liver and hepatocellular carcinoma. For this study they used massively parallel signature sequencing (MPSS) of miRNAs, which is able to provide an in-depth identification of miRNome, thus revealing differences in miRNA expression as well as the abundance characterizing individual miRNA. This group used MPSS in order to conduct an in-depth analysis of the miRNomes located in three normal tissues of the liver (distal normal liver tissue of hepatic hemangioma), a liver affected by severe chronic hepatitis B, a liver infected with HBV, an HCV-related HCC, two HBV-related HCCs and an HCC without either HBV or HCV infection. They furthermore normalized the abundance value of each known miRNA through the use of ‘‘transcripts per million (TPM)’’ in each library of small RNA. The results were as follows: 85.9% of miRNAs were poorly expressed (<10 TPM) in normal liver tissue, while _13.2% of miRNAs were modestly expressed (10–10,000 TPM) and a mere _0.9%

(9) of miRNAs were abundantly expressed (>10,000 TPM), yet these made up _88.2% of all miRNA reads conducted (Table 1). The three miRNAs that were expressed most abundantly were miR-122 (52.0% expression), miR-192 (16.9% expression), and miR-199a/b-3p (4.9% expression). Table 1 shows the other miRNAs found to be expressed in the normal human liver.

miRNA	**Avergage Ratio in miRNome**
miR-122	52.0%
miR-192	16.9%
miR-199a/b-3p	4.9%
miR-101	3.7%
let-7a	3.3%
miR-99a	2.2%
let-7c	2.1%
let-7b	1.7%
let-7f	1.5%
Total	**88.2%**

Table 1: the miRNAs that are most abundantly expressed in normal human liver

One of the first instances of a tissue-specific miRNA was miRNA-122. A trademark of liver tissues, miR-122 is highly expressed in the livers of all mammals, in which is completely conserved the sequence of the mature miRNA. It may be seen to chiefly define many of the characteristics specific to hepatocytes, characteristics such as lipid metabolism, tropism to hepatitis viruses, the production of cholesterol production, and so on (Norman et al, 2006; Diaz-Toledano, Ariza-Mateos et al, 2009). MiR-122 inhibition leads to the upregulation of hundreds of genes, several of which do not typically find expression in the healthy liver of an adult (Krutzfeldt, Rajewsky et al, 2005). The loss of miR-122 expression in liver cancer is correlated with the suppression of the liver phenotype and the acquirement of invasive properties (Coulouarn et al, 2009). The expression signature of microRNA in the liver also goes through changes during development: besides miR-122, many other microRNA (including miR-1, miR-16, miR-27b, miR-30d, miR-126, miR-133, miR-143, and the let-7 family) are also abundantly expressed in the adult liver, and miR-

483 and miR-92a are expressed in the liver of the fetus (Girard et al, 2008).

An extensive literature exists in relation to the miRNA 122, but there is not the same clear understanding of the specific roles this miR plays outside of disease-associated functions (such as hepatocellular carcinoma (HCC) and the hepatitis C virus (HCV)), particularly in relation to normal liver development. As demonstrated by Xu et al(2010), miRNA 122 is strongly upregulated for the duration liver embryonic development. In addition, it has been found that, during development, miR 122 gradually represses the transcriptional repressor CUTL1 that is understood as promoting proliferation and suppressing differentiation. As for miR-192, which is the human liver's second most abundant miRNA, there is yet little known about the roles it plays in liver biology and liver disease pathogenesis. As of today, there exists a relative scarcity of literature about the other miRNAs that the Chinese group found to be more expressed in the normal human liver.

1.11 miRNAs in liver diseases

Improvements in functional analysis techniques for and the characterization of miRNAs has not only revealed the role they play in multiple cellular processes, it has also uncovered abnormal miRNA expression patterns in several diseases, among them cancer(Lu et al, 2005), viral infections(Houzet et. al, 2008), inflammation(Baltimore et al., 2008), diabetes (Lynn et al, 2007), cardiovascular disease (Latronico et al., 2007) and Alzheimer's (Sethi et al., 2009). In responding to liver insults by antigens, viruses, toxins or bacteria, the interaction between immune and parenchymal cells plays a unique role in the liver. There is strong evidence to suggest that miRNAs can be characterized as key regulators in relation to both innate and adaptive immune responses (Baltimore et al., 2008; Bi et al., 2009); they may thus play a role in autoimmune, inflammatory or viral liver diseases (Bala et al.;2009). In virtually every aspect of cellular activity, miRNAs are found to be implicated. MiRNAs continue to be used as markers of disease and factors of prognosis. They can also determine the success of specific therapies and may function as

pharmacological targets. According to Jin et al (2009), miRNA expression changes over the course of non-alcoholic fatty liver disease and the inhibition of certain miRNAs, such as miR-122, have been found to potentially produce a deregulation of steroid and lipid metabolism as well as the development of hepatic steatosis. A long-term study of liver cancer patients also correlated poor prognosis with noteworthy changes in the level of expression of 19 miRNAs (out of 196 total measured); these included miR-21, miR-199a and miR-301 (Jiang et al, 2008). According to recent research, miRNAs are also involved in the regulation of HCV infection. HCV, an enveloped RNA virus that forms part of the Flavivirus family, is able to cause acute as well as chronic hepatitis in humans through the infection of liver cells. With approximately 170 million people infected on a global level, HCV is recognized as a significant cause of chronic liver disease. The disease is fatal, in that as much as 70% of patients end up presenting persistent infection following inoculation. The disease displays wide variation, with cases ranging from asymptomatic chronic infection to cirrhosis as well as hepatocellular carcinoma or HCC

(Hoofnagle et al, 2002). The replication of HCV appears to be associated with an increased expression of the cholesterol biosynthesis genes that are regulated by miR-122 and, as a result, it is considered to be a possible target for intervention through antivirals (Randall et al., 2007). MiR-122 was the first liver-specific cellular miRNA to be identified, and has been demonstrated to boost HCV replication through the targeting of the viral 5' non-coding region (Jopling et al., 2005). Given that cellular miRNAs have an important role to play in viral pathogenesis, they are also likely to be involved in HBV infection. In fact, the livers of HCV- and HBV-infected individuals suffering from hepatocellular cancer were found to contain a differential pattern of miRNAs expression (Ura et al., 2009). There was a clear differentiation of 19 total miRNAs between HBV and HCV groups and, in the HCV group 13 miRNAs out of 19 were found to be down regulated while in the HBV group, 6 were found to display a decreased expression. The miRNAs that were differentially regulated the HBV and HCV groups included miR-20, miR-134, miR-151, miR-190, miR-193 and miR-211. An interesting finding of the same study was that

a pathway analysis of targeted genes employing infection-associated miRNAs was able to differentiate the genes into two different groups. What remains to be evaluated is the question of whether differentially regulated miRNAs might be used as potential biomarkers in order to differentiate between HBV and HCV in early stages of pathogenesis. A study by Jiang et al (2008), did show an increased expression of miRNA in hepatitis-positive and cirrhotic liver samples, and the team proposed that significant changes in the expression of miRNA might occur while cirrhosis and chronic viral hepatitis are developing.

Chronic hepatitis is another live pathology of high importance. Chronic hepatitis is recognized as contributing to liver fibrosis; it has been linked to fibrosis caused by fibrin deposition by hepatic stellate cells, or HSC (Bataller et al., 2005). MiR-195 has been the focus of intense investigation in multiple experimental models in relation to hepatic fibrogenesis. It has been reported that the down-regulation of cyclin E1 and the upregulation of p21 expression carried out by miR-195 causes a proliferation of interferon beta (IFNβ)-driven HSC and successive

fibrogenesis (Sekiya et al.,2011). Additionally, the major driver of fibrosis in the liver has been identified as the activation of hepatic stellate cells. In a recent study, Roderburg and colleagues were able to systematically analyze miRNA regulation in a mouse model of carbon tetrachloride [CCl(4)]-induced hepatic fibrogenesis. In the livers of mice who were undergoing hepatic fibrosis, they found that a group of miRNAs were specifically deregulated. Of particular interest, they found that all three members of the miR-29 family turned out to be down-regulated to a significant degree in CCl(4)-treated mouse livers (Roderburg et al., 2011). The experimental data from this study were also found to correlate with the findings obtained from human patient material, in that a reduced expression of miR-29 was observed in the livers of patients suffering from advanced liver fibrosis. Roderburg and colleagues succeeded in demonstrating that, in terms of the mechanism, TLR signaling and activation of the NF-κB signaling cascade functioned to mediate the downregulation of miR-29 in murine HSC (Roderburg et al.,2011). On this basis, the authors came to the conclusion

that miR-29 works to mediate the regulation of liver fibrosis in cases that involve a TGF-β1- and NF-κB-dependent downregulation of the members of the miR-29 family within HSC. And lastly, a recent study showed that four different human and murine miRNAs (miR-200a, miR-200b, miR-199a and antisense miR-199a*) were very significantly upregulated in progressing mouse liver fibrosis, as compared to the controls in a CCl(4)-induced mouse model that were compared to animals treated with olive oil (Murakami et al., 2011). Again, these experimental results were successfully correlated with human data. The progression of hepatic fibrosis in this model driven by CCl(4) was found to be both linked to and significantly correlated with an over-expression of miR-200 as well as miR-199 (Murakami et al.,2011).

The literature being produced most recently displays an abundance of studies that profile liver tumors. A complex disease, liver cancer displays extraordinary heterogeneity in terms of cause and outcome; this involves epigenetic and chromosomal instability(El-Serag et al., 2007) as well as abnormalities in both coding and non-coding gene

expression, including the expression of miRNAs (Liang et al., 2007; Varnholt et al., 2008). Worldwide, HCC is recognized as the third most common cause of cancer death and the fifth most widespread malignancy (El-Serag HB et al., 2007). HCC's main etiologies include chronic liver disease caused by chronic hepatitis C or hepatitis B or virus infections, metabolic disorders such as nonalcoholic steatohepatitis or insulin resistance, hereditary hemochromatosis, alcoholic steatohepatitis and immune-related diseases including PBC and autoimmune Hepatitis (Thorgeirsson SS et al., 2002). Several studies exist which identify specific miRNA signatures in the formation (Braconi et al., 2008, Varnholt et al., 2008; Wang Yet al., 2008) and progression of HCC which might potentially be exploited for use as potential cancer biomarkers. In most studies, it has been reported that microRNA including miR-21, miR-18, miR-224miR-221, miR-301, miR-222, and miR-373 are upregulated (Jiang et al., 2008, Ladeiro et al., 2008; Wong et al., 2008) in HCC while miR-130a, miR-122, miR-150, miR-125, miR-200, miR-199, and members of the let-7 family (Wong et al., 2008; Murakami et al., 2006;

Gramantieri et al., 2008; Gramantieri et al., 2009; Fornari et al., 2009) were reported to undergo downregulation in HCC. The Chinese group (Hou et al., 2011) also found a similar result. Given that only a few specific miRNAs are expressed abundantly in the miRNome and these appear to play the most important role in liver biology as well as HCC, in this study only those miRNAs with a TPM of >2000 (which accounted for _99% of the total miRNome) and a greater than 1-fold alteration were treated as most likely to have importance in the pathogenesis of HCC. The third-ranked most abundant miRNA found in the human liver, miR-199a/b-3p, was found to display a marked decrease in every one of the sequenced HCC samples when compared to matched liver tissue that was non-neoplastic. An interesting aspect is that, in miRNoma, there was no significant difference found between miRNomes of normal liver tissue and HBV or HCV-infected liver tissues. MiR-221 is a popular example of an miRNA that plays a critical role in the development of HCC on the basis of apoptosis regulation. There are two contrasting roles that it fulfills in relation to regulating apoptosis: one role is pro-apoptotic

pathway and the other is anti-apoptotic pathway. MiR-221 was found on the one hand to be drastically up regulated in reaction to apoptosis mediated by death receptor; its ectopic expression might, on the other hand, protect both primary hepatocytes and hepatoma cells from apoptosis.

A study carried out by Laidero et al (2008) engaged in the analysis of miRNA profiling for two series of liver tumors that were fully annotated, with the aim of uncovering associations that may exist between clinical and pathological features and oncogene/tumor suppressor mutations. Researchers compared the expression levels of 250 miRNAs located in forty-six malignant and benign hepatocellular tumors to the levels in four normal liver samples. They thereby identified and validated miR-21, miR-200, miR-200c, miR-10b, miR-224, and miR-222-specific deregulation in benign or malignant tumors and the overexpression of miR-224 in all tumors. Furthermore, miR-96 overexpression was reported in HBV tumors, while miR-126* was found to be down-regulated in the case of alcohol-related hepatocellular carcinoma. Invasive growth and the spread of tumor cells throughout the body

represents a marker of malignancy. Researchers described miR-31 as a master regulator in relation to the metastasis of various types of cancer because it is able to control both metastasis-relevant genes as well as genes promoting proliferation, and because it was additionally found to control the cell cycle as well as apoptotic cell death (Schmittgen, 2010). miR-492 is another miRNA that was found to be connected with metastasis. It was in relation to hepatoblastomas that miR-492 was first found to have relevance, and it has also been shown to be up regulated in the case of metastatic hepatoblastoma (von Frowein et al., 2011).

1.12 The potential of miRNAs in diagnostics, disease prognosis and therapy

There is an increasing amount of evidence to suggest that miRNAs have an important role to play in a wide range of diseases of the liver, from viral hepatitis and cancers to metabolic diseases. Due to the unique expression profile that miRNAs display in different types of cancer and at different cancer, as well as in other diseases, these small molecules might be exploited for use as novel biomarkers in disease diagnostics and might furthermore represent a novel strategy in terms of miRNA gene therapy. Kota et al (Kota et al., 2009) recently suggested the possible therapeutic use of miRNAs when they demonstrated that, in a mouse model of HCC, administering miR-26a, (which down regulates cyclins D2 and E2 and is under-expressed in HCC cells) through the use of adeno-associated virus (AAV) functions to inhibit the proliferation of cancer cells and results in the induction of tumor-specific apoptosis as well as dramatically protecting from disease progression, without associated toxicity. This research further demonstrated that anti-miRNA compounds could be safely

and effectively delivered in vivo; the way is now open for these basic research findings to be translated into clinical applications. Other factors must also be taken into consideration: some miRNA genes (such as, for instance, miR-1) have been shown to undergo methylation-mediated regulation in HCC cell lines (Datta et al., 2008), which suggests a significant connection between the DNA methylome and the miRNome. More specifically, some reports exist which show that miRNA expression profiles vary between malignant cholangiocytes, malignant hepatocytes and benign forms of liver cancer(Ladeiro et al., 2008), which suggests that miRNA profiling might be employed as molecular diagnostic markers in relation to liver disease.

In relation to disease diagnosis, the analysis of circulating miRNAs has gained increasing importance recently. Hepatocytes are found to express a distinct set of miRNAs, and of these the most abundant is miRNA 122 (Lagos-Quintana et al., 2002). Recent studies employing rodents demonstrate that miRNA 122, in addition to other miRNAs that are hepatocyte-abundant, are released from cells in the

course of drug-induced injury to the liver (Wang et al., 2009; Laterza et al., 2009). It was possible to detect these hepatocyte-derived miRNAs (HDmiRs) in plasma or serum, and levels increased in relation to the dose as well as duration of drug exposure (Farid et al, 2011). Furthermore, HDmiRs were found to be correlated with aspartate transaminases (AST), serum transaminases and alanine transaminase (ALT) in addition to liver histology. Beyond the diagnostic potential represented by miRNA, experimental animal studies also show that miRNAs can be considered a feasible target for therapeutic intervention aimed at minimizing and even reversing severe tissue injury of the type caused by ischemic tissue insults (Bonauer et al., 2009). It has recently been demonstrated in humans that, in the HDmiRs, it is also possible to detect miRNA 122 in serum and its levels were found to be elevated in patients presenting hepatocyte injury produced by alcoholic, viral or chemical-related hepatotoxicity (Zhang et al., 2010; Bihrer et al., 2011). In these same patients, serum and plasma miRNA 122 were also closely correlated with both transaminases as well as liver

histology. Recent small animal model-based studies as well as studies in humans demonstrate that HDmiRs represent highly sensitive and stable serum biomarkers of injury in the liver (Wang et al., 2009; Laterza et al., 2009 Zhang et al.,2010; Bihrer et al., 2011). HDmiRs appeared, in both humans and rodents, to increase earlier and more quickly in serum than it did in AST and ALT. Specifically, levels of miR-122 were substantially elevated even in the case of subjects who had transaminases below. A study by Farid et al. (2011) demonstrated that the idea of miRNAs as hepatic injury biomarkers can be considered feasible in the setting of liver transplantation as well. Serum HDmiR levels were found to be elevated in patients presenting liver injury after liver transplantation as well as during acute rejection. On the other hand, hepatic levels of miRNA in liver graft biopsies were found to exhibit decreased expression with prolonged warm ischemic times. Serum HDmiRs showed similar kinetics during acute rejection; however, miRNA levels increased and decreased sooner than did transaminases. As was the case in prior studies as well (15, 18), miRNAs showed higher sensitivity than did

transaminases and the stability of miRNA was confirmed, following the results of previous studies (Chen et al.,2008; Mitchell et al., 2008; Li et al.,2011; Cortez et al.,2009; El-Hefnawy et al., 2004; Kosaka et al.,2010). It is possible that HDmiRs might offer a solution to the urgent need for superior non-invasive biomarkers that would be able to serve as earlier and more sensitive markers of rejection or liver graft dysfunction. Improved markers would greatly aid in the management of liver transplant recipients and would enable the safer reduction of immunosuppressive medication in order to achieve an improved equilibrium between desired effects (such as prevention of graft rejection) and side effects (such as toxicity, infection or malignancy).

There are also other miRNAs that might be evaluated for use as therapeutic targets:

- It has been suggested that miRNA-221 represent a possible therapeutic target for the treatment of hepatitis and liver failure (Sharma et al., 2011)

- miRNA 492 might function as a biomarker in evaluating hepatoblastoma progression.

- The induced expression of miRNA-29 has also been proposed as a possible therapeutic agent in treating liver fibrosis (Chau et al., 2011).

2. THE AIM

The main objectives of this thesis are as follows:

- The first objective is to study the aging of the human liver, evaluating the expression of miRNAs, which play a fundamental role in the regulation of genetic expression. The applied experimental strategy is to investigate the miRNA profiling in hepatic biopsies obtained from donors of different ages (from 13 to 90 years old) and identify miRNAs most significantly associated with aging. As of today, there are no available published data related to age-dependent miRNA human liver. Furthermore, clinical evidence from the organ transplant context suggests that there is a difference between the liver's chronological and biological ages. As a matter of fact, the use of 90 year-old donors leads to transplant engraftment results that are comparable to those achieved by using younger donors (40-50 y.o.).

- The second objective is to analyze the role of recipient microenvironment and to address the question of whether the new (younger or older) microenvironment may in some way affect the transplanted organ. To this regard the task is to study the effect produced by donor/recipient age mismatch on the miRNAs identified in the first objective. To this end, the research attempted to evaluate the effect of the greatest donor/recipient age mismatches, both when the recipient is much younger than the donor (24.6 ± 2.5 years) and vice versa (24.6 ± 7 years). In order to better to clarify the role of donor-recipient age-mismatch, this study foresees the analysis of control group in which donor/recipient age mismatch is absent (±4 years). No literature exists in relation to this issue.

- Lastly, the discussion has also a clinical perspective on the donor/recipient age mismatch effects in terms of transplant success, tolerance onset together with immunosuppressive therapy regulation.

This study was forms part of the 2008 PRIN project titled "*Pretransplant liver biological age and age-mismatch between donor and recipient as new predictors of transplant outcome,"* which aims to identify biomarkers of biological aging in the context of liver transplantation.

3. MATERIALS and METHODS

3.1 Liver samples

The biological samples for the study are the fragments of liver biopsies performed on donors undergoing liver transplant (by the unit of liver transplantation- S'Orsola Malpighi Hospital in Bologna, Italy) during 2008–2011 were used for this study.

The samples were cut, placed inside cryovials, immediately frozen in liquid nitrogen and stored at -80°C. The liver fragments, initially vary in size between 200 and 300 mg. These dimensions have been reduced to 30-40 mg in liquid nitrogen. The fragment thus obtained was then disintegrated with an electric immersion homogenizer.

The liver biopsies were collected before transplantation, from 50 donors for 4 age categories: young people from 20 to 30 years, 31 to 50 years, 51 to 70 years, more than 71 years. (Table 3.1)

AGE GROUP	GROUP 1 (< 30 years)	GROUP 2 (31-50 years)	GROUP 3 (51-70 years)	GROUP 4 (>71 years)
Number	9	7	18	16

Table 3.1: donors divided into 4 age groups

Table 3.2 shows all analyzed samples divided into 4 groups according to age.

Age Group 1 (20 -30 years)			
Sample Code	**donor sex**	**donor age**	**recipient age**
4	F	18	54
5	M	30	58
16	F	13	14
17	M	23	46
20	M	26	64
22	M	29	37
39	M	20	49
49	M	16	64
50	F	18	69
Age Group 2 (31-50 years)			
6	M	43	57
7	M	44	41
10	M	37	51
15	M	50	65
24	M	50	66
44	M	43	21
45	M	45	49
Age Group 3 (51-70 years)			
1	M	59	62
2	F	58	52
3	F	69	50
11	F	63	59
12	F	67	27
13	F	58	-
14	M	54	-
21	F	60	60
23	F	53	45
25	F	59	58
26	M	66	55
27	F	54	64
28	M	58	67
31	F	69	59
35	F	59	56
37	F	61	52
38	F	70	53
41	F	69	63

Age Group 4 (71-90 years)			
8	F	73	64
9	F	76	42
18	F	82	-
19	M	87	59
29	M	84	68
30	M	76	43
32	F	77	47
33	M	82	50
34	F	90	40
36	M	82	57
40	M	74	66
42	M	77	48
43	F	74	56
47	M	72	46
48	F	78	25
52	M	74	68

Table 3.2: 50 samples analyzed, divided into 4 age groups: donor age and sex, recipient age

3.2 Follow-up samples

In order to evaluate the effect of the mismatch of age donor/recipient in liver transplantation, we analyzed the samples follow-up. The follow up samples used for this study were from recipients several months after transplant(by the unit of liver transplantation- S'Orsola Malpighi Hospital in Bologna, Italy) performed during 2008–2011. This biopsy has a high net weight of approximately 3-4 mg. The follow-up samples were liver biopsies obtained at 7-23 months after transplantation. All analyzed samples, both donor and recipient, come from males.

