

# Chapter 2

## MicroRNAs in Disease

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### The Study of MicroRNAs

#### *Overview*

MicroRNAs or miRNAs are a newly described class of short non-coding RNA molecules with the distinctive role of fine-tuning the expression of mRNAs in the living cells of all organisms [1]. Their targets are usually mRNA molecules that bear specific miRNA recognition sites on their 3' UTRs, 5' UTRs or coding regions; miRNAs bind onto their target sites in a Watson–Crick base pairing manner and eliminate mRNA translation (Fig. 2.1a) [2]. In humans, such post-transcriptional regulation of gene expression cannot be overlooked as more than half of all genes have evolutionary conserved miRNA target sites [3]. Consequently, miRNAs are prime regulators of all kinds of physiological cellular processes; hence faulty regulation of mRNA expression can lead to disease.

#### *Target Prediction Algorithms*

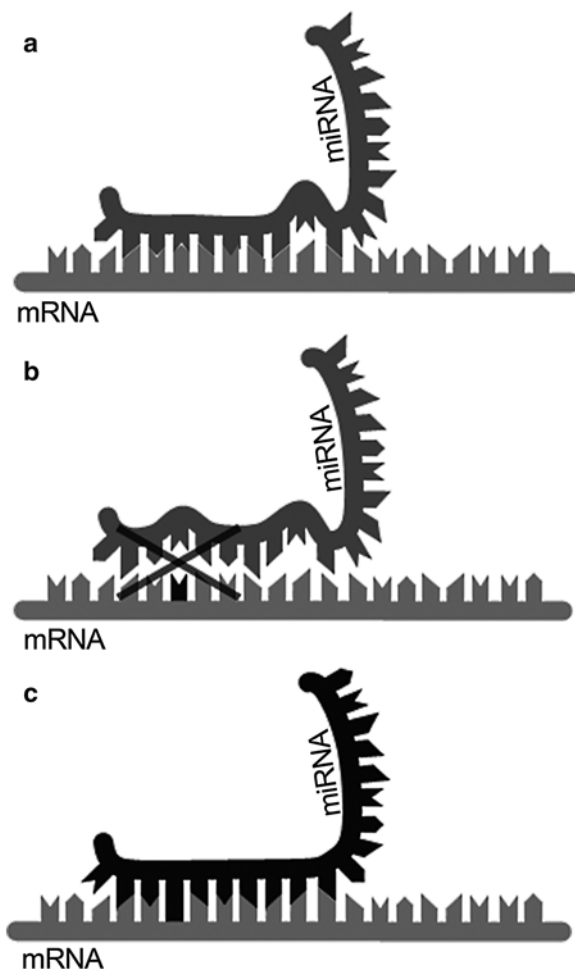
More than a handful of algorithms are available online and are elegantly designed to deliver predictions for miRNA binding sites on the 3' UTR of protein-coding mRNAs, or to predict target sites for any miRNA sequence. Their numbers keep growing in order to serve the emerging demands of the scientific community for customization and credibility of results. Search variables usually include the

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**Fig. 2.1** Polymorphisms on miRNA target sequences (miRSNPs). Normal binding of miRNAs (a) can be diminished when target nucleotides corresponding to the miRNAs seed region are altered (b). In some cases, miRSNPs serve as gain-of-function mutations, when they create new target sites for different miRNAs (c)



seed-region length and miRNA/mRNA species, while each algorithm allows for further customization of search criteria depending on the prediction approach or even a cutoff p-value in correspondence to the statistical processing followed in each case. One must always have in mind the philosophy of predicting a miRNA target site on the 3' UTR of an mRNA or elsewhere: a certain algorithm follows precise and predetermined criteria to predict such sites, which some are based solely on Watson–Crick complementarity between the miRNA and its target sites and other encompass more complicated approaches such as a learning algorithm or free energy values, therefore hits returned are destined to include false-positive results. Consequently, not all prediction results are valid and there is always a need for using a filtering approach in an effort to isolate the most useful and in a sense true miRNA–mRNA pairs. Filtering strategies can revolve around a wide or a narrow spectrum of criteria that are predominantly “*making sense*”; hence, both the miRNA