The weight of each sample is approximately 3 to 4 mg.

The follow-up samples were divided into three groups based on the mismatch of age donor / recipient. (Tab 3.3)

- In the first group, the samples analyzed are from matches in which the recipient is older than the donor and the age mismatch is greater than 14 years (RECIPIENT AGE > DONOR AGE)

- In the second group, the recipients were younger than the donors, with an age difference of 18 to 29 years (RECIPIENT AGE < DONOR AGE)
- In the third group, donors and recipients are the same age ± 4.

First group:

RECIPIENT AGE > DONOR AGE								
SAMPLE CODE	DONOR AGE	RECIPIENT AGE	DONOR SEX	RECIPIENT SEX	AGE MISMATCH (YEARS DON - YEARS RECIPIENT)	DISEASES RECIPIENT	HEPATITIS RECIPIENT	FOLLOW UP
6	43	57	M	M	-14	alcoholic cirrhosis		23 months
15	50	66	M	M	-16	hepatocellular carcinoma/cirrhosis	B+	18 months
24	50	65	M	M	-15	hepatocellular carcinoma/cirrhosis	C+	19 months
39	20	49	M	M	-29	amyloidosis		8 months

Second group:

RECIPIENT AGE < DONOR AGE								
SAMPLE CODE	DONOR AGE	RECIPIENT AGE	DONOR SEX	RECIPIENT SEX	AGE MISMATCH (YEARS DON - YEARS RECIPIENT)	DISEASES RECIPIENT	HEPATITIS RECIPIENT	FOLLOW UP
19	87	60	M	M	27	hepatocellular carcinoma/cirrhosis		12 months
42	77	48	M	M	29	post-necrotic cirrhosis	C+	13 months
46	75	57	M	M	18	hepatocellular carcinoma/cirrhosis		10 month

Third group:

RECIPIENT AGE = DONOR AGE								
SAMPLE CODE	DONOR AGE	RECIPIENT AGE	DONOR SEX	RECIPIENT SEX	AGE MISMATCH (YEARS DON - YEARS RECIPIENT)	DISEASES RECIPIENT	HEPATITIS RECIPIENT	FOLLOW UP
84	49	49	M	M	0	hepatocellular carcinoma/cirrhosis	B+	7 months
7	44	41	M	M	3	other disease		8 months
45	45	49	M	M	-4	post-necrotic cirrhosis	C+	7 months

Tab 3.3. three different groups of follow-up analyzed based on age of the recipient

All follow-up samples was performed using real time PCR (qRT PCR) to evaluate the expression of miRNAs. The same analysis was performed on biopsies of the donor.

3.3 Total and small RNA extractions

Total RNA and miRNA were extracted from liver biopsies and follow-up using two different protocols:

- mir Vana™ miRNA Isolation Kit (Ambion) for liver biopsies analysis

- AllPrep DNA/RNA/Protein (Qiagen) for follow up analysis

The mirVana™ miRNA Isolation Kit: employs an organic extraction followed by immobilization of RNA on glass-fiber filters to purify either total RNA, or RNA enriched for small species, from tissue samples. In particular, the sample is first lysed in a denaturing lysis solution which stabilizes RNA and inactivates RNases. The lysate is then extracted once with Acid-Phenol:Chloroform which removes most of the other cellular components, leaving a semi-pure RNA sample. This is further purified over a glass-fiber filter by one of two protocols to yield either total RNA or a size fraction enriched in miRNAs. The glass-fiber filter procedure uses solutions formulated specifically for miRNA retention to avoid the loss of small RNAs that is typically seen with glass-fiber filter methods.

The *mir*Vana miRNA Isolation includes the following steps:

- Weigh the biopsy fragment: all fragments weighing 40-80 mg

- Aliquot 10 volumes per tissue mass of Lysis/Binding Buffer into a homogenization vessel on ice
- Keeping the sample cold, thoroughly disrupt the tissue in Lysis/Binding Buffer using a motorized rotor-stator homogenizer.
- Add 1/10 volume of miRNA Homogenate Additive to the tissue lysate, and mix well by vortexing or inverting the tube several times.
- Leave the mixture on ice for 10 min.
- Add a volume of Acid-Phenol:Chloroform that is equal to the lysate volume before addition of the miRNA Homogenate Additive.
- Vortex for 30–60 sec to mix.
- Centrifuge for 5 min at maximum speed (10,000 x g) at room temperature to separate the aqueous and organic phases. After centrifugation, the interphase should be compact; if it is not, repeat the centrifugation.
- Carefully remove the aqueous (upper) phase without disturbing the lower phase, and transfer it to a fresh tube. Note the volume removed.

- Add 1.25 volumes of room temperature 100% ethanol to the aqueous phase
- Pipet the lysate/ethanol mixture (from the previous step) onto the Filter Cartridge
- Centrifuge for ~15 sec to pass the mixture through the filter. Centrifuge at RCF 10,000 x g (typically 10,000 rpm).
- Discard the flow-through, and repeat until all of the lysate/ethanol mixture is through the filter.
- Apply 700 µL miRNA Wash Solution 1 (working solution mixed with ethanol) to the Filter Cartridge and centrifuge for ~5–10 sec.
- Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.
- Wash the filter twice with 500 µL Wash Solution 2/3**:** Apply 500 µL Wash Solution 2/3 (working solution mixed with ethanol) and draw it through the Filter Cartridge as in the previous step.
- Repeat with a second 500 µL aliquot of Wash Solution 2/3.

- After discarding the flow-through from the last wash, replace the Filter Cartridge in the same Collection Tube and spin the assembly for 1 min to remove residual fluid from the filter.
- Elute RNA with 100 µL 95°C Nuclease-free Water: Transfer the Filter Cartridge into a fresh Collection Tube (provided of pre-heated (95°C) nuclease-free water to the center of the filter, and close the cap.
- Spin for ~20–30 sec at maximum speed to recover the RNA. below.
- Collect the eluate and place at -80 ° C

AllPrep DNA/RNA/Protein Mini Kit: allows simultaneous purification of DNA, RNA, and protein from the same precious sample (resume in flow chart Fig 3.4). The peculiarity of this kit is that it allows the extraction of DNA/RNA and protein fragment from the same small, up to 30 mg.

- All pieces weigh less than 10 mg
- Tissues are first lysed and homogenized, using a motorized rotor-stator homogenizer, in Buffer RLT

which immediately inactivates DNases and RNases as well as proteases to ensure isolation of intact DNA, RNA, and proteins.

- The lysate is then passed through an AllPrep DNA spin column. This column, in combination with the high-salt buffer, allows selective and efficient binding of genomic DNA. The column is washed and pure, ready-to-use DNA is then eluted.
- Ethanol is added to the flow-through from the AllPrep DNA spin column to provide appropriate binding conditions for RNA, and the sample is then applied to an RNeasy spin column, where total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in RNase-free water.
- Buffer APP, a novel aqueous protein precipitation solution, is added to the flow-through of the RNeasy spin column, and the precipitated proteins are pelleted by centrifugation. Intact total proteins are redissolved in an appropriate buffer and then ready to use in downstream applications. The kit includes

Buffer ALO, which is compatible with SDS-PAGE, for dissolving the protein pellet.

The **AllPrep DNA/RNA/Protein Mini Kit** includes the following steps:

- Tissues are first lysed and homogenized, using a motorized rotor-stator homogenizer
  Tutti I campioni pesano 4-5 mg
- Centrifuge the lysate for 3 min at maximum speed. Carefully remove the supernatant by pipetting, and transfer it to the AllPrep DNA spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm).
- **(STEP A)** Place the AllPrep DNA spin column in a new 2 ml collection tube, and store at room temperature (15–25ºC) or at 4ºC for later DNA purification. Use the flow-through for RNA purification.

Total RNA purification

- Add 350 µl of 100% ethanol to the flowthrough, and mix well by pipetting. Do not centrifuge.
- Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm).
- **(STEP B)** Transfer the flow-through to a 2 ml tube for protein purification in steps .
- Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
- Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
- Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at

≥8000 x g (≥10,000 rpm) to wash the spin column membrane.

- Place the RNeasy spin column in a new 2 ml collection tube and discard the old collection tube with the flowthrough. Centrifuge at full speed for 1 min.
- Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
- If the expected RNA yield is >30 µg, repeat step 12 using another 30–50 µl of RNase-free water.

Total protein precipitation

- Add 600 µl of Buffer APP to the flow-through from **STEP B**. Mix vigorously and incubate at room temperature for 10 min to precipitate protein.
- Centrifuge at full speed for 10 min.
- **(STEP C)** Transfer the supernatant to a new 2 ml tube.

- Add 500 µl of 70% ethanol to the protein pellet. Centrifuge at full speed for 1 min, and remove the supernatant by using a pipet or by decanting as much liquid as possible. It is not necessary to resuspend or incubate the pellet.
- Dry the protein pellet for 5–10 min at room temperature.
- Add up to 100 µl 8 M urea and mix vigorously to dissolve the protein pellet.
- Incubate for 5 min at 95°C to completely dissolve and denature the protein. Then cool the sample to room temperature.
- Centrifuge for 1 min at full speed to pellet any residual insoluble material. The supernatant can be stored at –20°C .

Purification of miRNA from cells and tissues using the AllPrep® DNA/RNA/Protein Mini Kit and RNeasy® MinElute® Cleanup Kit

- Add 1 volume of 100% ethanol to the supernatant from **STEP C**, and mix well by pipetting up and down.
- Transfer up to 700 µl of the sample to an RNeasy MinElute spin column placed in a
- 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Repeat this step until the entire sample has passed through the RNeasy MinElute membrane.
- Place the RNeasy MinElute spin column in a new 2 ml collection tube. Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flowthrough.
- Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.

- Place the RNeasy MinElute spin column in a new 2 ml collection tube. Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.
- Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane.
- Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

Genomic DNA purification

- Add 500 µl Buffer AW1 to the AllPrep DNA spin column from **STEP A**. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (10,000 rpm). Discard the flow-through.
- Add 500 µl Buffer AW2 to the AllPrep DNA spin column. Close the lid gently, and centrifuge for 2 min at full speed to wash the spin column membrane.
- Place the AllPrep DNA spin column in a new 1.5 ml collection tube. Add 100 µl Buffer EB directly to the

spin column membrane and close the lid. Incubate at room temperature (15–25ºC) for 1 min, and then centrifuge for 1 min at ≥8000 x g (10,000 rpm) to elute the DNA

- Repeat below with 50 µl Buffer EB to elute further DNA

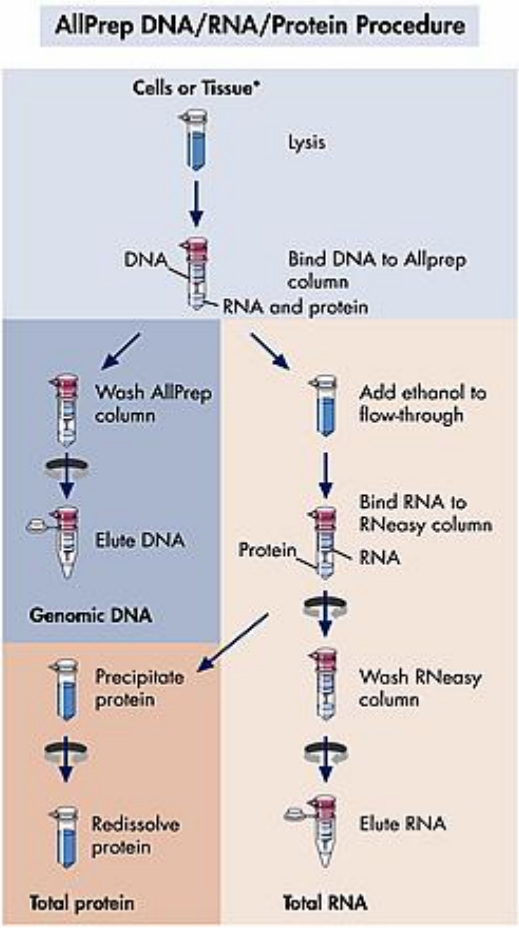


Fig. 3.4: Flowchart AllPrep DNA/RNA/Protein procedure

3.4 Analysis of total RNA extracted

- **Bioanalyzer analisys**

RNA integrity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies).

Qualitative and quantitative RNA testing was performed by capillary electrophoresis gel denaturant on equipment using microchips Agilent RNA 6000 Nano Assay (Agilent Technologies, USA). In particular, electrophoresis allows visualization of the two peaks of rRNA and 18S, 28S (Fig. 2.5), which together account for about 80% of total RNA, as well as a peak on RNA complex of low molecular lost. The integrated area under the two peaks rRNA allows an estimate of the concentration of total RNA which is a parameter great importance particularly in view of subsequent uses that require a high level of RNA quality. This quantification was performed only for the samples of RNA extracted from biopsies.

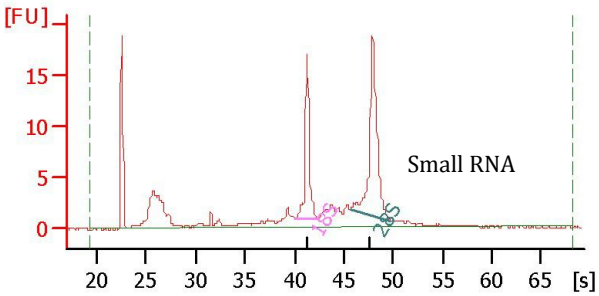


Fig. 3.5: Example of Electropherogram

- **Nanodrop analysis**

RNA extracted using the protocols described above was analyzed by spectrophotometer (NanoDrop-1000, Thermo Scientific, USA) to verify the quantity using 1 µl of extracted RNA. This quantification was performed both for the samples of RNA extracted from biopsies at time zero and for the follow-up RNA samples.

3.5 Affymetrix GeneChip Arrays

The methodology used for the analysis of the transcriptome, is that of Affymetrix microarrays (Human Genome U133 Plus 2.0). The Affymetrix chip allows to analyze simultaneously transcribed approximately 47.000 in order to study the different gene expression in different age-groups.

The laboratory where I conducted this research is not equipped with the necessary platform for reading Affymetrix chips. For this reason, RNA extracts were sent to the laboratory of the Department of Biomedical Sciences at the University of Modena. I personally (though not operationally) oversaw the Modena team in the process of preparing the chips.

Biopsy samples analyzed were 35, including 13 females and 22 males. The following table 3.6 shows the data for the 35 biopsies divided into 4 age groups based on age of the donor:

TRASCRIPTOME ANALYSIS	**Age Group 1 (20 -30 years)**			
	Sample Code	**donor sex**	**donor age**	**recipient age**
	5	M	30	58
	16	F	13	14
	17	M	23	46
	20	M	26	64
	22	M	29	37
	39	M	20	49
	Age Group 2 (31-50 years)			
	6	M	43	57
	7	M	44	41
	10	M	37	51
	15	M	50	65
	24	M	50	66
	44	M	43	21
	45	M	45	49
	Age Group 3 (51-70 years)			
	1	M	59	62
	2	F	58	52
	3	F	69	50
	11	F	63	59
	12	F	67	27
	14	M	54	-
	21	F	60	60
	23	F	53	45
	26	M	66	55
	27	F	54	64
	28	M	58	67
	31	F	69	59
	Age Group 4 (71-90 years)			
	8	F	73	64
	9	F	76	42
	19	M	87	59
	29	M	84	68
	30	M	76	43
	32	F	77	47
	33	M	82	50
	34	F	90	40
	36	M	82	57
	42	M	77	48

Table 3.6: samples used for transcriptome analysis

GeneChip® Expression Analysis

Total RNA (1 μg to 15 μg) is first reverse transcribed using a T7- Oligo(dT) Promoter Primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA is purified and serves as a template in the subsequent in vitro transcription (IVT) reaction. The IVT reaction is carried out in the presence of T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets are then cleaned up, fragmented, and hybridized to GeneChip expression arrays.

The following major steps outline GeneChip expression analysis:

1. Target Preparation: double-stranded cDNA is synthesized from total RNA. An in vitro transcription (IVT) reaction is then done to produce biotin-labeled cRNA from the cDNA. The cRNA is fragmented before hybridization.

2. Target Hybridization: A hybridization cocktail is prepared, including the fragmented target, probe array controls, BSA, and herring sperm DNA. It is then hybridized to the probe array during a 16-hour incubation. The hybridization process is described in the respective sections for the different probe array types.

3. Fluidics Station Setup: The fluidics station is then prepared for use by priming with the appropriate buffers.

4. Probe Array Washing and Staining: Immediately following hybridization, the probe array undergoes an automated washing and staining protocol on the fluidics station.

5. Probe Array Scan: Once the probe array has been hybridized, washed, and stained, it is scanned. Each workstation running Affymetrix Microarray Suite or GCOS can control one scanner. The software defines the probe cells and computes an intensity for each cell. Each complete probe array image is stored in a separate data file identified

by the experiment name and is saved with a data image file (.dat) extension.

6. Data Analysis: The .dat image is analyzed for probe intensities; results are reported in tabular and graphical formats.

The protocol is summarized in the following flowchart (Fig. 3.7)

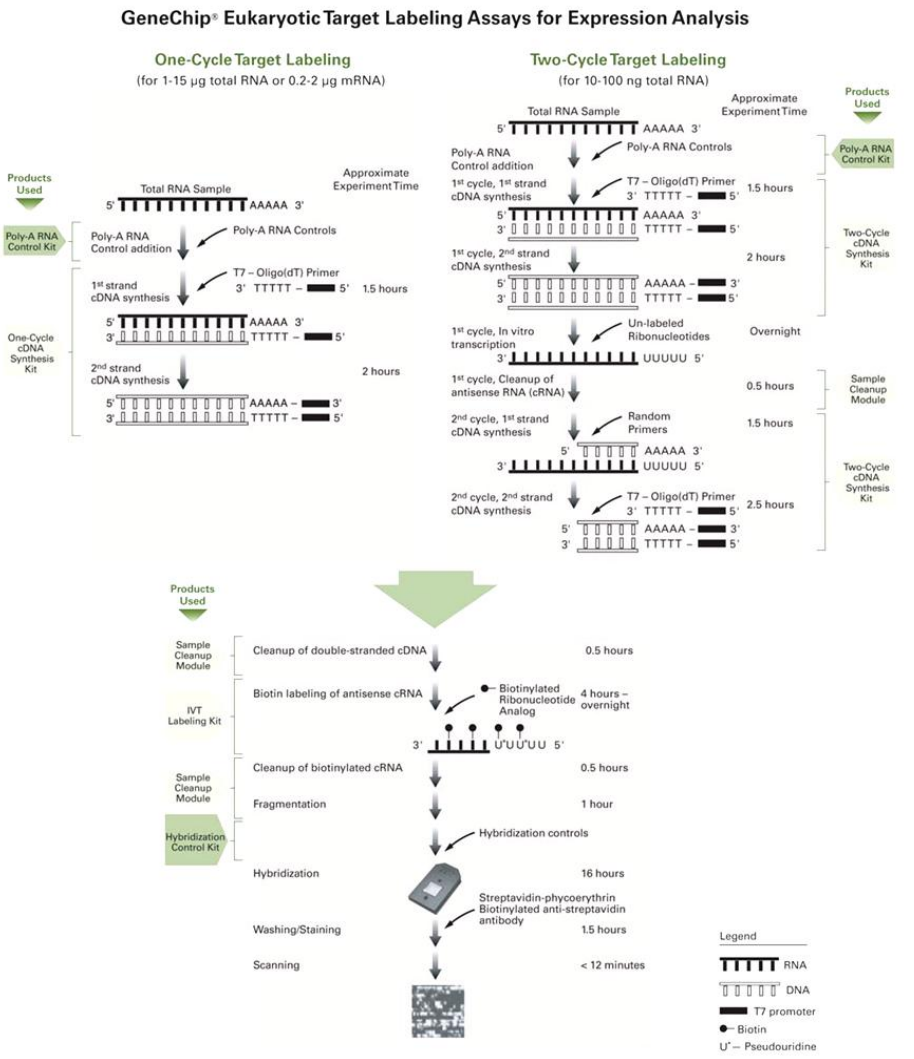


Fig. 3.7: GeneChip Eukaryotic Labeling Assays for Expression Analysis

3.6 MicroRNA profiling

365 human mature miRNAs were profiled in 12 liver sample of male subjects of different age (age range 18-90 y). MiRNA analysis was performed using an Applied Biosystem 7900 HT real-time PCR instrument and human MicroRNA Array pool A (TaqMan, Applied Biosystem), containing 365 different human miRNA assays in addition to selected small nucleolar RNAs (snoRNAs). The table below (Table 2.8) shows the distribution of samples in the 4 age groups.

Age Group 1 (20 -30 years)			
Sample Code	**donor sex**	**donor age**	**recipient age**
5	M	30	58
17	M	23	46
20	M	26	64
Age Group 2 (31-50 years)			
6	M	43	57
7	M	44	41
10	M	37	51
Age Group 3 (51-70 years)			
14	M	54	-
26	M	66	55
28	M	58	67
Age Group 4 (71-90 years)			
19	M	87	59
29	M	84	68
30	M	76	43

Table 3.8: distribution of samples in the 4 age groups

Following the protocol:

Reverse transcription (RT) Reaction

RNA was converted to cDNA by priming with a mixture of looped primers using MegaPlex kit (Applied Biosystems, Foster City CA).

RT Reaction Mix

- Combine the following in a 1.5-mL microcentrifuge tube:

RT Reaction Mix Components	Volume for One Sample (µl)
Megaplex RT primers (10X)	0.8
dNTPs with dTTP (100mM)	0.2
MultiScribe Reverse (50 U/µl)	1.5
10X RT Buffer	0.8
MgCl2 (25 mM)	0.9
Rnase Inhibitor (20 U/µl)	0.1
Nuclease-free water	0.2
TOTAL	**4.5**

- Invert the tube six times to mix, then centrifuge the tubes briefly.

- In a Tube Strip, pipette 4.5 µL of the RT reaction mix into each tube
- Add 6 µL (1 to 350 ng) total RNA into each tube containing RT reaction mix.
- Then invert the tubes six times to mix. Spin briefly.
- Incubate the plate on ice for 5 min.
- Set up the run method using the following conditions:

Stage	Temp	Time
Cycle (40 cycles)	16 °C	2 min
	42 °C	1 min
	50 °C	1 sec
Hold	85 °C	5 min
Hold	4 °C	∞

Preamplification Reactions

In this step, preamplify specific cDNA targets to increase the quantity of desired cDNA for gene expression analysis using TaqMan® MicroRNA Arrays.

PreAmp Reaction Mix:

PreAmp Reaction Mix Components	Volume for One Sample (µl)
TaqMan preAmp Master Mix, 2X	12.5
MegaPlex PreAmp Primers (10X)	2.5
Nucelase-free water	7.5
TOTAL	**22.5**

- Invert the tube six times to mix, then centrifuge the tubes briefly.
- Into each tube, pipette 2.5 µL of each RT product
- Dispense 22.5 µL of PreAmp reaction mix into each tube containing the RT product.
- Then invert the tubes six times to mix. Spin briefly.
- Incubate the tubes on ice for 5 min.
- Set up the run method using the following conditions:

Stage	Temp	Time
Hold	95 ℃	10 min
Hold	55 ℃	2 min
Hold	72 ℃	2 min
Cycle (12 Cycles)	95 ℃	15 sec
	60 ℃	4 min
Hold	99.9 ℃	10 min
Hold	4 ℃	∞

- Remove the tubes from the thermal cycler.
- Briefly centrifuge the tubes or plate.
- Dilute the sample add 75 μL of 0.1× TE pH 8.0 to each tube.
- Seal the tubes, then invert six times to mix, and spin briefly.

Real-Time PCR Reactions

Prepare the TaqMan® MicroRNA Array: To bring the card to room temperature before use

- Thaw the diluted, stored PreAmp product on ice.
- Mix by inverting six times, then centrifuge the tube or plate briefly.

- Mix the TaqMan Universal PCR Master Mix by swirling the bottle.
- Prepare the PCR reaction mix in a 1.5-mL microcentrifuge tube:

Component	Volume for One Array
Taqman Universal PCR Master Mix, No AmpErase UNG 2X	450
Diluited PreAmp product	9
Nuclease-free water	441
Total	900

- Invert the tube six times to mix, then centrifuge the tubes briefly.
- Dispense 100 µL of the PCR reaction mix into each port of the CARD A v 2 TaqMan MicroRNA Array (Fig. 3.9)
- Centrifuge, then seal the array.
- Load and run the array using the 384 –well TaqMan Low density Array default thermal- cycling (conditions in Applied Biosystem 7900HT Fast Real-Time PCR System instrument)

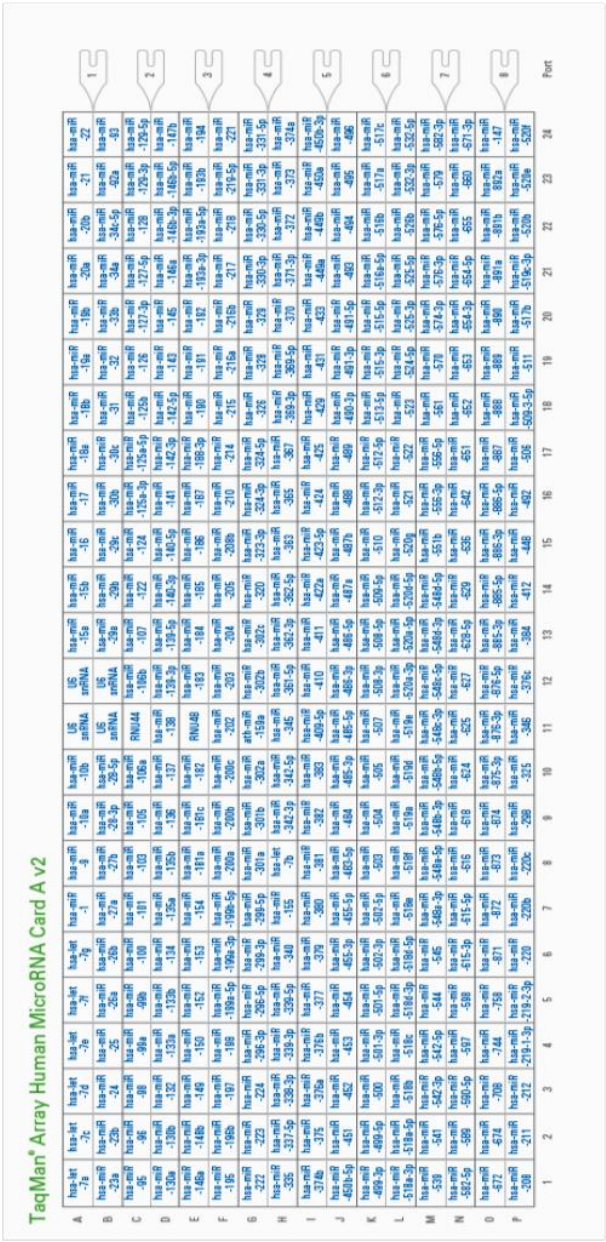


Fig 3.9. TaqMan® Array Microfluidic Card A (Applied Biosystem)

3.7 Real Time PCR (qRT PCR)

Expression levels of specific mature miRNAs were assessed by real-time PCR analysis using a TaqMan Human MicroRNA Assay kit (Applied Biosystems, Foster City, CA).

Quantification using the TaqMan MicroRNA Assays was carried out using two-step RT-PCR:

1. In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using specific miRNA primers from the TaqMan MicroRNA Assays and reagents from the TaqMan®miRNA Reverse Transcription Kit.
2. In the PCR step, PCR products are amplified from cDNA samples using the TaqMan MicroRNA Assay together with the TaqMan® Universal PCR Master Mix.

   The two steps are summarized in Figure 3.10

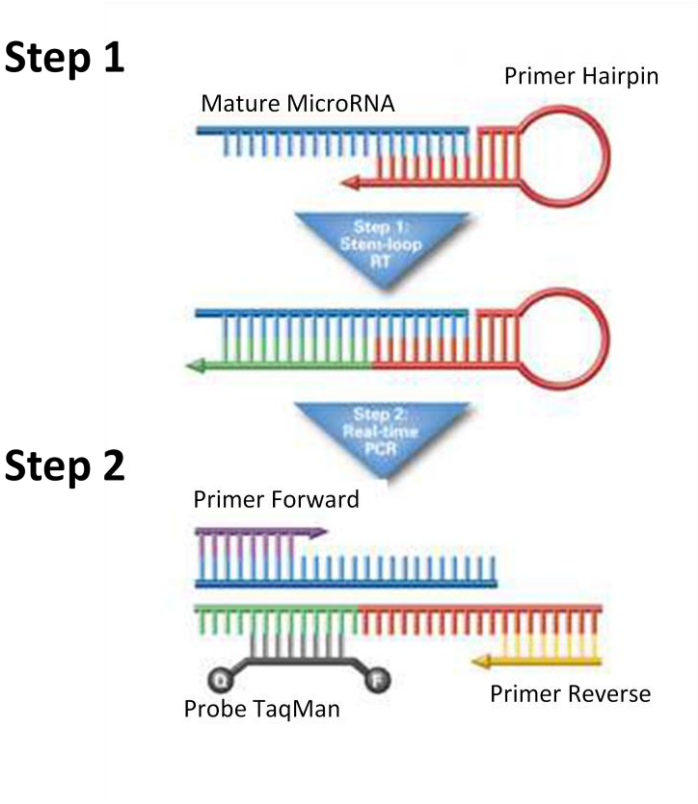


Figure. 3.10: Real-time PCR kit 'TaqMan MicroRNA Assay'.

Step 1

- In a test tube add 0.2 ml volume use of 10 ng of total RNA per 15 µL RT reaction
- Prepare the mix for reverse transcription using reagents of the kit 'TaqMan ® MicroRNA Reverse

Transcription 'primer and 5X contained in the kit' TaqMan MicroRNA Assay.

Component Master Mix	**Master mix Volume/15 µl**
100mM dNTPs (with dTTP)	0.15 µl
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00 µl
10X Reverse Transcription Buffer	1.50 µl
RNase Inhibitor, 20U/µL	0.19 µl
Nuclease-free water	4.16 µl
Total	**7.00 µl**

- Each 15-µL RT reaction consists of 7 µL master mix, 3 µL primer, and 5 µL RNA sample.
- Mix gently. Centrifuge to bring solution to the bottom of the tube.
- Before opening the RT Primer tubes, thaw the tubes on ice and mix by vortexing, then centrifuge them.
- For each 15-µL RT reaction, combine RT master mix with total RNA in the ratio of: 7 µL RT master mix to 5 µL total RNA.

- Mix gently. Centrifuge to bring the solution to the bottom of the tube.
- For each 15-µL RT reaction, dispense 12.0 µL of RT master mix containing total RNA into a 0.2-mL polypropylene reaction tube.
- Transfer 3 µL of RT primer (tube labeled RT Primer) from each assay set into the corresponding RT reaction tube.
- Incubate the tube on ice for 5 min and keep on ice
- Placed the samples in thermal cycler and run the following program.

Stage	Temp	Time
Hold	16 °C	30 min
Hold	42 °C	30 min
Hold	85 °C	5 min
Hold	4 °C	∞

Step 2

After the reverse transcription reaction, place the cDNA samples on ice and prepare the reaction mix for real-time PCR.

Component	Volume (µl)/ 20 µl Reaction
TaqMan MicroRNA Assay (20X)	1
Product from RT reaction (Minimum 1:15 Dilution)	1.33
TaqMan 2x Universal PCR FAST Master Mix, No	10
Nuclease-free water	7.67
Total Volume	20

- Mix gently. Centrifuge to bring solution to the bottom of the tubes.
- Prepare the PCR reaction tube by dispensing 20 µL of the complete PCR master mix into each tube.
- Load the reaction tubes into the rotor in real time PCR cycler Rotor Gene-Q and start. The samples were analyzed in duplicate. Instrument used : real-time PCR cycler Rotor Gene-Q (Qiagen)
- Set up the run method using the following conditions:

Stage	Temp	Time
Hold	95°C	20 sec
Cycle	60 °C	1 sec
Hold	85 °C	20 sec

- The Real Time PCR results were normalized in two different ways:

- miRNA 21 was used to normalize the results obtained from the donor.
- RNU44 was used to normalize the results obtained from the follow up.

Subsequently, the relative expression of the miRNAs was evaluated by inserting data into an Excel data sheet using the $2^{-(Ct)}$ method.

3.8 Protein extraction and western blot analysis

- Total proteins were extracted from 200 mg frozen liver tissue.
- Western blot analysis was performed in 4 young subjects compared with 7 older subjects (Table 3.11)

sample code	age (years)	sex
88	12	M
80	37	M
77	44	M
24	50	M
78	71	M
113	73	M
100	75	M
46	75	M
36	82	M
114	83	M
98	83	M

Table 3.11: samples analyzed by western blot analysis

- Keeping the sample thoroughly disrupt the tissue in 500 µl of RIPA buffer using a motorized rotor-stator homogenizer
- Leave the mixture on ice for 10 min
- Vortex for 30–60 sec to mix.
- Leave the mixture on ice for 10 min
- Centrifuge for 1 hour at maximum speed (25,000 x g) at 4°C
- The supernatant were quantified using the Bradford assay (BioRad Laboratories).

Western Blot analysis:

- Take a volume of 40 µg protein extract.
- Add 2,5 µl 2X Laemmli buffer.
- Add water to a final volume of 10 µl
- placing the tubes in the thermocycler at 95 ° C for 5 minutes
- With a Hamilton syringe by loading the samples and markers in quantities by default on polyacrylamide gel.
- Prepare the gel with the following composition for resolving and stacking gel:

RESOLVING GELS 12%	
Components Gel	**Volume (ml)**
Water	3.3
30% acrylamide- Bisacrilamide solution 37.5: 1	4
1,5M Tris (PH 8,8)	2.5
10% SDS	0.1
10% Ammonium persolfate	0.1
TEMED	0.004

STACKING GEL 5%	
Components Gel	**Volume (ml)**
Water	2.2
30% acrylamide- Bisacrilamide solution 37.5: 1	0.67
1,0M Tris (PH 6,8)	1
10% SDS	0.04
10% Ammonium persolfate	0.04
TEMED	0.004

- Perform electrophoresis in 1X Running Buffer at 30 mA for 1 h at RT.
- Equilibrate the gel for 1 'in 1X Transfer Buffer.
- Assemble the transfer apparatus and transfer of membrane 1X transfer buffer at 250 mA for 2.5 h at 4°C.
- Disassemble the apparatus and stain the membrane with Ponceau red to verify the advent of protein transfer.
- Decolorize the membrane in TBS Tween 20 0,1%
- Membranea Wash in TBS Tween 20 0.1% more (TBS-T)
- Block the membrane for 1 hour at RT gentle agitation.

- Wash the membrane for 1 'in TBS-T.
- Incubate the membrane overnight at 4 ° C in agitation with the primary antibody:

  - SCL1A2 (LSBio, USA) diluted 1:250 in 5% (w/v) milk powder in TBS- Tween 20 0.01%

  - actin: diluted 1:1000 in 5% (w/v) milk powder in TBS- Tween 20 0.01%
- Wash the membrane 3 times in TBS-T-7 'at RT in agitation.
- Incubate the membrane for 1 hour at RT in agitation with the secondary antibody:

  - anti-mouse diluted 1:1000 in 5% (w/v) milk powder in TBS- Tween 20 0.01% for SCL1A2

  - anti–goat diluted 1: 1500 in 5% (w/v) milk powder in TBS- Tween 20 0.01% for Actin
- Detected with ECL Advance Western Blotting Detection Kit (Santa Cruz Biotechnology) in the dark room to add a photographic plate and, after 30 minutes for SCL1A2, 1 minute for Actin, the best time to impress, to proceed with the development of the photographic plate.

- Acquiring a digital image of the plates through the scanner and the program PhotoShop and quantifying the bands using densitometric software Quantity-One (Biorad).

3.9 Softwares

Analysis of Gene Chip Affymetrix microarray was performed by the group of computer science and statistics of the Department of Physics, University of Bologna, by the use of the software MATLAB. T student Test and Anova were used to assess significance among groups of different ages. P values less than 0.05 were considered significant

Analysis of miRNAs putative targets: SID1.0 (Simple String Identifier) was used for identification of miRNAs targets. SID1.0 is Fortran program, based on the strategy of exhaustive search and specifically designed to screen shared data (target genes, miRNAs and pathways) available from PicTar and DIANA-MicroT 3.0 databases (Albertini et al., 2011). computational tools for determining the most energetically favored hybridization sites of small to large

RNAs. PicTar (Krek et al., 2005) is capable of identifying common targets of known miRNAs. DIANA-microT (Kiriakidou et al., 2004) system utilizes experimentally derived miRNA/mRNA binding rules.

3.10 Statistical analysis of CARD A

MiRNAs from CARD A expressed at detectable level (80% of CARD raw data) were included in the final data analysis. miRNAs expression was normalized comparing their expression to the endogenous control, i.e the MummU6 average . The overall miRNA expression on each array is normalised by using miRNA21 or RNU44 median (ΔCt). MiRNA fold changes higher than 2 and lower than 0.5 were selected (ΔΔCt > 2 and < 0.5). Fold-change was calculated based on the estimated mean difference ($2^{(-\Delta\Delta CT)}$). To test the significance of miRNA expression among samples, independent or paired sample T-test was used. P values less than 0.05 were considered significant.

3.11 Statistical analysis of qRT-PCR

To validate miRNA profiling analysis, qRT-PCR was performed. Linear regression was applied to data and independent or paired samples T test was used to determine statistical significance of miRNA or mRNA expression among groups. P values less than 0,05 were considered significant.

4. RESULTS

4.1 MicroRNA expression patterns

To identify miRNAs differentially expressed in human liver during aging. liver specimens were first screened using miRNA CARD A. 12 liver samples obtained from male subjects of different age were analysed as follows: 6 subjects with an age range of 18-50 years. 3 subjects with an age range of 50-70 years and 3 subjects with an age range of 70-90 years. Table 4.1 show the age group and the mean of each group.

MALE SAMPLES	GROUP1	GROUP2	GROUP3	GROUP4
YEARS	23;26;30	37;43;44	54;58;66	76;84;87
MEAN	26.3	41.3	59.3	82.3

Table 4.1: 12 liver sample in age group and mean of the years for each age group

miRNAs expressed at detectable level in more than 80% of samples were included in the analysis. Using these filtering criteria, 290 miRNAs of the 365 analysed were included in the final analysis, shown in Table 4.2. The first part of the table displays the values of the 4 (MammU6) endogenous

controls and their mean, the standard deviation and the number of miRNAs actually detected. The second part of the table, on the left, shows the miRNAs' number identification (Detector) and the Ct value of 12 samples, which were labeled with abbreviations: S01. S02. etc. The Delta Ct (ΔCt) was normalized with the average of the (Mumm U6) endogenous control: Delta Ct (ΔCt) = Ct – Average MummU6. The Ct value is listed from smallest to largest. The third part of the table contains those miRNAs were not detected in any of the 12 samples.

		Endogenous Control											
	Detector	**S01**	**S02**	**S03**	**S04**	**S05**	**S06**	**S07**	**S08**	**S09**	**S10**	**S11**	**S12**
11	MammU6-4395470	6,47	6,21	7,42	6,45	6,64	6,97	6,64	6,11	6,32	6,30	6,55	6,95
12	MammU6-4395470	6,73	6,19	7,46	6,30	6,92	6,92	6,46	6,06	6,44	6,26	6,57	6,40
35	MammU6-4395470	6,20	6,13	7,11	6,19	6,72	6,96	6,37	6,07	6,31	6,18	6,59	6,17
36	MammU6-4395470	6,31	5,97	7,20	6,13	6,65	6,83	6,47	6,06	6,23	6,14	6,55	6,60
	Average CT	**6,43**	**6,13**	**7,30**	**6,27**	**6,73**	**6,92**	**6,49**	**6,07**	**6,33**	**6,22**	**6,56**	**6,53**
	StDev	0,23	0,11	0,17	0,14	0,13	0,06	0,11	0,02	0,08	0,07	0,02	0,33
	Number of expressed miRNA	303	308	301	294	296	300	304	307	303	296	306	303

		Delta Ct = (Ct) - (Average Ct MummU6)											
#	**Detector**	**S01**	**S02**	**S03**	**S04**	**S05**	**S06**	**S07**	**S08**	**S09**	**S10**	**S11**	**S12**
107	RNU48-4373383	2,15	2,04	0,89	1,72	1,54	1,11	1,50	0,84	1,46	1,19	1,16	1,60
20	hsa-miR-19b-4373098	2,22	2,06	3,02	2,59	2,85	1,92	2,05	2,48	3,28	2,80	2,27	2,50
67	hsa-miR-126-4395339	2,50	2,19	2,75	2,39	3,03	2,45	2,91	2,26	3,04	2,40	2,26	2,16
41	hsa-miR-30c-4373060	2,85	3,64	3,49	4,46	4,45	3,50	4,07	3,98	4,27	4,11	3,43	4,25
62	hsa-miR-122-4395356	3,11	3,07	2,99	4,14	3,13	2,99	3,37	3,74	3,79	3,94	3,60	3,25
116	hsa-miR-192-4373108	3,19	2,55	2,81	2,90	3,21	2,78	3,58	2,66	2,72	2,90	2,77	2,59
95	hsa-miR-146b-5p-4373178	3,26	5,71	5,57	3,50	4,79	2,28	3,69	4,63	4,31	4,66	4,11	3,79
37	hsa-miR-29a-4395223	3,31	3,85	4,51	4,15	4,44	3,89	3,89	4,33	4,34	3,91	3,93	4,97
146	hsa-miR-223-4395406	3,31	4,95	4,55	4,42	4,10	2,58	3,27	4,17	3,21	4,19	3,61	3,95
27	hsa-miR-24-4373072	3,53	2,77	2,92	3,28	3,08	3,24	2,91	3,14	3,11	3,66	3,29	3,26
40	hsa-miR-30b-4373290	3,84	3,81	3,71	4,50	3,87	3,37	3,85	3,90	4,08	4,18	3,83	4,12
115	hsa-miR-191-4395410	4,62	4,44	4,59	4,49	4,74	4,57	4,76	4,40	4,31	4,63	4,74	4,26
21	hsa-miR-20a-4373286	4,64	4,01	4,81	4,74	4,91	3,96	3,86	4,73	5,11	5,11	4,57	5,03
16	hsa-miR-17-4395419	4,70	4,23	4,93	4,52	5,04	4,34	4,47	4,56	4,77	5,12	4,92	4,64
58	hsa-miR-106a-4395280	4,82	4,21	4,80	4,55	5,27	4,58	4,39	4,73	4,73	5,14	4,93	4,61
120	hsa-miR-194-4373106	4,87	4,61	4,59	5,21	4,84	4,70	4,99	4,70	4,92	5,16	4,61	4,57
92	hsa-miR-145-4395389	4,98	4,53	4,87	5,50	6,04	3,95	4,42	5,05	5,75	4,97	4,29	5,05

23	hsa-miR-21-4373090	5,27	5,66	5,06	5,40	4,93	4,75	5,41	5,16	4,76	5,46	5,16	5,51
93	hsa-miR-146a-4373132	5,28	4,38	4,60	4,54	4,59	4,70	4,88	4,53	4,49	4,70	3,95	5,01
59	RNU44-4373384	5,42	5,80	5,39	5,54	5,51	5,10	5,42	5,38	5,60	5,48	5,53	5,33
39	hsa-miR-29c-4395171	5,63	5,38	6,17	6,19	6,55	5,72	5,69	6,47	6,26	6,17	5,82	6,55
177	hsa-miR-342-3p-4395371	5,88	5,84	5,69	5,60	5,90	5,71	5,91	5,33	5,13	5,60	5,40	4,94
85	hsa-miR-139-5p-4395400	5,91	5,44	5,75	6,04	6,62	5,56	6,31	5,93	6,15	5,68	6,02	5,76
15	hsa-miR-16-4373121	5,98	5,69	6,20	6,00	6,30	5,89	6,37	6,13	6,02	6,32	6,24	5,82
308	hsa-miR-574-3p-4395460	6,09	5,60	5,40	5,75	6,59	5,20	6,18	5,44	6,00	6,03	6,06	5,79
350	hsa-miR-885-5p-4395407	6,29	5,41	5,39	6,27	5,78	5,72	6,31	5,85	5,91	6,04	6,18	6,01
119	hsa-miR-193b-4395478	6,30	5,77	5,39	5,59	5,94	5,15	6,06	5,91	5,66	5,63	5,79	5,52
66	hsa-miR-125b-4373148	6,30	6,46	6,07	7,40	6,62	5,63	6,16	6,77	7,24	6,94	6,25	6,75
225	hsa-miR-484-4381032	6,43	6,67	6,40	5,77	6,81	5,82	6,81	6,01	6,01	5,69	6,32	5,96
100	hsa-miR-150-4373127	6,44	5,53	5,93	6,27	4,65	5,98	5,13	4,90	5,76	5,68	3,93	5,45
97	hsa-miR-148a-4373130	6,49	6,47	6,66	7,43	6,65	6,32	6,51	6,97	7,09	7,22	6,91	6,76
91	hsa-miR-143-4395360	6,50	6,38	7,25	7,23	7,64	6,10	6,24	7,00	7,81	6,94	5,94	6,65
29	hsa-miR-26a-4395166	6,58	6,66	6,78	7,46	6,78	5,79	6,31	6,91	7,28	6,99	6,56	6,75
89	hsa-miR-142-3p-4373136	6,94	7,64	7,56	8,24	6,83	6,62	6,83	7,30	7,11	7,68	6,31	7,36
111	hsa-miR-186-4395396	6,96	6,60	6,89	6,68	7,01	6,51	6,74	6,00	6,25	6,33	6,57	6,49
145	hsa-miR-222-4395387	7,04	6,94	7,17	6,91	6,30	6,60	6,44	6,79	6,51	6,53	5,90	6,44
54	hsa-miR-100-4373160	7,05	6,83	6,68	7,79	6,93	6,37	6,70	7,02	7,66	7,65	6,80	7,96
121	hsa-miR-195-4373105	7,07	7,29	7,30	7,89	7,17	6,53	6,50	7,62	7,79	7,68	6,82	7,40
52	hsa-miR-99a-4373008	7,14	7,13	6,67	8,02	7,25	6,31	6,97	7,37	7,69	7,97	7,00	7,42
167	hsa-miR-331-3p-4373046	7,17	6,86	6,54	6,94	6,55	6,34	6,95	6,72	6,53	6,92	6,48	6,85
126	hsa-miR-199a-3p-4395415	7,20	7,79	7,77	8,05	7,75	6,29	6,90	7,83	8,62	8,11	7,10	7,68
47	hsa-miR-92a-4395169	7,37	6,88	6,82	7,36	7,25	6,07	7,23	8,04	7,74	7,76	7,02	7,49
87	hsa-miR-140-5p-4373374	7,50	7,77	7,93	8,28	8,08	7,47	7,57	7,46	7,97	8,04	7,35	7,44
137	hsa-miR-214-4395417	7,66	8,15	7,74	7,64	8,39	6,64	7,51	8,12	8,61	8,06	7,33	7,83
192	hsa-miR-374a-4373028	7,72	7,51	7,23	7,48	7,80	7,03	7,50	7,32	7,56	7,46	7,55	7,42
60	hsa-miR-106b-4373155	7,75	8,11	7,68	7,88	7,72	7,28	7,83	7,65	7,56	8,04	7,63	7,89
4	hsa-let-7e-4395517	7,78	8,03	7,04	8,36	7,64	7,12	7,58	7,99	8,33	8,40	7,84	7,97
24	hsa-miR-22-4373079	7,81	8,51	8,26	8,15	7,86	8,03	8,35	8,18	7,92	8,47	8,34	8,00
19	hsa-miR-19a-4373099	7,89	8,02	8,70	8,51	8,75	7,91	7,78	8,30	8,61	8,61	8,23	8,23
158	hsa-miR-320-4395388	7,96	8,05	8,10	7,89	8,64	7,74	8,15	7,85	8,28	8,21	8,38	8,09
30	hsa-miR-26b-4395167	7,97	7,81	7,81	8,91	7,84	7,12	7,41	8,06	8,20	8,15	7,64	7,92
32	hsa-miR-27b-4373068	7,98	7,70	7,53	8,60	7,45	7,60	7,41	8,24	8,02	8,51	7,66	7,88
48	hsa-miR-93-4373302	8,01	8,31	8,08	8,23	8,49	7,76	7,96	8,15	7,86	8,61	8,17	8,30
31	hsa-miR-27a-4373287	8,03	8,18	8,26	8,26	8,37	7,34	7,60	8,24	8,47	8,57	7,32	8,14
6	hsa-let-7g-4395393	8,04	8,31	8,21	8,61	8,37	7,95	8,08	8,15	8,48	8,48	8,24	8,17
132	hsa-miR-203-4373095	8,09	7,78	7,25	7,73	9,21	8,38	7,97	7,04	7,66	7,93	8,12	8,15
33	hsa-miR-28-3p-4395557	8,14	7,54	7,49	8,20	8,04	7,59	8,17	7,76	7,77	7,86	7,49	7,98
101	hsa-miR-152-4395170	8,23	8,34	7,89	8,41	8,23	7,85	7,86	8,02	8,13	8,03	7,86	7,87
184	hsa-miR-365-4373194	8,49	7,65	7,51	8,38	7,96	7,54	8,48	7,81	8,16	8,13	8,17	7,90
22	hsa-miR-20b-4373263	8,67	7,37	8,16	8,02	8,49	7,70	8,07	8,09	8,47	8,57	8,20	8,29
34	hsa-miR-28-5p-4373067	8,74	8,69	8,12	9,20	8,65	8,11	8,61	8,58	8,61	9,01	8,19	8,66
194	hsa-miR-375-4373027	8,78	8,38	8,57	7,78	6,89	6,92	7,74	9,26	8,55	8,64	7,62	7,63
142	hsa-miR-218-4373081	8,90	8,85	9,38	9,37	9,20	8,68	8,68	8,96	9,70	8,88	7,81	9,07
176	hsa-let-7b-4395446	9,06	10,00	8,59	10,24	9,21	8,85	9,25	9,79	10,09	10,08	9,35	9,94
45	hsa-miR-34a-4395168	9,18	10,14	9,20	9,12	8,91	9,29	8,51	9,70	8,66	9,12	8,34	8,27
179	hsa-miR-345-4395297	9,21	8,79	8,88	9,52	9,37	8,51	8,83	8,84	8,43	8,83	8,96	8,99
218	hsa-miR-451-4373360	9,30	8,51	9,29	9,56	9,97	7,36	9,30	9,45	10,17	9,12	8,53	9,59
75	hsa-miR-132-4373143	9,35	9,71	9,26	8,96	9,35	8,46	8,90	9,16	9,65	9,47	8,81	8,89
28	hsa-miR-25-4373071	9,35	9,45	9,29	9,86	9,39	9,00	9,21	9,60	9,35	9,74	9,25	9,58
288	hsa-miR-532-5p-4380928	9,37	9,46	9,01	9,19	9,46	8,83	9,29	9,22	9,24	9,09	9,06	9,01
221	hsa-miR-454-4395434	9,43	9,22	9,67	8,97	9,56	9,17	9,05	8,54	8,49	9,05	9,11	8,78
73	hsa-miR-130a-4373145	9,44	9,89	9,85	10,14	9,54	8,67	9,44	9,46	9,51	10,02	9,35	9,77
1	hsa-let-7a-4373169	9,52	9,93	8,31	10,42	9,32	8,85	9,13	9,73	9,95	9,93	9,72	9,68
136	hsa-miR-210-4373089	9,59	9,78	10,20	10,04	9,08	10,94	11,02	10,11	10,32	10,05	10,50	9,83
133	hsa-miR-204-4373094	9,67	9,75	9,68	10,48	10,13	8,53	8,66	10,01	10,33	10,03	9,39	9,50
14	hsa-miR-15b-4373122	9,76	10,20	8,97	10,09	9,28	8,91	9,85	9,93	9,27	10,32	9,59	9,96
65	hsa-miR-125a-5p-4395309	9,78	10,03	9,97	10,30	11,00	9,38	10,25	10,32	10,75	10,18	9,82	10,86
26	hsa-miR-23b-4373073	9,82	9,92	9,46	10,22	9,77	9,26	10,16	10,03	9,70	10,20	10,13	9,87

2	hsa-let-7c-4373167	9,84	10,27	8,95	10,72	9,59	9,13	9,65	10,12	10,45	10,71	9,82	10,09
193	hsa-miR-374b-4381045	9,95	9,85	9,40	10,78	10,09	9,38	9,92	10,35	10,44	10,63	10,14	10,16
224	hsa-miR-483-5p-4395449	9,95	9,89	9,26	10,31	10,24	9,82	9,96	9,71	9,60	9,86	9,77	10,04
340	hsa-miR-744-4395435	9,96	10,12	9,41	9,92	9,80	9,42	9,63	9,88	9,69	10,48	9,72	9,71
372	hsa-miR-376c-4395233	9,97	9,81	10,44	10,50	11,23	9,51	10,33	9,90	10,76	10,20	10,03	9,98
9	hsa-miR-10a-4373153	10,02	10,01	10,23	10,85	10,29	9,21	9,85	10,60	11,03	10,53	10,13	10,19
68	hsa-miR-127-3p-4373147	10,20	10,25	10,35	11,05	11,08	9,33	10,23	10,32	11,01	10,45	9,88	10,75
335	hsa-miR-660-4380925	10,27	10,33	10,17	10,51	10,34	9,81	10,15	10,43	10,39	10,35	10,10	10,25
53	hsa-miR-99b-4373007	10,31	10,45	9,89	11,03	10,35	9,36	10,15	10,67	10,84	10,71	9,95	12,60
38	hsa-miR-29b-4373288	10,33	10,43	10,67	10,71	10,33	9,87	9,91	10,74	10,36	10,65	10,08	10,64
3	hsa-let-7d-4395394	10,34	11,08	9,67	11,27	10,71	9,70	10,30	10,65	10,92	11,02	10,74	10,10
223	hsa-miR-455-5p-4378098	10,38	10,25	10,37	10,83	10,50	10,14	10,24	10,52	10,93	10,85	10,48	10,42
56	hsa-miR-103-4373158	10,41	10,36	9,98	10,69	10,32	9,93	10,22	10,50	10,23	10,53	10,44	10,31
287	hsa-miR-532-3p-4395466	10,63	10,77	9,91	10,78	10,47	9,69	10,60	10,41	10,08	10,22	10,38	10,49
128	hsa-miR-200a-4378069	10,65	10,17	10,60	11,71	8,17	10,58	9,89	10,26	11,15	10,52	9,47	10,40
17	hsa-miR-18a-4395533	10,70	10,94	10,71	10,18	10,95	10,04	10,09	10,71	10,42	11,02	10,91	11,05
315	hsa-miR-590-5p-4395176	10,71	10,17	10,79	10,44	10,92	10,42	10,34	10,08	10,35	10,55	10,49	10,20
118	hsa-miR-193a-5p-4395392	10,74	10,36	9,61	11,33	10,19	10,18	10,36	10,72	11,36	11,32	11,00	11,13
13	hsa-miR-15a-4373123	10,74	11,07	11,24	11,57	11,15	11,15	11,07	11,20	10,98	11,25	11,08	10,98
55	hsa-miR-101-4395364	10,95	10,82	11,40	11,88	11,40	10,67	10,80	11,43	11,79	11,51	11,13	11,21
195	hsa-miR-376a-4373026	10,98	10,77	10,76	10,69	11,28	9,67	10,34	10,16	10,86	10,50	9,93	10,18
74	hsa-miR-130b-4373144	10,99	11,79	11,03	12,39	11,15	10,16	10,65	10,17	9,60	10,35	11,04	10,98
174	hsa-miR-340-4395369	11,00	10,26	10,65	11,51	11,10	10,42	10,89	10,90	11,58	11,72	10,93	11,03
351	hsa-miR-886-3p-4395305	11,10	12,42	12,71	12,13	10,93	10,66	9,37	13,75	12,90	12,91	11,08	10,61
104	hsa-miR-181a-4373117	11,12	12,04	11,16	12,39	11,57	10,79	11,47	11,92	12,34	12,45	11,37	11,73
110	hsa-miR-185-4395382	11,14	11,53	11,24	11,04	11,05	11,33	11,48	11,20	11,04	11,13	11,23	10,98
161	hsa-miR-324-5p-4373052	11,24	11,53	11,10	11,75	11,28	10,73	11,28	11,28	11,00	11,67	11,33	11,56
209	hsa-miR-425-4380926	11,28	11,26	10,82	11,52	11,11	11,05	11,72	10,96	10,99	11,28	11,57	11,21
129	hsa-miR-200b-4395362	11,29	11,06	11,41	13,17	9,50	11,32	10,59	11,21	11,91	11,35	10,28	11,50
99	hsa-miR-149-4395366	11,34	12,10	12,04	11,97	12,62	10,84	11,55	11,63	12,42	11,91	11,74	11,98
49	hsa-miR-95-4373011	11,47	11,46	11,11	11,53	11,47	10,62	11,23	11,14	11,67	11,82	11,17	11,25
222	hsa-miR-455-3p-4395355	11,56	11,20	10,72	12,66	11,37	10,62	11,31	12,34	12,45	12,49	11,66	11,80
10	hsa-miR-10b-4395329	11,61	12,12	12,50	12,58	12,61	10,28	11,68	12,43	13,73	12,87	12,02	12,11
205	hsa-miR-411-4381013	11,66	11,70	11,60	11,95	12,31	10,73	11,56	11,46	12,23	11,71	11,27	11,46
173	hsa-miR-339-5p-4395368	11,76	11,83	12,31	12,66	11,55	11,18	11,15	12,18	11,79	12,20	11,16	11,41
160	hsa-miR-324-3p-4395272	11,88	12,12	11,60	12,19	11,73	11,53	11,94	11,92	11,90	12,21	11,91	11,95
352	hsa-miR-886-5p-4395304	11,93	12,80	13,79	12,07	11,50	10,97	8,96	13,50	13,30	12,54	10,87	10,40
84	hsa-miR-139-3p-4395424	11,97	11,76	11,54	12,29	12,67	12,05	12,25	12,36	12,76	12,39	12,12	12,24
163	hsa-miR-328-4373049	12,09	11,90	11,01	12,76	11,56	10,38	11,51	12,09	12,16	12,26	11,41	12,04
123	hsa-miR-197-4373102	12,24	11,81	11,07	12,24	11,65	11,01	11,89	12,02	11,75	12,31	11,47	11,59
180	hsa-miR-361-5p-4373035	12,24	11,96	11,48	12,37	12,29	11,29	11,49	11,97	12,04	12,44	11,81	12,12
172	hsa-miR-339-3p-4395295	12,37	11,70	12,60	12,09	12,20	12,41	12,22	12,16	12,07	12,19	11,98	11,77
76	hsa-miR-133a-4395357	12,38	10,35	12,54	11,79	13,78	10,65	10,70	11,81	13,00	10,97	9,67	11,27
169	hsa-miR-335-4373045	12,38	12,29	11,70	13,21	12,18	11,38	12,79	13,35	13,25	13,47	12,70	11,91
152	hsa-miR-301a-4373064	12,43	12,59	12,44	13,10	12,35	11,89	12,16	12,28	11,99	12,89	12,12	12,45
325	hsa-miR-628-5p-4395544	12,44	11,68	12,14	11,97	12,20	11,90	11,62	11,90	11,70	12,45	12,16	12,01
198	hsa-miR-379-4373349	12,53	12,61	12,69	12,94	13,56	12,08	12,81	12,77	13,50	13,07	12,57	12,87
8	hsa-miR-9-4373285	12,58	12,75	12,91	13,40	13,93	12,35	11,90	12,64	13,36	12,92	11,94	12,70
5	hsa-let-7f-4373164	12,61	12,85	11,85	12,98	12,69	11,93	12,26	12,53	12,72	12,98	12,48	12,66
330	hsa-miR-652-4395463	12,80	13,37	13,05	13,64	13,11	12,28	12,64	13,03	12,93	13,05	12,55	13,18
144	hsa-miR-221-4373077	12,92	12,40	12,04	13,87	13,36	11,47	12,29	13,12	12,44	13,52	12,34	12,47
208	hsa-miR-424-4373201	12,96	13,87	12,60	14,20	12,36	12,41	13,20	13,18	13,54	13,61	14,12	13,83
201	hsa-miR-382-4373019	13,14	13,23	12,45	14,08	13,19	12,32	12,98	13,61	13,78	13,71	12,86	13,38
202	hsa-miR-383-4373018	13,24	13,78	14,10	14,71	15,07	12,21	14,05	15,04	14,75	14,61	14,33	14,31
147	hsa-miR-224-4395210	13,33	14,31	13,45	12,35	10,66	12,95	12,73	12,92	13,07	12,98	12,22	11,85
125	hsa-miR-199a-5p-4373272	13,47	14,21	12,49	14,32	14,15	12,52	13,22	14,01	14,90	14,42	13,30	14,82
149	hsa-miR-296-5p-4373066	13,48	14,16	13,64	14,82	14,44	12,91	13,43	14,22	15,35	14,12	13,16	13,66
61	hsa-miR-107-4373154	13,53	13,25	13,07	14,61	13,82	13,02	13,47	13,36	13,59	13,74	13,74	13,11
86	hsa-miR-140-3p-4395345	13,54	13,33	13,60	13,41	13,60	13,24	12,91	13,20	13,45	13,08	12,64	13,05
289	hsa-miR-539-4378103	13,54	13,35	13,36	14,33	14,48	12,69	13,53	13,86	13,92	13,87	13,36	13,47
80	hsa-miR-135b-4395372	13,56	13,55	14,24	14,32	14,45	13,92	13,34	14,01	15,45	14,08	13,12	13,28

188	hsa-miR-370-4395386	13,58	13,68	13,30	13,69	14,32	12,75	13,85	13,33	13,71	13,70	13,21	13,44
236	hsa-miR-491-5p-4381053	13,62	13,29	13,21	14,01	13,56	13,32	13,44	13,28	13,88	13,72	13,96	13,43
90	hsa-miR-142-5p-4395359	13,65	13,82	13,85	14,16	12,83	13,27	13,33	13,51	13,45	14,08	12,46	13,40
181	hsa-miR-362-3p-4395228	13,77	13,97	13,48	14,02	13,92	13,16	13,60	13,81	13,74	13,81	13,56	13,77
250	hsa-miR-505-4395200	13,82	14,17	13,14	13,99	13,58	13,61	14,42	13,86	14,18	14,29	14,36	13,89
238	hsa-miR-494-4395476	13,89	13,45	13,58	14,20	14,66	13,08	14,02	13,84	14,30	14,12	13,71	13,85
379	hsa-miR-511-4373236	13,93	13,72	13,38	14,12	13,92	13,49	14,24	14,07	13,50	13,75	13,61	13,64
70	hsa-miR-128-4395327	13,95	14,17	13,33	14,28	13,57	13,02	14,02	14,18	13,37	14,30	13,83	13,88
98	hsa-miR-148b-4373129	13,97	14,24	13,88	14,70	14,02	13,06	13,64	13,87	13,61	14,15	13,66	13,81
243	hsa-miR-500-4395539	13,98	14,42	14,01	14,21	14,35	13,85	13,99	14,00	13,72	13,67	14,12	14,06
18	hsa-miR-18b-4395328	14,00	14,32	13,81	13,29	14,08	13,52	13,18	13,83	13,51	14,38	14,16	14,04
239	hsa-miR-495-4381078	14,02	14,00	13,76	15,01	14,96	13,06	14,21	13,95	14,45	14,05	13,81	13,94
51	hsa-miR-98-4373009	14,06	14,23	13,43	14,42	13,80	13,70	14,10	13,32	13,67	13,81	14,28	13,83
317	hsa-miR-598-4395179	14,06	14,29	15,03	15,08	15,12	13,99	14,50	14,67	14,90	14,83	14,07	14,45
363	hsa-miR-212-4373087	14,07	13,81	13,74	14,00	13,85	12,65	13,30	13,48	14,06	13,56	13,03	13,57
117	hsa-miR-193a-3p-4395361	14,10	14,47	13,96	13,64	14,23	14,69	13,91	14,22	14,43	14,45	14,22	13,70
42	hsa-miR-31-4395390	14,11	10,87	14,05	12,61	12,83	13,82	11,47	13,89	15,07	11,35	9,91	12,42
309	hsa-miR-576-3p-4395462	14,14	14,17	14,41	14,95	15,00	14,24	14,28	14,38	14,71	14,75	14,54	14,51
248	hsa-miR-503-4373228	14,19	14,62	14,18	14,79	14,24	13,61	13,44	14,18	14,19	14,39	14,18	14,74
233	hsa-miR-489-4395469	14,34	14,08	15,67	14,05	15,19	14,79	13,84	14,52	15,31	14,99	14,30	14,31
182	hsa-miR-362-5p-4378092	14,34	15,09	14,07	14,65	14,95	14,07	14,43	14,33	14,10	14,04	14,49	14,33
170	hsa-miR-337-5p-4395267	14,36	14,71	15,47	15,47	16,15	13,99	14,73	14,74	15,09	14,78	14,47	15,15
316	hsa-miR-597-4380960	14,37	14,39	14,73	14,99	14,84	13,97	13,67	14,74	13,85	14,83	14,11	14,19
207	hsa-miR-423-5p-4395451	14,42	14,35	14,10	15,15	14,36	14,07	14,15	14,64	14,56	14,79	14,89	14,77
130	hsa-miR-200c-4395411	14,46	9,34	13,85	13,48	14,60	12,35	12,14	13,74	14,13	10,89	9,88	11,80
219	hsa-miR-452-4395440	14,52	14,82	14,71	14,32	13,15	14,56	14,43	14,47	14,73	14,70	14,24	13,75
94	hsa-miR-146b-3p-4395472	14,54	17,13	15,91	15,42	15,40	13,41	14,74	16,02	15,51	16,53	14,99	15,03
190	hsa-miR-372-4373029	14,64	16,52	15,75	14,89	17,76	12,54	14,98	15,19	16,19	15,75	14,22	14,03
43	hsa-miR-32-4395220	14,78	14,18	14,88	14,67	14,88	14,33	14,45	14,25	14,36	14,74	14,68	14,51
83	hsa-miR-138-4395395	14,79	15,36	14,89	15,89	14,91	14,58	14,17	13,75	16,03	14,87	14,41	15,05
210	hsa-miR-429-4373203	14,86	15,09	15,13	16,61	12,84	14,82	14,43	14,88	15,83	15,38	14,30	15,01
321	hsa-miR-618-4380996	14,88	14,53	15,18	14,65	14,86	14,33	14,35	14,69	15,41	15,74	14,69	14,06
246	hsa-miR-502-3p-4395194	15,05	15,62	14,69	15,58	15,06	14,75	15,01	15,38	15,28	15,28	15,08	15,25
339	hsa-miR-708-4395452	15,05	15,27	15,91	15,98	15,09	14,83	13,54	14,89	16,08	15,27	14,72	14,90
159	hsa-miR-323-3p-4395338	15,21	14,72	14,14	14,87	15,81	13,81	14,98	14,97	15,06	14,63	14,79	14,39
140	hsa-miR-216b-4395437	15,22	13,87	15,81	16,21	13,64	17,15	14,78	15,02	15,18	15,73	14,95	15,20
247	hsa-miR-502-5p-4373227	15,24	15,66	15,01	15,35	15,26	14,84	15,24	15,07	14,86	15,00	15,05	15,17
206	hsa-miR-422a-4395408	15,41	14,60	14,91	15,71	15,43	14,88	15,29	15,40	15,11	15,28	15,65	14,63
231	hsa-miR-487b-4378102	15,42	15,34	15,17	15,67	16,24	14,35	15,30	15,04	15,82	15,08	14,95	14,97
349	hsa-miR-885-3p-4395483	15,61	16,07	15,37	16,25	15,76	15,84	16,44	15,88	15,87	16,42	16,17	16,28
215	hsa-miR-450a-4395414	15,66	16,16	15,70	16,58	15,38	14,87	15,84	15,86	16,54	16,32	16,43	16,72
204	hsa-miR-410-4378093	15,72	15,14	15,62	15,74	16,22	14,40	15,22	15,09	15,33	15,28	15,14	15,22
323	hsa-miR-625-4395542	15,72	16,37	15,87	16,62	16,46	15,88	16,00	15,64	16,18	16,26	15,60	16,02
291	hsa-miR-542-3p-4378101	15,76	16,44	15,77	16,87	15,09	14,84	15,96	16,34	16,52	16,52	16,31	16,50
362	hsa-miR-211-4373088	15,80					15,45						
336	hsa-miR-671-3p-4395433	15,80	15,28	14,82	15,79	15,10	14,44	14,51	15,67	14,82	15,63	14,87	15,07
217	hsa-miR-450b-5p-4395318	15,80	16,40	15,21	16,65	14,69	14,82	15,29	16,16	16,40	16,50	16,98	15,49
127	hsa-miR-199b-5p-4373100	15,82	16,48	16,37	16,85	16,74	14,88	15,76	16,35	17,24	17,00	15,69	16,42
311	hsa-miR-579-4395509	15,84	15,43	15,72	16,35	16,08	15,54	15,47	15,90	15,73	16,03	15,99	15,95
211	hsa-miR-431-4395173	16,12	16,08	16,02	15,32	16,84	15,64	15,96	14,94	14,85	15,23	15,60	15,29
114	hsa-miR-190-4373110	16,14	16,27	16,40	16,94	16,40	14,96	15,60	16,13	16,57	16,47	15,98	16,32
183	hsa-miR-363-4378090	16,18	16,26	16,14	16,56	16,52	15,18	16,33	16,15	16,10	16,53	15,82	16,32
168	hsa-miR-331-5p-4395344	16,21	15,52	15,80	15,10	15,95	16,12	16,03	15,85	15,97	16,24	16,12	15,35
341	hsa-miR-758-4395180	16,26	16,02	15,80	16,67	16,93	15,29	16,02	15,91	16,42	16,44	16,00	16,10
229	hsa-miR-486-5p-4378096	16,48	15,02	15,35	15,93	16,33	13,67	15,98	15,46	16,47	15,10	14,92	15,86
171	hsa-miR-338-3p-4395363	16,54	15,97	17,44	16,98	16,14	15,79	16,44	16,55	16,97	16,54	15,62	16,25
79	hsa-miR-135a-4373140	16,55	15,86	18,04	18,70	15,26	16,63	15,24	17,51	17,80	16,59	15,12	16,92
328	hsa-miR-642-4380995	16,56	16,46	16,76	18,01	16,74	15,81	15,10	16,32	16,34	16,67	14,95	16,69
245	hsa-miR-501-5p-4373226	16,58	16,77	15,53	16,05	16,02	15,98	17,06	16,17	15,48	15,66	16,60	16,39
151	hsa-miR-299-5p-4373188	16,59	16,75	16,17	17,01	17,50	15,47	16,26	16,64	17,41	16,59	16,16	16,55
292	hsa-miR-542-5p-4395351	16,63	17,44	17,60	17,00	17,01	17,15	16,89	16,65	17,49	17,33	17,50	17,33

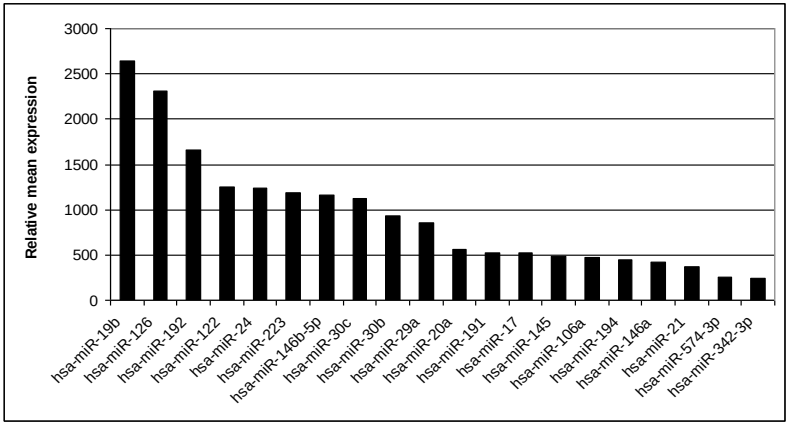
165	hsa-miR-330-3p-4373047	16,63	17,05	16,69	17,40	16,68	16,60	17,05	16,62	16,23	16,90	16,60	16,88
153	hsa-miR-301b-4395503	16,74	17,42	16,11	17,50	16,25	16,57	16,32	15,06	14,92	15,55	17,16	16,96
212	hsa-miR-433-4373205	16,77	16,53	16,42	17,41	17,90	15,69	16,79	16,96	17,51	17,13	16,69	16,87
88	hsa-miR-141-4373137	16,78	12,18	16,25	15,77	15,95	14,42	14,07	16,16	16,59	13,33	11,77	13,99
294	hsa-miR-545-4395378	16,85	16,72	16,60	17,03	17,02	16,60	16,64	16,70	16,59	17,41	17,05	16,87
333	hsa-miR-654-5p-4381014	16,85	17,26	17,37	17,37	18,51	16,72	17,46	17,19	17,73	17,73	17,59	17,08
200	hsa-miR-381-4373020	16,95	17,32	17,24	18,30	18,71	16,37	17,35	17,05	17,69	17,21	16,88	17,39
64	hsa-miR-125a-3p-4395310	16,98	16,95	17,19	16,71	18,21	16,96	17,06	16,89	18,09	17,14	16,58	17,29
355	hsa-miR-889-4395313	17,04	16,66	16,66	17,60	17,48	16,08	17,12	17,33	17,53	17,45	17,12	17,24
332	hsa-miR-654-3p-4395350	17,12	17,22	16,83	18,03	18,47	16,26	17,65	17,29	18,35	17,60	17,27	17,79
237	hsa-miR-493-4395475	17,14	16,86	17,38	17,65	17,87	16,05	17,54	16,92	17,36	17,19	16,89	16,59
112	hsa-miR-187-4373307	17,17	16,88	17,82	17,76	18,79	16,77	17,54	17,77	18,69	17,74	17,16	17,48
105	hsa-miR-181c-4373115	17,23	17,62	16,80	18,16	17,17	16,56	17,16	17,36	18,22	17,90	17,09	17,34
242	hsa-miR-499-5p-4381047	17,32	16,37	17,08	18,03	17,99	16,65	16,80	16,73	17,35	17,92	18,06	17,77
334	hsa-miR-655-4381015	17,39	17,00	17,44	18,53	18,36	16,58	17,60	17,65	18,55	17,83	17,46	17,69
186	hsa-miR-369-3p-4373032	17,69	18,09	18,11	19,07	18,96	16,92	17,86	18,06	18,55	18,44	17,84	18,18
187	hsa-miR-369-5p-4373195	17,74	18,15	18,23	18,76	18,56	16,87	17,99	18,14	18,81	18,05	17,52	17,90
82	hsa-miR-137-4373301	17,90	18,43	18,38	17,75	19,76	16,36	16,36	17,43	16,48	17,20	17,34	16,79
234	hsa-miR-490-3p-4373215	18,03	17,94	18,41	18,48	19,62	18,28	18,49	18,69	19,61	18,79	18,58	19,85
78	hsa-miR-134-4373299	18,09	13,25	13,84	13,95	14,63	13,19	13,52	13,53	13,94	13,35	13,28	13,39
139	hsa-miR-216a-4395331	18,13	17,63	19,12	19,77	17,31	20,60	17,73	18,44	18,31	18,99	18,37	18,34
143	hsa-miR-219-5p-4373080	18,28	18,14	17,80	18,66	18,02	17,81	17,61	18,25	18,03	18,55	18,07	18,43
326	hsa-miR-629-4395547	18,34	19,44	18,72	18,70	18,91	18,34	18,11	17,96	18,23	18,21	17,71	18,48
312	hsa-miR-582-3p-4395510	18,39	18,28	19,13	18,92	19,47	18,47	18,41	19,27	19,09	19,28	19,08	18,96
103	hsa-miR-154-4373270	18,44	19,16	18,87	19,98	19,22	17,81	18,63	18,95	19,65	18,73	18,27	19,03
256	hsa-miR-512-3p-4381034	18,48	19,71	19,62	19,10	20,53	19,30	19,44	19,01	19,06	19,41	19,50	19,73
320	hsa-miR-616-4395525	18,48	17,82	17,94	17,38	18,31	17,16	17,10	18,11	16,77	17,69	17,58	17,40
77	hsa-miR-133b-4395358	18,50	16,89	18,64	18,60	19,92	17,07	16,87	18,50	19,79	17,65	16,16	17,63
46	hsa-miR-34c-5p-4373036	18,52	20,03	19,13	19,37	18,57	19,03	18,39	19,43	18,93	19,98	18,06	18,76
249	hsa-miR-504-4395195	18,57	20,60	19,19	19,97	20,09	16,75	19,01	18,95	19,68	19,42	17,77	20,32
178	hsa-miR-342-5p-4395258	18,69	18,08	18,26	18,70	17,61	19,29	18,90	18,27	18,81	19,71	18,30	18,31
226	hsa-miR-485-3p-4378095	18,69	18,33	17,71	19,29	18,83	16,60	18,62	18,65	19,19	18,59	18,11	18,70
272	hsa-miR-518f-4395499	18,71	17,27				19,84	18,58	20,04	20,81	20,75	21,83	20,12
298	hsa-miR-548b-5p-4395519	18,74	19,42	18,78	18,50	17,62	19,21	19,64	19,56	19,28	20,10	18,90	17,93
313	hsa-miR-582-5p-4395175	18,90	18,85	19,49	19,12	18,93	18,55	18,58	19,36	19,14	19,39	18,76	18,65
303	hsa-miR-551b-4380945	18,97	18,33	19,17	20,97	16,94	17,70	16,75	16,78	17,75	18,36	16,44	17,93
141	hsa-miR-217-4395448	19,12	19,10	20,30	21,89	17,90	21,58	19,08	20,56	19,93	20,31	19,66	19,62
244	hsa-miR-501-3p-4395546	19,16	19,25	18,15	20,26	18,49	18,65	19,52	21,33	18,88	18,91	18,87	18,95
300	hsa-miR-548c-5p-4395540	19,18	20,66	19,60	19,35	18,53	20,23	20,29	20,70	20,37	21,74	20,16	19,43
7	hsa-miR-1-4395333	19,22	17,19	18,64	19,51	20,12	16,79	17,10	18,87	20,22	17,90	16,40	18,76
324	hsa-miR-627-4380967	19,43	18,96	19,77	18,75	19,34	18,69	18,80	17,94	18,19	18,40	18,74	18,43
302	hsa-miR-548d-5p-4395348	19,49	20,27	19,38	19,63	19,23	19,71	20,54	20,59	20,18	21,64	19,95	18,97
122	hsa-miR-196b-4395326	19,51	19,27	19,27	20,22	20,64	19,37	20,14	17,96	20,17	20,15	18,72	19,40
196	hsa-miR-376b-4373196	19,62	20,71	20,88	20,87	20,99	18,92	20,26	20,07	20,71	21,05	20,57	20,57
203	hsa-miR-409-5p-4395442	19,62	19,19	19,01	19,99	20,70	18,61	19,28	19,48	19,79	19,61	19,55	19,53
109	hsa-miR-184-4373113	19,65	19,55	18,77	19,86	17,48	19,44	18,77	20,07	19,36	20,31	20,15	19,79
191	hsa-miR-373-4378073	19,82	19,33	22,31	20,33	23,62	18,17	21,39	20,43	22,01	20,73	18,37	20,88
307	hsa-miR-570-4395458	20,26	19,30	18,83	20,79	19,15	18,28	18,63	19,02	19,59	20,28	20,02	18,63
164	hsa-miR-329-4373191	20,31	20,34	20,11	21,30	20,89	19,38	20,76	20,54	21,26	21,58	20,21	20,49
232	hsa-miR-488-4395468	20,34	20,49	20,95	21,86	20,62	19,31	20,08	20,74	21,48	21,40	20,18	20,46
44	hsa-miR-33b-4395196	20,40	20,99	21,33	22,38	21,33	20,75	20,25	20,85	20,54	21,42	20,23	20,41
364	hsa-miR-219-1-3p-4395206	20,40	19,96	19,61	20,26	18,97	19,65	18,26	20,23	19,44	20,44	19,92	19,62
213	hsa-miR-449a-4373207	20,47	20,74	19,30	18,85	20,48	20,31	20,35	18,59	19,47	20,12	19,86	18,98
189	hsa-miR-371-3p-4395235	20,54		21,38	20,25		17,12	20,58	20,89	22,89		19,61	19,41
106	hsa-miR-182-4395445	20,57	21,63	19,81	19,09	18,17	20,24	20,49	19,14	18,16	20,21	20,31	20,85
327	hsa-miR-636-4395199	20,75	20,13	19,91	19,72	20,22	19,75	20,22	20,74	19,64	19,84	20,71	19,98
329	hsa-miR-651-4381007	20,79	20,15	20,61	21,51	20,09	19,63	20,11	20,38	20,48	21,35	20,31	20,33
214	hsa-miR-449b-4381011	20,81	20,80	19,50	19,82	20,73	20,15	20,82	19,61	20,02	20,30	20,51	19,93
230	hsa-miR-487a-4378097	20,85	20,48	20,58	20,80	20,88	19,48		20,49	20,92		21,20	20,62
310	hsa-miR-576-5p-4395461	20,87	20,37	20,46					20,99				21,21
197	hsa-miR-377-4373025	20,88	20,61	20,92	23,12	22,12	20,05	20,10	21,44	22,76	21,97	20,78	22,83

50	hsa-miR-96-4373372	20,98	22,38	20,50	20,59	18,34	20,28	20,71	21,00	19,09	22,56	21,02	22,09
162	hsa-miR-326-4373050	20,99	21,12	20,53	21,96	21,07	19,93	20,98	21,24	21,49	21,16	20,66	20,82
228	hsa-miR-486-3p-4395204	21,24	20,19	20,44	21,24	21,60	19,12		20,74		20,28	19,73	
166	hsa-miR-330-5p-4395341	21,32	21,52	21,37	20,92	21,17	20,73	21,26	20,45	21,04	20,95	21,03	21,52
267	hsa-miR-518b-4373246	21,56											
314	hsa-miR-589-4395520	21,62	21,62	21,33	22,29	21,57	21,50	24,17	22,34	22,49	22,22	21,53	22,39
71	hsa-miR-129-3p-4373297	21,65	20,82	20,43	22,64	20,54	21,43	21,16	19,28	19,28	22,22	19,84	21,08
301	hsa-miR-548d-3p-4381008	21,73	22,14	22,51	23,07	22,91	22,49	21,76	22,18	21,72	23,32	22,11	22,84
69	hsa-miR-127-5p-4395340	21,73	22,62	21,85	23,06	23,52	21,06	22,33	21,82	23,11	23,28	21,82	22,11
148	hsa-miR-296-3p-4395212	22,07	24,12	22,38	23,61	23,66	22,36	21,74	23,51		22,84	21,09	22,16
63	hsa-miR-124-4373295	22,07	24,80	22,88	25,27	23,64		23,02	23,15	24,87	24,53	24,01	
282	hsa-miR-523-4395497	22,15						21,86	22,28	21,70		22,38	21,56
263	hsa-miR-517a-4395513	22,28	22,65	21,74	22,43	22,56	22,53	22,70	22,49	22,15	22,67	23,34	22,33
108	hsa-miR-183-4395380	22,32		21,07	22,07	19,31	21,64	22,37	21,44	20,28	22,35	21,87	22,75
124	hsa-miR-198-4395384	22,44	23,15			22,43		21,16	23,74			22,25	21,55
274	hsa-miR-519d-4395514	22,57	23,33	22,67	23,91	24,23	25,17	23,96	23,55	23,85	24,19	23,93	24,82
297	hsa-miR-548b-3p-4380951	22,61	20,42	22,98	21,91	21,58	22,14	22,71	20,88	21,86	23,09		21,57
81	hsa-miR-136-4373173	22,63	21,63	23,86	22,23	23,74	22,42	21,80	21,72	23,16	21,94	21,67	21,27
185	hsa-miR-367-4373034	22,71	22,71	23,14		23,56	21,81	22,18		22,83		20,93	24,68
264	hsa-miR-517c-4373264	22,81	23,04	23,23	23,86	23,89	23,45	23,16	22,83	22,73	23,26	22,48	23,87
175	has-miR-155-4395459	22,95	22,65	21,65	23,19	22,09	22,52	21,60	22,56		23,51	21,02	22,66
331	hsa-miR-653-4395403	22,96	23,98	23,50	23,74	26,80	23,72	23,57		26,70	23,88	23,46	25,24
360	hsa-miR-147-4373131	23,09	23,88	24,71	24,02	24,14	24,43	24,01		24,66	25,30	22,86	23,90
96	hsa-miR-147b-4395373	23,30	23,40	21,73		21,56	23,54		23,26	23,97	24,13	23,75	23,22
273	hsa-miR-519a-4395526	23,37	22,26	22,12	23,22	23,08	22,82	22,19	22,71	22,84	23,54	22,60	20,53
293	hsa-miR-544-4395376	23,43	23,72	23,95	23,81	23,47	21,89	22,53	22,17	22,77	22,47	22,65	23,37
131	hsa-miR-202-4395474	23,44	23,31	21,92	23,80	22,33	21,67	21,86	24,91	24,87		23,92	22,88
199	hsa-miR-380-4373022	23,46	24,43		23,98		22,47	22,99	24,35	24,99		22,83	23,45
150	hsa-miR-299-3p-4373189	24,07	23,95	23,34	24,57	24,57	23,52		23,76	24,07	22,84	22,57	23,46
344	hsa-miR-873-4395467	24,25	26,93	26,05		25,76		23,95		27,24		25,72	
304	hsa-miR-556-3p-4395456	24,33	25,19	23,83		23,75	24,87	24,70	24,78	23,77	22,93	25,79	24,04
102	hsa-miR-153-4373305	24,41	23,33	23,33	23,88	24,28	21,50	22,19	23,43	24,33	25,01	22,55	23,22
271	hsa-miR-518e-4395506	24,83	24,27		24,67	25,01		23,67	24,93	23,53			24,99
305	hsa-miR-556-5p-4395455	25,28	25,81					24,65		25,35	25,37		24,74
347	hsa-miR-876-3p-4395336	25,34		27,16	28,08	24,49	25,92	25,18	26,73			26,49	
157	hsa-miR-302c-4378072	25,36	24,04					24,22			25,36		24,05
382	hsa-miR-520b-4373252	25,52	25,12	24,52	26,01	26,08	24,10	25,08	26,16	26,61	25,51	25,52	25,58
259	hsa-miR-515-3p-4395480	25,70	25,35		24,91				27,15	27,83	27,98	27,21	24,51
240	hsa-miR-496-4386771	25,79	23,67	24,68		25,15			26,25	25,73			24,16
281	hsa-miR-522-4395524	26,43	26,04	24,82		24,82	24,41		25,63	25,13			22,77
306	hsa-miR-561-4380938	26,97							27,38				
295	hsa-miR-548a-3p-4380948	27,40	24,87	24,30		26,92	25,48	25,85		26,96			24,40
113	hsa-miR-188-3p-4395217		23,28							23,48			
134	hsa-miR-205-4373093		24,42		23,66		22,49	23,49	23,14			24,14	
154	hsa-miR-302a-4378070			22,55				23,27			23,40	23,38	
156	hsa-miR-302b-4378071		23,70	24,14	24,83	22,67	22,84	23,59	23,28		22,98	21,95	22,45
220	hsa-miR-453-4395429		24,29	25,04	23,26		23,10	25,22	23,63			22,76	23,50
235	hsa-miR-491-3p-4395471		23,90					23,39		24,50		23,89	
252	hsa-miR-508-3p-4373233		23,39	24,07	24,64	22,07	23,32	21,83	23,47	25,24	25,70	21,24	24,61
254	hsa-miR-509-5p-4395346								23,83			20,88	
260	hsa-miR-515-5p-4373242											26,64	
262	hsa-miR-516b-4395172									26,10			
265	hsa-miR-518a-3p-4395508								27,27				
275	hsa-miR-519e-4395481		26,38										
277	hsa-miR-520a-5p-4378085								27,42				
290	hsa-miR-541-4395312								24,27	23,71			
296	hsa-miR-548a-5p-4395523			28,14		27,89	25,31				28,93		
299	hsa-miR-548c-3p-4380993		26,21	25,94	25,33		26,53		27,08	27,04			
322	hsa-miR-624-4395541										25,66		
345	hsa-miR-874-4395379		15,98	14,86		14,70	15,70	14,77	15,55	15,84	15,98	14,96	15,69
353	hsa-miR-887-4395485		19,95		19,12		19,70	19,63	18,86	19,71	19,26	18,69	18,79
354	hsa-miR-888-4395323		22,28	24,38	22,55		19,27	16,60	20,78	22,25	22,19	21,63	20,59
357	hsa-miR-891a-4395302		24,92					24,73	24,11	25,05	20,89	25,47	
373	hsa-miR-384-4373017							28,97					
377	hsa-miR-506-4373231											24,88	
380	hsa-miR-517b-4373244			24,98									
384	hsa-miR-520f-4373256							25,45			24,71	25,92	24,73

Never expressed miRNA	
25	hsa-miR-23a-4373074
57	hsa-miR-105-4395278
72	hsa-miR-129-5p-4373171
135	hsa-miR-208b-4395401
138	hsa-miR-215-4373084
155	ath-miR159a-4373390
241	hsa-miR-499-3p-4395538
251	hsa-miR-507-4373232
216	hsa-miR-450b-3p-4395319
253	hsa-miR-508-5p-4395203
227	hsa-miR-485-5p-4373212
255	hsa-miR-510-4395352
257	hsa-miR-512-5p-4373238
258	hsa-miR-513-5p-4395201
261	hsa-miR-516a-5p-4395527
266	hsa-miR-518a-5p-4395507
268	hsa-miR-518c-4395512
269	hsa-miR-518d-3p-4373248
270	hsa-miR-518d-5p-4395500
276	hsa-miR-520a-3p-4373268
278	hsa-miR-520d-5p-4395504
279	hsa-miR-520g-4373257
280	hsa-miR-521-4373259
283	hsa-miR-524-5p-4395174
284	hsa-miR-525-3p-4395496
285	hsa-miR-525-5p-4378088
286	hsa-miR-526b-4395493
318	hsa-miR-615-3p-4386777
319	hsa-miR-615-5p-4395464
337	hsa-miR-672-4395438
338	hsa-miR-674-4395193
342	hsa-miR-871-4395465
343	hsa-miR-872-4395375
346	hsa-miR-875-3p-4395315
348	hsa-miR-876-5p-4395316
356	hsa-miR-890-4395320
358	hsa-miR-891b-4395321
359	hsa-miR-892a-4395306
361	hsa-miR-208-4373091
365	hsa-miR-219-2-3p-4395501
366	hsa-miR-220-4373078
367	hsa-miR-220b-4395317
368	hsa-miR-220c-4395322
369	hsa-miR-298-4395301
370	hsa-miR-325-4373051
371	hsa-miR-346-4373038
374	hsa-miR-412-4373199
375	hsa-miR-448-4373206
376	hsa-miR-492-4373217
378	hsa-miR-509-3-5p-4395266
381	hsa-miR-519c-3p-4373251
383	hsa-miR-520e-4373255

Table 4.2: miRNAs detected with CARD A

In Fig 4.3 the most highly expressed miRNAs obtained by all the liver biopsies are reported.



*Relative expression level. calculated by the median normalized ΔCt values.

Fig 4.3. The most highly expressed miRNAs in human liver

290 out of 384 miRNAs resulted at detectable levels (quality assessment); analysing age-dependent effects four miRNAs were found up-regulated comparing the oldest (70-90 years) with the others three age groups. In the Fig. 4.4 ΔΔCT higher than 1 and lower than -1.5 are reported. On the whole, significantly down-regulated miRNAs in terms of fold change were observed. It is necessary to

remind that MiRNA fold changes higher than 2 and lower than 0.5 were considered significant for further analysis.

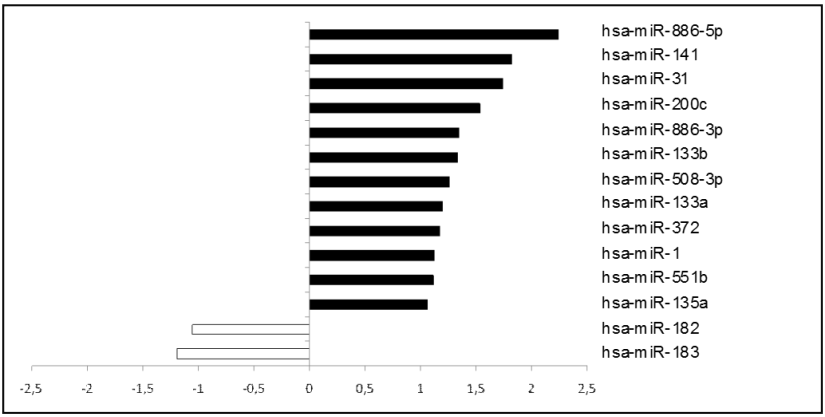


Fig 4.4. MiRNA profiling results in 12 males (n. 3 older than 70 years and n. 9 younger than 70 years).

Mir-31 -200c. -141 and -886-5p resulted upregulated in old compared to young subjects as shown in Fig 4.5.

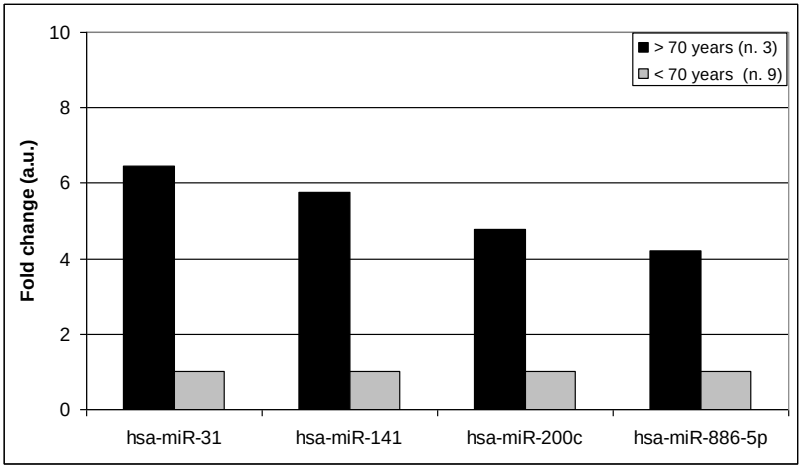


Fig 4.5. upregulated MiRNA: results in 12 males from miRNA profiling (old *vs* young)

Because there were no significant differences in miRNAs expression between the group of subjects with an age range of 50-70 year and the group of subjects with an age range of 18-50 years, these two age-groups were analysed together. Thus, the subsequent analysis were performed compared two groups of subjects: older than 70 years and younger than 70 years.

4.2 Validation of miRNA array results

MicroRNA array results were validated *via* qRT-PCR. MiRNA-31, miRNA-141 and miRNA- 200c and -886-5p. miRNA validation was performed in 45 liver samples: 23 males liver samples, including specimens from males aging from 18 to 90 years and 22 females liver samples, the same age. All qRT-PCR data were firstly analyzed as unadjusted Ct values and secondarly normalized to miRNA21.

In table 4.6 shows the values of expression of miRNAs 31, 141 and 200c normalized (in green):

							NORMALIZATION		
SAMPLE	SEX	AGE	Ct 21	Ct 31	Ct 141	Ct 200c	31-21	141-21	200-21
2	F	58	15,717	28,353	30,111	29,087	12,636	14,394	13,689
3	F	69	15,543	27,251	28,372	27,447	11,708	12,829	12,548
4	F	18	15,244	30,179			14,935		
8	F	73	16,465	29,627	33,908	28,752	13,162	17,443	12,287
12	F	67	16,33	26,76	30,811	25,352	10,43	14,481	9,022
13	F	58	15,97	29,177	28,639	28,536	13,207	12,669	13,138
16	F	13	15,364	29,311	31,747	28,437	13,947	16,383	13,291
18	F	82	15,8	28,485	28,72	26	12,8	13	13,889
21	F	60	16,03	23,436	32,076	28,374	7,406	16,046	12,576
23	F	53	15,839	30,035	34,127	28,065	14,196	18,288	12,226
25	F	59	17,26	28,257	29,779	26,409	10,997	12,519	9,149
27	F	54	16,09	27,49	29,817	26,704	11,4	13,727	11,188
31	F	69	14,218	25,526	28,255	24,047	11,308	14,037	9,829
32	F	77	17,572	26,968	29,509	25,366	9,396	11,937	7,794
34	F	90	16,141	30,399	31,413	28,41	14,258	15,272	12,269
35	F	59	16,352	26,809	28,495	25,085	10,457	12,143	8,733
37	F	61	15,433	27,082	29,131	25,209	11,649	13,698	9,776
38	F	70	16,422	27,909	30,583	26,466	11,487	14,161	10,044
41	F	69	16,844	26,613	29,301	26,024	9,769	12,457	9,18
43	F	74	16,319	24,826	26,364	24,315	8,507	10,045	7,996
48	F	78	17,289	26,784	27,583	24,397	9,495	10,294	7,108
50	F	18	15,995	25,809	26,919	23,206	9,814	10,924	7,211
1	M	59	15,798	29,305	32,372	30,011	13,507	16,574	13,921
5	M	30	15,448	30,065	31,612	29,351	14,617	16,164	13,321
7	M	44	15,859	30,002	32,247	29,243	14,143	16,388	13,795
10	M	37	14,579	27,664	30,293	28,01	13,085	15,714	13,628
14	M	54	14,899	27,726	28,696	28,605	12,827	13,797	12,635
15	M	50	15,398	27,465	30,335	29,341	12,067	14,937	13,624
19	M	87	15,516	27,103	29,562	26,616	11,587	14,046	10,98
20	M	26	14,382	26,858	30,072	29,337	12,476	15,69	14,668
24	M	50	15,122	28,079	29,663	26,832	12,957	14,541	11,71
26	M	66	14,477	29,945	31,091	28,626	15,468	16,614	14,047
28	M	58	14,669	25,654	28,128	25,97	10,985	13,459	10,606
29	M	84	16,181	25,522	31,716	26,804	9,341	15,535	10,623
30	M	76	16,15	28,177	30,128	27,637	12,027	13,978	11,487
33	M	82	15,636	24,832	27,125	24,224	9,196	11,489	8,844
36	M	82	15,415	28,12	30,089	25,125	12,705	14,674	9,71
39	M	20	15,409	28,651	29,753	27,226	13,242	14,344	11,817
40	M	74	15,247	26,446	29,575	26,021	11,199	14,328	10,774
42	M	77	15,94	27,336	30,496	26,458	11,396	14,556	10,518
44	M	43	15,229	29,048	32,189	28,145	13,819	16,96	12,916
45	M	45	16,392	28,023	30,78	27,272	11,631	14,388	10,88
46	M	75	15,244	25,45	26,993	22,701	10,206	11,749	7,457
47	M	72	15,231	26,701	29,607	25,751	11,47	14,376	10,52
49	M	16	15,922	29,308	30,442	25	13,386	14,52	9,078

Tab 4.6: All qRT-PCR: data analyzed as unadjusted Ct values (withe columns). Data standardized to miRNA21 (green columns)

RT-PCR analysis confirmed the results obtained by microarray for miRNA 31, -141 and -200c. In particular miRNA 31 expression levels resulted 4.5 fold higher in the old compared to the young subjects. (P=0.007) (Fig.4.7). MiRNA-141 expression level was 2.66 fold higher in the old compared to the young (P=0.034), and miRNA200c expression level was 6.7 fold higher in the old compared to the young (P=0.017) (Fig. 4.7). MiRNA -886-5p was not confirmed as differential expressed in old vs young. All these differences were not observed in females sample (n. 22). Samples were normalized to miRNA-21 expression and analyzed using the ΔΔCT .

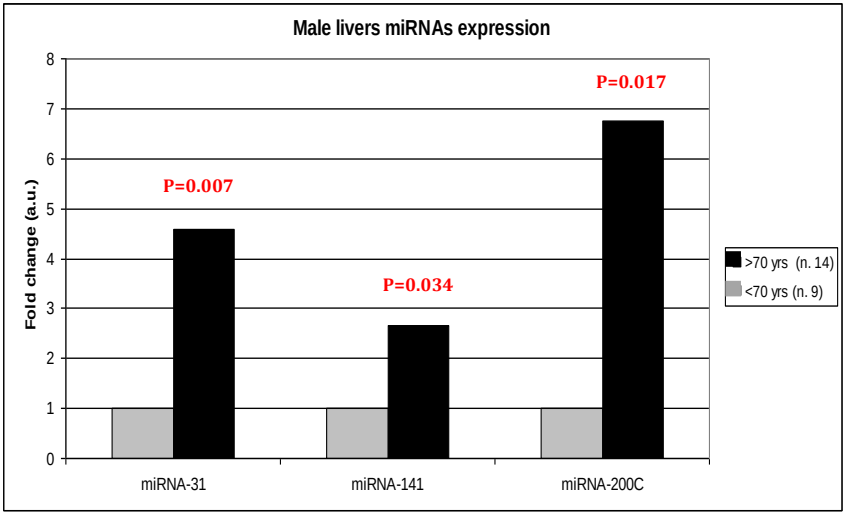


Fig 4.7: MiRNA Real time PCR validation results.

4.3 Linear regression analysis

In Fig 4.8 the linear regression analysis on male samples is reported and it confirms the age-dependent increase of three miRNAs expression. The same type of analysis has been performed for females, but the correlation has not been confirmed due to low statistical power (data not shown).

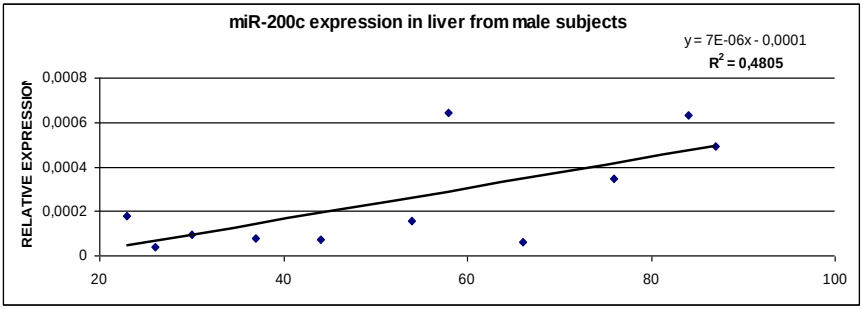


Fig 4.8: Linear regression analysis fom male samples

4.4. mRNA expression patterns

Global liver gene expression profiles were assessed by Affymetrix array in 12 males subjects (the same analyses in the CARD A). From this analysis about 90 mRNAs resulted significantly down-regulated in the old group compared with young group (young/elderly ratio <0.67. log(ratio) = -0.4. ANOVA $p<0.05$). The results are reported in Table 4.9.

Symbol	Description	Pval	log(O/Y)
SLC1A2	solute carrier family 1 (glial high affinity glutamate transporter), member 2	0,00170445	**-1,79726**
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	0,022262	**-1,35002**
SLC1A2	solute carrier family 1 (glial high affinity glutamate transporter), member 2	0,000782043	**-1,11444**
ELL2	elongation factor, RNA polymerase II, 2	0,0074845	**-1,08558**
SPINK1	serine peptidase inhibitor, Kazal type 1	0,0417602	**-1,02997**
PAPPA2	pappalysin 2	0,00479133	**-1,00324**
USP9Y	ubiquitin specific peptidase 9, Y-linked (fat facets-like, Drosophila)	0,0390819	**-0,98779**
TBL1Y	transducin (beta)-like 1Y-linked	0,0279074	**-0,94949**
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	0,0211862	**-0,93055**
VIL1	villin 1	0,0269864	**-0,91751**
TSPYL5	TSPY-like 5	0,012896	**-0,88258**
PCSK6	proprotein convertase subtilisin/kexin type 6	0,0422326	**-0,87952**
TTMA	two transmembrane domain family member A	0,0134268	**-0,7921**
STAG3	stromal antigen 3	0,0366479	**-0,77449**
LOC440731	hypothetical LOC440731	0,0394613	**-0,77421**
FLJ10986	hypothetical protein FLJ10986	0,00797767	**-0,74766**
SLC13A3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	0,0301963	**-0,74273**
FNDC3B	fibronectin type III domain containing 3B	0,0334357	**-0,72413**
KIAA1729	KIAA1729 protein	1,62E-05	**-0,72305**
TMCC1	transmembrane and coiled-coil domain family 1	0,0119792	**-0,70207**
MBNL2	muscleblind-like 2 (Drosophila)	0,0224731	**-0,70203**
C10orf116	chromosome 10 open reading frame 116	0,0165622	**-0,6932**
C1orf179	chromosome 1 open reading frame 179	0,0059995	**-0,68464**
SOX5	SRY (sex determining region Y)-box 5	0,0221148	**-0,67805**
FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor	0,0338911	**-0,67741**
ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide	0,0409988	**-0,65465**
P4HA1	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I	0,0189868	**-0,6463**
INSIG1	insulin induced gene 1	0,047368	**-0,64382**
VIL1	villin 1	0,0358748	**-0,64318**
MAPK13	mitogen-activated protein kinase 13	0,0314373	**-0,61196**
ZDHHC11	zinc finger, DHHC-type containing 11	0,00608822	**-0,60078**
SERINC5	serine incorporator 5	0,032969	**-0,59287**
NR0B2	nuclear receptor subfamily 0, group B, member 2	0,0496508	**-0,58774**
AMDHD1	amidohydrolase domain containing 1	0,00998431	**-0,58722**
FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor	0,0437986	**-0,58471**
STAB2	stabilin 2	0,0404152	**-0,58241**
VIL1	villin 1	0,0127921	**-0,57754**
COL12A1	collagen, type XII, alpha 1	0,0357375	**-0,57477**
ACOT12	acyl-CoA thioesterase 12	0,0141419	**-0,57061**
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	0,0116402	**-0,56667**
MGC88374	similar to CG32662-PA	0,0342419	**-0,56572**
MAPK13	mitogen-activated protein kinase 13	0,0219773	**-0,56222**
DPP4	dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	0,000191708	**-0,56079**
DPP4	dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	0,0217048	**-0,55942**
EPB41L4B	erythrocyte membrane protein band 4.1 like 4B	0,036748	**-0,55406**
BCAR3	breast cancer anti-estrogen resistance 3	0,0351837	**-0,54476**
ADRB2	adrenergic, beta-2-, receptor, surface	0,0197168	**-0,53615**
BEX2	brain expressed X-linked 2	0,0186238	**-0,53555**
FNDC3B	fibronectin type III domain containing 3B	0,0150789	**-0,53148**
PPARG	peroxisome proliferator-activated receptor gamma	0,0328753	**-0,52985**
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	0,025658	**-0,52258**
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0,0263896	**-0,52155**
PPP1R1A	protein phosphatase 1, regulatory (inhibitor) subunit 1A	0,00325051	**-0,50781**
LOC253039	hypothetical protein LOC253039	0,0274617	**-0,49777**
HIST2H2AA3	histone cluster 2, H2aa3	0,0468608	**-0,49393**
TMCC1	transmembrane and coiled-coil domain family 1	0,0145276	**-0,49315**
LRP6	low density lipoprotein receptor-related protein 6	0,0411374	**-0,49253**
BTG1	B-cell translocation gene 1, anti-proliferative	0,00572153	**-0,49149**

PANK1	pantothenate kinase 1	0,025233	**-0,48285**
CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9	0,0278149	**-0,47437**
LOC388323	hypothetical LOC388323	0,0250584	**-0,46775**
CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9	0,0137265	**-0,46442**
APOF	apolipoprotein F	0,0163829	**-0,46265**
LGR5	leucine-rich repeat-containing G protein-coupled receptor 5	0,0408925	**-0,46064**
RFTN1	raftlin, lipid raft linker 1	0,00617195	**-0,46054**
ABHD12	abhydrolase domain containing 12	0,0497302	**-0,45535**
ALPK3	alpha-kinase 3	0,00688071	**-0,45346**
SOX12	SRY (sex determining region Y)-box 12	0,0343179	**-0,45298**
LRRC61	leucine rich repeat containing 61	0,0138211	**-0,44759**
TSPAN16	tetraspanin 16	0,0322856	**-0,44744**
FAM79B	family with sequence similarity 79, member B	0,0411973	**-0,44348**
BTG1	B-cell translocation gene 1, anti-proliferative	0,0371544	**-0,4416**
TPPP	brain-specific protein p25 alpha	0,0347178	**-0,43646**
MGA	MAX gene associated	0,0194358	**-0,4356**
MRPL19	mitochondrial ribosomal protein L19	0,0256339	**-0,43446**
PPP1R1A	protein phosphatase 1, regulatory (inhibitor) subunit 1A	0,0161259	**-0,43053**
INADL	InaD-like (Drosophila)	0,0106325	**-0,42973**
JMJD2B	jumonji domain containing 2B	0,0490932	**-0,42895**
GGH	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	0,0470066	**-0,42824**
HIBCH	3-hydroxyisobutyryl-Coenzyme A hydrolase	0,020817	**-0,42407**
MGC26963	sphingomyelin synthase 2	0,025762	**-0,42345**
SULT1A4	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 4	0,0392332	**-0,41975**
ARRDC3	arrestin domain containing 3	0,0423418	**-0,41577**
HAL	histidine ammonia-lyase	0,00765585	**-0,41094**
CREM	cAMP responsive element modulator	0,0494307	**-0,4087**
TGIF2	TGFB-induced factor homeobox 2	0,0188084	**-0,40552**
SMPD1	sphingomyelin phosphodiesterase 1, acid lysosomal (acid sphingomyelinase)	0,0208767	**-0,40424**

Table 4.9 . Most significantly down-regulated mRNAs in liver from old vs young male subjects

4.5. miRNAs and their predicted targets

In principle, the mRNAs that are predicted to be targets of specific miRNAs are expressed at significantly lower levels. This is likely caused by miRNA-mediated destabilization of target mRNA. The predicted targets for each miRNA were downloaded from TargetScan. Therefore, a specific

software (SID1, Albertini et al. 2011) was used to look for matching between the three identified miRNAs and the 90 genes found to be significantly down-regulated. The putative targets gene were identified alone or in combination among the 90 down-regulated mRNAs those targeting mir-141. -200c and -31 mRNA. Three out of 90 mRNAs were identified having conserved 3'UTR sites targeted from the selected miRNAs (Table 4.10) as listed below:

1) solute carrier family 1-glial high affinity glutamate transporter member 2 (**SCL1A2/GLT1**): target of miRNA 31 and 200c

2) elongation factor RNA polymerase II (**ELL2**): target of miRNA141 and 200c

3) arrestin domain containing 3 (**ARDD3**): target of miRNA 31.

GENE	DESCRIPTION	GO	Log OLD/YOUNG	miRNA
GLT1 (SCL1A2)	solute carrier family 1 (glial high affinity glutamate transporter), member 2	GO:0015813: glutamate transport	-1,79726	**mir-31 mir-200c**
ELL2	RNA polymerase II elongation factor (liver)	GO:0006368:RNA elongation from RNA polymerase II	-1,08558	**mir-141 mir-200c**
ARDDC3	Arrestin domain containing 3	protein interacts with ubiquitin ligase	-0,415766	**mir-31**

Tab. 4.10 miRNA-putative mRNAs target correlation in 12 males liver samples. The most highly down-regulated mRNAs in liver from old vs younger male subjects and predicted miR target sites

4.6 Validation of microarray results

The identified three targets (Tab 4.10) were validated via qRT-PCR in liver specimens from 14 young and were compared to 13 old males subjects. An age-dependent trend of decrease was evidenced in all the the three mRNA candidates but only GLT1 (SCL1A2) was found significant (P=0.009) (Fig. 4.11).

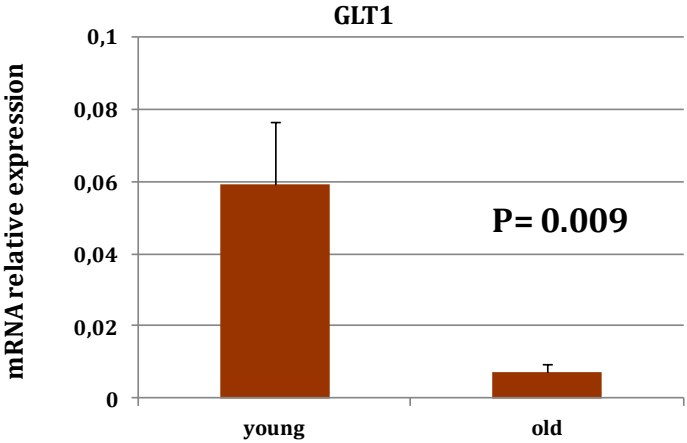


Fig 4.11. GLT1 mRNA expression in liver from young (n. 14) and old male subjects (n. 13)

ELL2 and ARDCC3 mRNA validation comparing 7 old with 7 young males is reported in Fig.4.12 (ELL2 P=0.2; ARDCC3 P = 0.17)

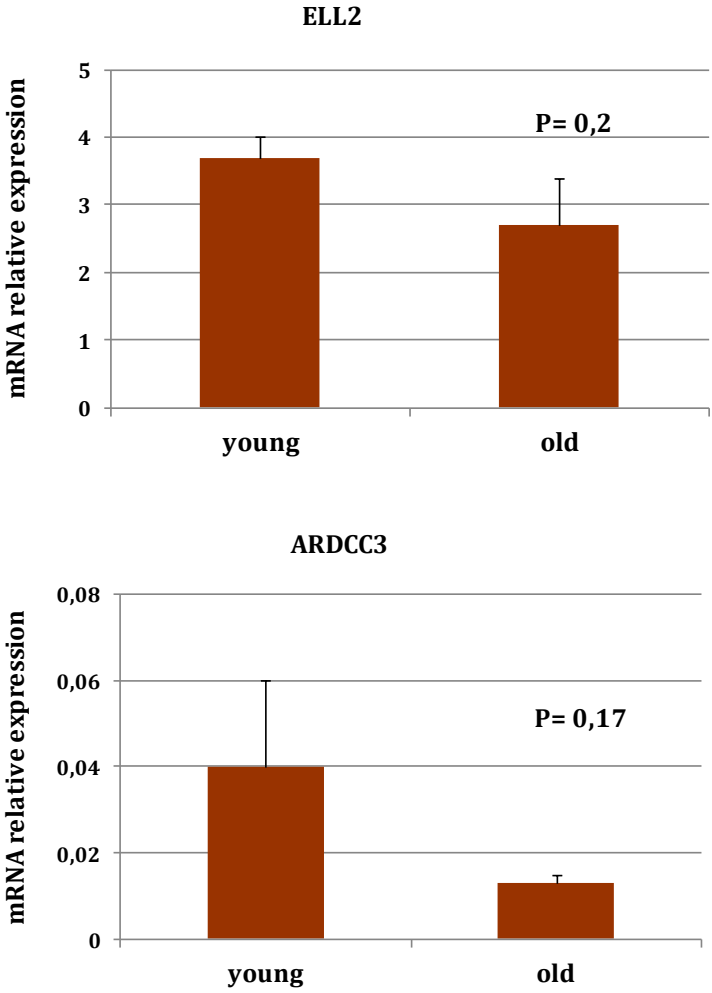


Fig. 4.12 ELL2 and ARDCC3 mRNA expression in liver from young (n. 7) and old male subjects (n. 7)

4.7 GLT1 (SCL1A2) protein quantification

Following the decrease in the relative expression of GLT-1 mRNA, these data were then validated by quantifying GLT1 protein through the use of Western blot analysis.
This test was performed in 4 young subjects compared with 7 older subjects. The result was normalized with actin (Fig 4.13). Hela was used as positive control.

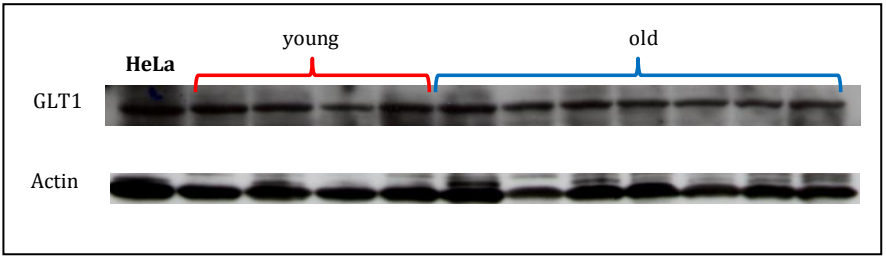


Fig. 4.13 Western blot analysis. Upper lane GLT1: HeLa, positive control, 4 young subjects compared with 7 older subjects. Bottom lane Actin: The same samples for GLT1

T test analysis revealed no significant differences between young and old samples (Tab 4.14)

SAMPLE	AVERAGE GLT1 /ACTIN	T Test young vs old
4 YOUNG	0.5	P = 0.10
7 OLD	0.5	

Tab 4.14:Results of T test analysis

This result was unexpected, as we had previously observed a significant decrease in GLT1 mRNA.

4.8 Follow up analysis

A follow-up analysis was performed in order to evaluate the effect of donor/recipient age mismatch. As previously described, the samples were divided into three groups according to the age of the recipient. miRNAs were normalized with the RNU 44 Ct average. The ΔCt was subsequently obtained in the following manner:

$$\Delta Ct = (\text{sample } Ct - \text{RNU44 average})$$

The relative expression was calculated using the formula

$2^{(-\Delta Ct)} \pm$ s.e.m

The results obtained are reported as follows:

miRNA 31:

- **Recipient age > donor age**: Fig 4.15 displays the mean (±; s.e.m) of the relative expression of miRNA in donor and follow-up samples in cases where the recipient's age is greater than that of the donor. It is clear from the figure that there is an increase in the relative expression of miRNA 31 in the follow-up samples. The data was analyzed with a paired data T Test and found to be significant (P = 0.041).

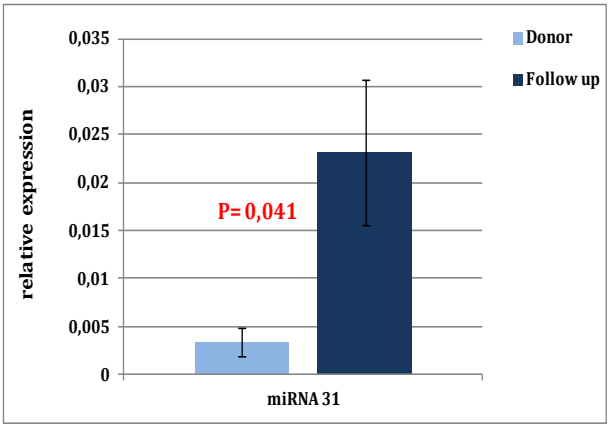


Fig 4.15: mean (± s.e.m) of the relative expression of miRNA 31 in donors and follow up (p= 0.041). The recipient is older than the donor

- **Recipient age < donor age:** the figure below (Fig. 4.16) shows the relative expression of miRNA 31 (mean value ±s.e.m) of the samples in which the recipient is younger than the donor. In this case, there is no significant variation in expression.

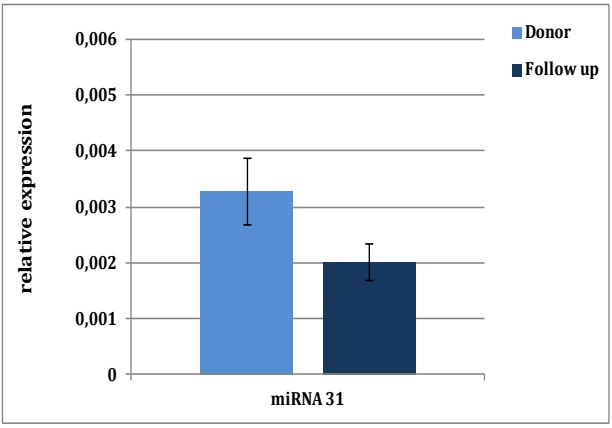


Fig 4.16: the mean (± s.e.m) of the relative expression of miRNA 31 in donors and follow up. The recipient is younger than the donor.

- **Recipient age =(± 4 years) donor age:** the figure below (Fig. 4.17) shows the mean values (±s.e.m) of the expression of miRNA 31 in cases where the recipient is the same age (± 4 years) as the donor, hypothesizing that, at a biological level, this difference approximates zero. The value of the T Test turned out to be significant in this case as well.

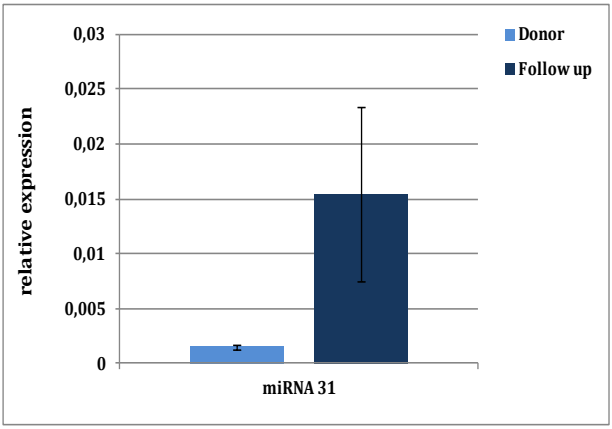


Fig 4.17: mean (± s.e.m) relative expression of miRNA 31 in donors and follow up. The recipient is the same age (±4 years) as the donor

miRNA 141:

- **Recipient age > donor age:** Figure 4.18 shows the mean (±s.e.m) of the relative expression of miRNA 141 in donor and follow up samples in cases where the recipient's age is greater than that of the donor. It is clear from the figure that there is an increase in the relative expression of miRNA 141 in follow up samples, as was found in the previous case with miRNA 31 analyzed in the same sample group. The increase in the relative expression found in the

follow up samples turns out to be significant. P = 0.034 (paired data T Test).

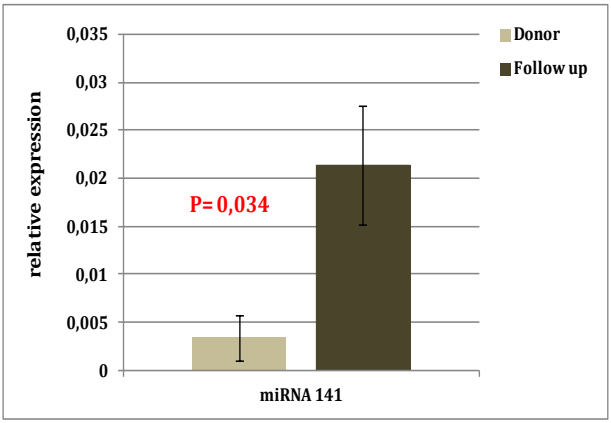


Fig 4.18: mean (± s.e.m) of the relative expression of miRNA 141 in donor and follow up samples (p= 0.034). The recipient is older than the donor

- **Recipient age < donor age:** the figure below (Fig. 4.19) shows the mean value of the relative expression (±s.e.m) of miRNA 141 in follow up and donor samples in cases where the recipient age is lesser than that of the donor. The analysis was conducted with a T Test and detected no significant

variation, although the value P= 0.056 can be considered very nearly significant.

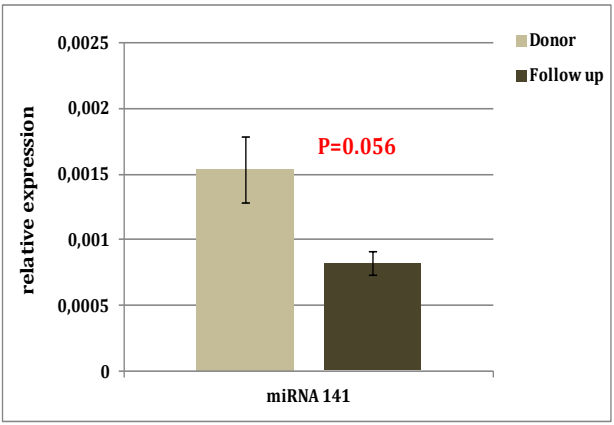


Fig 4.19: mean (± s.e.m) relative expression of miRNA 141 in donor and follow up samples. The recipient is younger than the donor (P= 0.056)

- **Recipient age =(± 4 years) donor age:** the figure below (Fig. 4.20) shows the mean values (±s.e.m) of the expression of miRNA 141 in cases where the recipient is the same age (± 4 years) as the donor, hypothesizing that, at a biological level, this difference approximates zero. The value of the T Test in this case did not turn out to be significant.

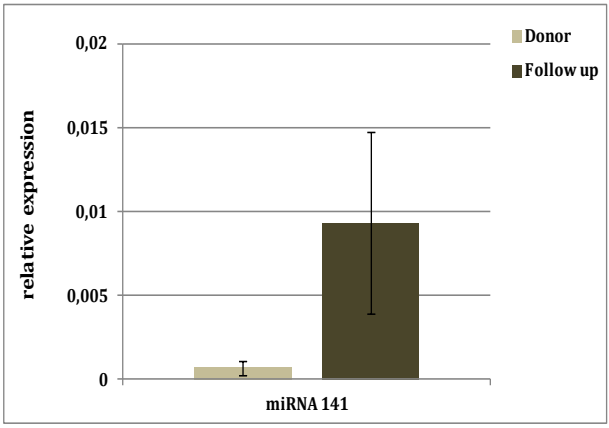


Fig 4.20: mean (± s.e.m) relative expression of miRNA 141 in donor and follow up samples. The recipient is the same age (±4 years) as the donor

miRNA 200c:

- **Recipient age > donor age:** Figure 4.21 shows the mean (±s.e.m) of the relative expression of miRNA 200c in donor and follow up samples in cases where the recipient's age is greater than that of the donor. The significant s.e.m of the results is clear from the figure, which is due to the variability of the obtained data. Unlike the two previous cases (miRNA 31 and miRNA 141) in this case there is no significant variation between donor and follow up samples in the expression of miRNA 200c, although the

progression does follow the same trend as the two previously analyzed miRNAs.

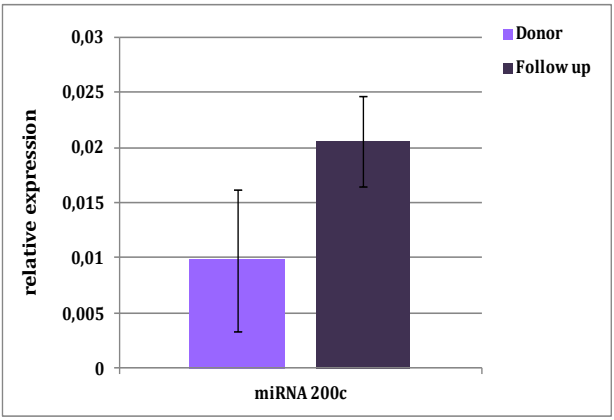


Fig 4.21: mean (± s.e-m) of the relative expression of miRNA 200c in donor and follow up samples. The recipient is older than the donor.

- **Recipient age < donor age:** the figure below (Fig. 4.22) shows the mean values (± s.e.m) of the relative expression of miRNA 200c in samples in which the recipient's age is lesser than that of the donor. There are no significant variations in the expression of this miRNA between the samples analyzed.

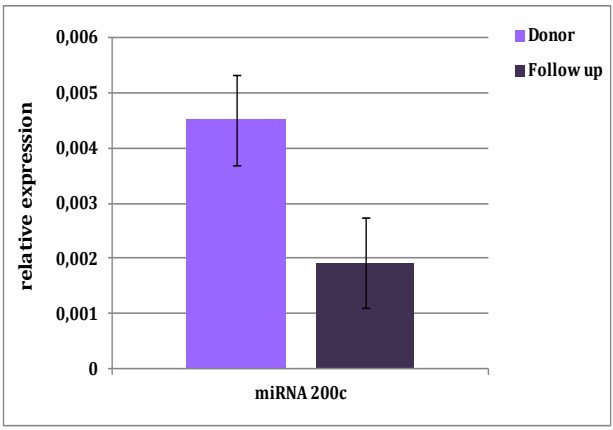


Fig 4.22: mean (± s.e.m) of the relative expression of miRNA 200c in donor and follow up samples.The recipient is younger than the donor.

- **Recipient age =(± 4 years) donor age:** the figure below (Fig. 4.23) shows the mean values (±s.e.m) of the expression of miRNA 200c in cases where the recipient is the same age (± 4 years) as the donor. The T Test in this case did not turn out to be significant either.

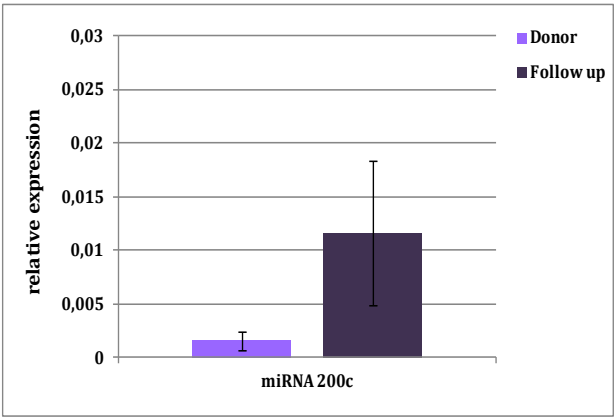


Fig 4.23: mean (± s-e-m) of the relative expression of miRNA 200c in donor and follow up samples. The recipient is the same age (±4 years) as the donor.

5. DISCUSSION

The study described in this thesis is part of a more general topic regarding the use of marginal organ donors, and more specifically the transplant of livers from “elderly” donors. The clinical data and in particular the transplant unit of S. Orsola-Malpighi hospital (Bologna, Italy) started the use of over-60 year-old donors in their protocol since some years (Cescon et al., 2008; Cescon et al., 2003), as already done in other European and American transplant units (United Network for Organ Sharing). Some clinical data demonstrate that the use of elderly donors (even 90 years old) leads to engraftment and tolerance results that are similar to those obtained using younger donors (40-50 years old) (Cescon et al., 2003).

In this context, the present study aimed at two main goals: the first one was to characterize the physiological aging process of the liver from a molecular point of view, while the second was to study the effects produced by donor/recipient age mismatch. The research was therefore aimed at identifying the changes in human liver gene expression associated with age and possible modulations in this process when the organ is engrafted into a younger or

older microenvironment (recipient). This last question is similar to the one addressed by parabiosis experiments in which elderly mice were exposed to serum obtained from young mice (heterochronic parabiosis). In these experiments, an increase of the regenerative capacity of old organs and tissues have been observed in old animals exposed to a "young" microenvironment, in particular as far as hepatocytes and muscle (Conboy et al.,2005). Using heterochronic parabiosis, Villeda and colligues (2001), show that blood-borne factors present in the systemic milieu can inhibit or promote adult neurogenesis in an age-dependent fashion in mice. Accordingly, exposing a young mouse to an old systemic environment or to plasma from old mice decreased synaptic plasticity, and impaired contextual fear conditioning and spatial learning and memory (Villeda et al., 2011).

The first part of this thesis is focused on evaluating the age-related changes in expression profile of both miRNAs and mRNA. Although there is a wide and longlasting tradition of research on liver aging, and in particular on its morphology and metabolism, or even mitochondrial function, the

literature on this specific topic is relatively scarce. Recently, miRNA expression in various organs such as the brain, liver and lung was evaluated in mice of different ages, and this study revealed tissue-specific age-related miRNA changes (Maes et al., 2007; Li et al., 2009; Williams et al., 2007). Williams and co-authors report in mice that miRNA transcription remains unchanged during lung aging, which suggests that the stable expression of miRNAs might work to buffer age-related changes in the expression of protein-encoding genes (Williams et al., 2007). Other authors found an increase in the expression of specific miRNAs in liver and brain (Maes et al., 2007; Li et al., 2009). Bates et al. (2010) used the Ames dwarf mouse model, an animal model that is interesting in the search for the genomic factors which control or affect the aging process. Mice belonging to this strain are known to live up to 70% longer than their wild-type equivalents due to a deficiency in 3 pituitary hormones (growth hormone, GH, thyreotropin and prolactin). This study investigated variations in protein and miRNA expression in dwarf mice of different ages and their wild-type counterparts using global proteomic

profiling together with miRNA microarray analyses, followed by a functional analysis of specific miRNA/target relationships. This study revealed the up-regulation of ten miRNAs in dwarf mouse livers, with miRNA-27a clearly emerging as the leading significant species of miRNA in this context. In the liver of the dwarf mouse, the expression of miRNA-27a increases with age. Moreover, miR-34a and miR-93 are up-regulated in the liver of wild type aged rats, with inverse down-regulation of their predicted targets, Sirt1 and Mgst1. Signaling networks involving Sirt1 and Mgst1 are the two major components of the stress response mechanism to counteract the damaging effect of reactive oxygen species (ROS). MiR-34a and miR-93 target among others two transcription factors, such as Sp1 and Nrf2 (Li et al., 2011). The age-dependent decreased expression of specific genes, as well as of the transcription factors known to activate these genes, suggests that with age, the intertwining signaling involving deacetylation and detoxification processes is attenuated in older liver (Li et al., 2011). On the whole, the vast majority of liver miRNAs exhibiting variation in aged mice are up-regulated (Bates et

al., 2010 ;Maes et al., 2007; Li et al., 2009; Li et al., 2011). The identification of miRNAs involved in the aging process is expected also in humans, and may correspond to progressive age-related deregulation in their expression (Wang, 2007). However data concerning miRNAs expression in human tissues, such as basal skeletal muscle, suggested an up-regulation of tissues specific miRNA in the old men compared to young men (Drummond et al., 2008).

In the research presented here, we took advantage of high throughput techniques by using different types of arrays, i.e Affymetrix Genechip (54K) for transcriptome analysis and card A system from Applyed Biosystem platform (384 miRNAs). In agreement with published data, in this research any miRNAs resulted down-regulated in human old liver samples, confirming data previously reported on murine aged liver (Bates et al., 2010 ;Maes et al., 2007; Li et al., 2009; Li et al., 2011).Three different miRNAs, such as miRNA-31 and miRNA-200c and -141, resulted up regulated more than 4 fold in old (>70 years) vs young (<70 years) subjects.

One interesting result is that the up-regulation of these three miRNAs was more pronounced in males. Initially, the profiling of the miRNAs was carried out on 12 male samples, and when validating the results with qRT-PCR, the significance previously found was confirmed even thought to a lesser extent. The validation was carried out on 47 subjects, 23 of which were women. Stratifying the 47 samples by sex, the up-regulation of the three miRNA (31, 151 and 200c) was confirmed only in the samples from males. In the female samples, there is a similar trend even if none of them reached statistical significance. This unexpected result is explained by the lack of homogeneity of the female samples: the young women (aged less than 70 y.o.) were too few in comparison to the older women (aged over 70) and this caused a lack of statistical power.

From global analysis, liver gene expression profiles were assessed by Affimetrix array in 12 male subjects, and 90 mRNAs were identified as most significantly down-regulated in the livers of elderly subjects (young/elderly ratio <0.67. log(ratio) = -0.4. ANOVA $p<0.05$).

Subsequently, the predicted target sequences for each miRNA were downloaded from TargetScan Mapping. Therefore, a specific software (SID1, Albertini et al. 2011) was used to look for matching between the three identified miRNAs and the 90 genes found to be significantly down-regulated. The putative targets gene were identified alone or in combination among the 90 down-regulated mRNAs those targeting miRNA-141, -200c and -31 mRNA.

By this analysis, three mRNA targets were identified, i.e. GLT1 (solute carrier family 1 (glial high affinity glutamate transporter), member 2), which is targeted by miRNA 31 and 200c, ELL2 (RNA polymerase II elongation factor (liver), which is targeted by miRNA 141 and 200c, and ARRD3 (Arrestin domain containing 3), which is targeted by miRNA 31. Validation experiments to confirm the decreased concentration of target mRNAs were performed by qRT-PCR, but a significant down regulation was confirmed only for GLT1 mRNA comparing 70 yrs old donors with younger donors. Nevertheless, at a protein level, the expression of GLT-1 remained unchanged when comparing 70 yrs old donors with younger donors. It is at

present unknown whether this is due to an increased stability of the protein or to other mechanisms.

GLT1, the glutamate transporter, is a Na+/K+-ATPase and functions to force glutamate to enter the cell against the concentration gradient thanks to the creation of an ionic gradient: a molecule of glutamate is exchanged through the entrance of 3Na+ into the cell and the exit of 1 K+ from the cell (Kanner, 2006). The glutamate transporter is very important at the hepatic level given that the entire organism's nitrogen is metabolized in the liver. Glutamate is produced by glutamine thanks to the activity of glutaminase, an enzyme that is found in high concentrations associated with mitochondria. Like glycemia, hematic concentrations of glutamine must be maintained at constant levels in blood to ensure vital functions such as central nervous system, renal, intestinal and hepatic activity as well as the activity of immune system and pancreatic beta-cells. Glutamine is the most abundant amino acid found in plasma and an important precursor of peptides; it is therefore essential for the synthesis of proteins, nucleotides, and nucleic acids.

Glutamate has an intercellular concentration that is nearly 30 times higher than the L-glutamine located in the extracellular compartment. Since glutamate has a negative charge and thus cannot be transported outside the cell, some transporters are located (at a low density, with the exception of SNC) on the plasmatic membrane (GLT-1). Glutamate is highly important for trans-deamination, as glutamate can remove the amino group for the synthesis of other amino acids. Glutamate can lose the –NH4+ group and become 2-oxoglutarate. In some tissue, such as the liver, glutamate and NH4+ can combine and, thanks to glutamine synthetase, produce glutamine, which will then be exported from the cell (Newsholme et al., 2003). The metabolism of glutamine in the liver occurs in different spaces: glutamine enters the periportal hepatocytes where the enzyme glutaminase has an elevated rate of activity; the glutamate produced in periportal cells can be further metabolized to produce other amino acids through transamination, may enter the cycle of tricarboxylic acids, or may enter into the gluconeogensis pathway, forming phosphoenolpyruvate from oxaloacetic acid. In this way,

the process of gluconeogenesis beginning from glutamine utilizes a large quantity of glutamate in the liver, which causes the production and export of glucose (De-Souza et al., 2001). In addition, arginine catabolism also provides glutamate for the synthesis of glutamine (O'Sullivan et al.; 1998).

Thus, GLT1 is a key regulator of glutamate metabolism. In the human liver, GLT-1 mRNA and transmembrane protein was strongly expressed by perivenous hepatocytes (Berger et al., 2006). In contrast to the brain, where GLT1 is a crucial player in glutamatergic neurotransmission, GLT1 expressions in the peripheral organs such as the liver may play a role in glutamate uptake for metabolic purposes or in the conversion of glutamate to glutamine (Berger et al., 2006). As of today, it is not yet understood how these pathways are regulated during human aging and how they are regulated by the action of miRNAs. The results present in the literature only report experiments conducted on mice. Our results on miRNAs profiling are in accordance with previous data on dwarf mouse liver, displaying an increased expression with age (Bates et al., 2010). As far as

the regulation of the protein, no data are available to our knowledge as far as human liver. Some data reported in the literature suggested an up-regulation of GLT-1 as a consequence of the beta-catenin activation (Cadoret et al. 2002). These results suggested that GLT-1 may be involved in the proliferative response of the liver to beta-catenin by providing glutamate to the cell as a precursor for glutamine. An increase in GLT-1 expression in liver has also been shown in growing versus non-growing lambs (Howell et al. 2003). We observed an up-regulation of miRNAs targeting GLT1, that is not mirrored by a decreased level of protein. This could be accounted for by a yet unidentified mechanism that counteract the action of miRNAs, due to the extreme importance of this protein, as discussed, that must likely be maintained constant is its transmembrane expression throughout lifespan.

In the second part of the study, we sought to determine whether or not the transplanted liver is affected by the new microenvironment of the recipient. In particular, we studied whether or not the expression of the three miRNAs (miRNA 31, 141 and 200c) in the transplanted liver could

be affected on the basis of the donor/recipient age mismatch. The analyzed samples were sub-divided into three groups according to recipient age: in the first group, the recipient is much older than the donor (an average difference of 24.6 ±7 years); in the second group, the recipient is much younger than the donor (an average difference of 24.6 ± 2.5 years); the third group, studied as a control group, is composed by patients who received an organ from a donor of similar age (±4 years). The analysis of the relative expression of miRNAs 31, 141, 200c produced very interesting results. For miRNA 31, the data previously obtained in this study showed that it is up-regulated in elderly subjects (> 70 years old) but not in young subjects (< 70 years old). For the sample group in which the recipient is older than the donor, the relative expression of miRNA 31 was found to be increased ($p = 0.041$) in the follow-up liver as compared to the same organ immediately before transplant. This change in expression is a very interesting result, as it is likely due to the effect of the recipient microenvironment, in this case of an elderly subject. For cases in which the recipient is

younger than the donor, the progression has an opposite trend, that is to say that the relative expression of miRNA 31 decreases, although not significantly, in the follow-up. At any rate, the tendency of the observed effect is in accordance with the hypothesis of recipient microenvironment effect on the transplanted liver: as the recipient is younger, there is a (trend to a) decrease in the expression of miRNA. The expression of miRNAs 141 and 200c in recipients displays a trend that is analogous to the expression of miRNA 31 described above. As for miRNA 141, its relative expression is found to be significantly increased in follow-up samples as compared to donor (P=0.034) in samples for which the recipient is older than the donor. In relation to the same type of sample, miRNA 200c is also found to have the same type of tendency, although in this case it is not significant. In cases where the recipient is younger than the donor, the variation in the relative expression of miRNA shows a decrease in follow-up samples that is very close to being significant, as for miRNA 141 (P=0.056), whereas it is not significant in relation to miRNA 200c. Lastly, for all three miRNAs studied, the

analysis of the control group lacking age mismatch detected a progression similar to that of the first group in which the recipient is older. For this last case, we expected to find a similar miRNA expression when comparing the two conditions analyzed, that is before and after transplant, in that the initial assumption was that age mismatch could have an effect on gene expression except in cases lacking such mismatch. This unexpected finding leads us to hypothesize that there is a significant effect on miRNA expression (a decrease) only when the recipient is much younger than the donor. Vice versa, miRNAs would have a tendency to increase in cases where the recipient is the same age or much older than the donor.

These experiments are surely affected by the small number of analyzed samples, but it is necessary to keep in mind that the very nature of this experimentation involves particularly challenging collection and analysis timeframes owing to its relatively rare occurrence.

Despite being conducted on a small number of samples for each group, the second part of the study does therefore

allow us to conclude that the younger microenvironment produces a significant effect on the expression of miRNAs 31 and 141.

The roles of miRNA 31 and miRNA 141 have been widely described in the literature. Both are found to be hyper-expressed in various types of tumor. For miRNA 31, a recent study by Karakatsanis et al., (2011) that aimed at evaluating the deregulation of miRNAs in patients with hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC) identified a significant upregulation in HCC of miRNA 31 among others, including miRNA 122. The same study also found a down regulation of miRNA 200c. In this report, the authors showed that a high level of expression of miRNA 21, miRNA 221, miRNA 31 and miRNA 122 was correlated with cirrhosis but only high levels of miRNA 21 and miRNA 221 were found to be associated with tumor stage. The aberrant expression of miRNAs in ICC has not been the subject of as much research as has HCC, perhaps owing to the infrequency of this pathology. MiRNA 21, miRNA 31, and miRNA 223 were found to be significantly up-regulated in ICCs in

comparison with normal samples from cancer-free individuals, while miRNA 122, miRNA 145, miRNA 146a, miRNA 200c, miRNA 221, and miRNA 222 were instead down-regulated. Karakatsanis and colleagues have shown that the expression of miRNA 21, miRNA 31, miRNA 122, and miRNA 221 in HCC tissues is correlated with cirrhosis, and that miRNA 21and miRNA 221 are also associated with both tumor stage and poor prognosis in HCC patients. It was found that miRNA 21, miRNA 31, and miRNA 223 are overexpressed in ICC samples but no correlation was found with clinico-pathological features. It was determined that the miRNA 31 functions to regulate several metastasis-related genes in breast cancer tissues and cells. A variety of approaches were used to demonstrate that cellular levels of miR-31 are correlated with the cell's capacity to invade and metastasize; cells having increased levels of this miRNA, on the other hand, were less metastatic (Schmittgen et al., 2011). MiRNA 141 was also found to be disregulated in various types of tumors. The upregulation of miRNA 141 is closely associated with tumorigenesis in relation to prostate cancer (Xiao et al., 2012 Epub ahead of print). A

very recent study revealed that plasma miR-141 is a sensitive marker and complements CEA for the detection of stage IV colon cancer (Cheng et al, 2011). Importantly, Chen and colleagues demonstrated that higher plasma levels of miRNA 141 is associated with shorter survival rates and that miRNA 141 is an independent prognostic indicator in relation to colon cancer. Along these lines, in addition to cancers of the prostate and colon, circulating levels of miRNA 141 have also been found to be associated with other pathophysiological conditions such as pregnancy (Chim et al., 2008) and ovarian cancer (Taylor et a., 2008). An interesting study by Banaudha K et al., (2011) reports a direct role for miRNA 141 in suppressing DLC-1, a gene that is frequently deleted in HCC as well as other solid human tumors (Xue et al., 2008). They point out that DLC-1 encodes a Rho-GTPase activating protein and represents a candidate tumor suppressor gene which is located on chromosome 8p21.3-22. HCV's intracellular induction of miRNA 141 appears to translationally inhibit the tumor suppressor DLC-1, whose depletion works to promote the proliferation of cells. Additionally, the efficient replication

of HCV is found to be correlated with a miRNA 141–mediated reduction of the DLC-1 protein in virus-infected cells. The reciprocal relationship that exists between miRNA 141 and DLC-1 protein levels in cells infected with HCV suggests that virus replication is favored in cells that display reduced levels of the DLC-1 protein, though the exact mechanism through which miRNA 141 or DLC-1 work to modulate virus replication remains unclear (Banaudha et al., 2011). In relation to miRNA 200c, there has been extensive investigation of the miRNA 200 family in cancer cells' epithelial-to-mesenchimal transition (EMT) (Brabletz et al., 2010); in EMT, down-modulation of the miRNA 200 family functions to enhance cancer aggressiveness and metastases, while the reintroduction of miRNAs belonging to the miRNA 200 family inhibits their growth in some tumors. Furthermore, available literature indicates that this miRNA is down regulated in subjects suffering from hepatocarcinoma (Karakatsanis et al., 2011). Laidero et al., (2008), analyzing miRNA profiling in both benign and malign tumors, identified an overexpression of miRNA-224 in all tumors and a down-regulation of miRNA200c,

miRNA-200, miRNA-21, miRNA-224, miRNA-10b, and miRNA-222 in benign and malign tumors. All three identified miRNAs up-regulated in this research project are therefore been observed to be involved in hepatic pathology. Nevertheless, it is to be underlined that the livers analysed in this study were considered healthy and suitable for transplant, therefore we surmise that these miRNAs change their expression not only during pathological events, but also during physiological aging.

In this research project, some recipients were HBV and/or HCV positive, but it was not possible to stratify the analyzed samples according to HCV- or HBV-positivity due to the small number of samples (power size effect). The recipient's condition is very important, for instance the fact that he or she might be infected with a virus such as HBV and HCV. It is well known that the development of chronic hepatitis (e.g. induced by HBV of HCV) and HCC are inter-connected (Haybaeck et al., 2009; Pikarsky et al.,2004; Karin et al.,2005; Karin et al.,2005). Using sequencing as well as bioinformatics data, the deregulation of miRNA transcriptome was identified in HCC development driven

by hepatitis-B (Mizuguchi et al., 2011). The microRNAs miRNA-7, miRNA-433, miRNA-511 and miRNA-196b in humans are known to affect the process of viral polymerase and the S gene of HBV, whereas miRNA-205 has an effect on the X-gene of HBV, which makes it a potentially useful therapeutic tool in treating HBV-induced hepatitis (Mizuguchi et al., 2011; Wu et al., 2011). Moreover, miRNA-345 seems to target the HBV pre-C gene and the down-regulation of miRNA-345 is known to facilitate the protein expression of HBV pre-C, a precursor of HBeAg (Wu et al.,2011). MiRNA-122 was also found to be importantly involved in controlling hepatitis C virus (HCV) infection, along with cholesterol metabolism and the formation of HCC (Mizuguchi et al., 2011). HCV is known to be a positive sense, single-stranded RNA virus that can result in chronic infection, leading to liver cirrhosis, chronic hepatitis, and, in many cases, HCC as well (Yang et al., 2010). MiRNA-122 was found to be essential in the replication of HCV in cultured human hepatocyte cell lines (Huh7). Furthermore, miRNA-122 binds directly to two adjacent sites along the 5´-UTR of HCV RNA (Mizuguchi et al., 2011). It is this

specific binding that is responsible for an increase in viral replicon amplification and the synthesis of RNA. It appears that miRNA-122 chiefly has a more indirect stimulatory effect on the synthesis of viral RNA rather than a direct effect on the synthesis of protein (Filipowicz et al., 2011). Research by Roberts and colleagues showed that miRNA-122 works to stimulate the accumulation and translation of HCV RNA through the HCV 5`-UTR. Additionally, miRNA-122 seems to modulate a second replication cycle through a mechanism that is not yet clear (Filipowicz et al., 2011). One way that miRNA-122 is known to act on its targets would be by influencing the stability of viral RNA. Nonetheless, miRNA 122 has been discussed as a possible target candidate for the control of HCV replication. Regrettably, quantitative analyses of miRNA 122 and HCV-RNA carried out in specimens of infected human liver showed a complicated interaction mechanism, given that the activation of HCV translation is driven by Argonaute rather than taking place through structural transformation in the HCV internal ribosomal entry site (IRES) (Vazquez-Del Mercado et al., 2010.;Roberts et al., 2011).

By now the important role of miRNA regulation in a wide range of hepatic diseases such as hepatitis, hepatic tumors and metabolic diseases is well know. These findings may turn out to be extremely useful in clinical practice: thanks to the miRNA expression profile, which is characteristic and unique for some of the most well-known pathologies, miRNAs can act as new biomarkers in diagnosing disease, as previously mentioned in this chapter. By now the literature has thoroughly identified the panel of miRNA most expressed in hepatocytes, among which miRNA 122 is the most abundant (Lagos-Quintana et al., 2002). In addition to playing an important role in controlling the hepatitis-C virus (HCV) as thoroughly described above, miRNA 122 is involved in regulating the metabolism of cholesterol and more generally in hepatic lipid metabolism: by silencing this miRNA, it was possible to observe a reduction in hepatic steatosis in mice fed on a diet rich in fats (Krutzfeldt et al., 2005; Esau et al., 2006; Filipowicz et al., 2011). It is also involved in the formation of hepatic carcinoma (Mizuguchi et al., 2011). Recent studies also carried out on mice reveal that miRNA 122, together with

other miRNAs expressed in the hepatocytes, are released into blood following drug-induced hepatic damage (Wang et al., 2009; Laterza et al., 2009). It was possible to detect that levels of miRNA (hepatocyte-derived miRNAs, HDmiRNAs) in plasma or serum increase strictly according to the dose and duration of drug exposure (Farid et al, 2011). In addition, HDmiRNAs are found to be correlated with levels of aspartate transaminase (AST), serum transaminase and alanine transaminase (ALT). It has been recently demonstrated in humans that the level of miRNA 122 in serum is elevated in patients presenting hepatic lesions owing to alcohol abuse, chemical or viral hepatotoxicity (Zhang et al.,2010; Bihrer et al., 2011). In these same patients, the dosages of miRNA 122 in serum and plasma were strictly correlated with both transaminase and hepatic histology. Like human studies, recent animal model-based studies demonstrate that HDmiRNAs represent highly sensitive and stable serum biomarkers for liver lesions (Wang et al., 2009; Laterza et al., 2009 Zhang et al.,2010; Bihrer et al., 2011). It was found both in humans and in rodents that increases in HDmiRNAs

levels occur much more rapidly in serum than in AST and ALT.

HDmiRNAs hold a fundamental importance in the context of liver transplantation. A study by Farid et al (2011) has demonstrated that serum levels of HDmiRNA are elevated in patients presenting liver lesions, both following liver transplant and during acute rejection. Serum HDmiRNA has shown similar kinetics during acute rejection; however, miRNA levels increase before transaminase. HDmiRNAs could be considered to be hepatic biomarkers capable of providing a tool for evaluating the possibility of rejection or dysfunction in the transplanted liver through non-invasive and highly sensitive methods. Finding reliable biomarkers is extremely important because, as of today, there is no parameter that would significantly improve the management of liver transplantand indisputably enable the reduction of immunosuppressive pharmaceutical dosages, thus enabling the achievement of a better equilibrium between desirable effects (such as the prevention of transplant rejection) and collateral effects (such as toxicity, infection and malign tumors). Farid and colleagues (2011)

have carried out an important study, testing 15 different kinds of hepatocyte- and cholangiocyte-abundant and control miRNAs which were selected from among other studies (Wang et al., 2009; Chen et al., 2009; Hand et al., 2009). This group includes miRNA 30a, miRNA 30c, miRNA 30e, miRNA 122, miRNA 133a, miRNA 148a, miRNA 191, miRNA 192, miRNA 194, miRNA 198, miRNA 200c, miRNA 222, miRNA 296, miRNA 710 as well as miRNA 711; however, the three HDmiRs were found to be significantly elevated during acute rejection wich HDmiRs, miRNA 122, miRNA 148a and miRNA 194.

Given that the use of much older donors is undergoing an enormous increase, the evaluation of liver transplant outcomes on the basis of donor/recipient age mismatch is gaining increasing importance. Traditionally, using liver allografts obtained from older donors was considered to be a risk factor for poor graft function (Marino et al., 1995). In contrast with other organs, however, the liver is known to undergo only minor loss of function in the majority of healthy elderly subjects (Schmucker et al., 2005; Timchenko et al., 2009). Using elderly donors for hepatic

transplantation continues to be controversial owing to considerations regarding inferior outcomes. Several studies about successful transplantations using grafts from very old donors were recently published. Andormo and colleagues utilized a graft obtained from an 84 y.o. donor (Andorno et al., 2001). Moreover, other groups reported liver transplantations from donors who were even older than 90 y.o. (Grazi et al., 2008; Romagnoli et al., 2001; Karpen et al., 2010). By other authors, it has been shown that liver function in older donors can be considered comparable to that of younger donors. Comparing the outcome of liver transplantation from donors who are older and younger than 60 y.o., Zhao et al. showed equivalent graft function between the groups (Zhao et al., 2004). Oh et al. report no differences in graft survival or the incidence of primary nonfunction when comparing organs obtained from donor groups aged below or above 50 y.o. (Oh et al., 2000). According to Gastaca et al, no differences were found in primary disfunction or acute re-transplantaton rate when comparing the outcomes of donors aged over 70 y.o. with donors who were younger (Gastaca et al., 2005).

6. CONCLUSIONS

Through this research:

- For the first time, 3 miRNAs that we propose as biomarkers of human liver aging have been identified; miRNA 31, 141 and 200c are found to be up-regulated in old people, especially in males aged > 70 years. In relation to females, it is not possible to provide the same result as the analyzed sample was deficient in young subjects and this resulted in a lack of statistical power.

- For the first time, this research highlights the effect of an age mismatch, where a young (recipient) microenvironment induced in old liver a modification of the expression of miRNA31 and 141 similar to that observed in young organs. It is not yet known whether this modification in miRNAs expression is mirrored by histological or functional outcomes and how long it lasts in time.

This study offers quite significant opportunities for further research which are of noteworthy importance for the study of transplant tolerance and the possibility

of reducing immunosuppressive therapy, on the basis of the donor/recipient age mismatch effect as well.

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