and its potential mRNA targets should be expressed in the same tissue for example, or they are encountered in the same pathway or developmental stage. Moreover, all algorithms are enforced with a powerful statistical evaluation of prediction results, which in most cases can itself filter out correct pairs. In some studies, researchers repeat prediction analyses using a number of available algorithms and align prediction results to pick up only the pairs predicted by the most of them.

All algorithms predict target sites on 3' UTR mRNA sequences, while some of them expand their predictions to include the gene's coding sequence, its 5' UTR, sequences located upstream the transcription start points and even on the mitochondrial genome [4, 5]. The miRWalk algorithm works by "walking" on the gene sequence in a window of seven or more nucleotides and a miRNA is predicted to target a specific mRNA based only on seed region complementarity [4]. TargetScan on the other hand, is searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA, while it "ranks" a predicted miRNA target based on site length, type, context and accessibility [6]. The miRanda algorithm considers the miRNA sequence as input and searches a sequence dataset for potential target regions and successful predictions are made based on the alignment score and the minimum free energy of the miRNA bound to the potential target sequence [7]. The miRDB algorithm uses a completely different approach, as it is based on an SVM algorithm which is trained by a wiki database, in which users are able to input sequences of validated miRNA/mRNA couples [8], while the RNA22 algorithm uses a reverse approach as it first examines gene sequences for putative miRNA binding sites and then identifies a miRNA that could target an identified 3' UTR site [9]. Predictions can be easily made by visiting the appropriate web location of each algorithm and placing a query about your miRNA or mRNA of choice. Results will be returned instantly and will depict the target site, its length, and a statistical score describing the likelihood this interaction is true based on the parameters on which each algorithm operates. The validity of prediction algorithms has been the number of many studies, all indicating an increased rate of false-positive results emerging from predictions, therefore a good bioinformatics analysis and filtering of predicted targets should be then supported by functional experiments [10].

### ***Validation of miRNA Targets***

As prediction algorithms can be a starting point in miRNA target discovery, direct interaction between a miRNA and an mRNA can only be valid when at least proven in vitro. Luciferase reporter constructs have been widely used as a straight-forward solution in studying direct binding of a miRNA on its target sequence. Albeit a useful and reliable tool, luciferase reporter constructs can only serve in examining one particular miRNA-mRNA target site and can be laborious at times. Target sites are introduced into the 3'-untranslated ending of the luciferase gene and plasmids are transfected into cell lines together with miRNA mimics or inhibitors (also called antagomirs). miRNA-analogous oligonucleotides are commercially

available, relatively low in price and ready to use in cell cultures in various types; “mimics” that share the same sequence as a mature miRNA, “inhibitors” that are used to silence a specific miRNA, or “target site protectors” that prohibit miRNA binding onto its target sequence. Commercially available mimics and inhibitors are frequently chemically modified to LNAs (Locked Nucleic Acids) by the insertion of a 2′O-5′C Methyl-bridge, which helps the RNA oligonucleotide to keep an open conformation and to be thermally more stable than conventional oligos. (The reader is encouraged to see [11] and references within for a good presentation of currently used techniques in miRNA research and their limitations).

A good miRNA–mRNA couple can significantly reduce luciferase expression levels and in the same time a target site mutation, preferably at the nucleotides corresponding to the miRNA’s seed region, can abolish miRNA binding ability and therefore increase luciferase expression; this is a useful approach when investigating the effects of SNPs occurring at miRNA target sites.

As miRNAs are considered as post-transcriptional regulators, further investigation of their properties can include the determination of the protein levels of target mRNAs. This can be achieved by performing protein assays, i.e. western blots, amino acid stable isotope labelling or proteomics, after the overexpressing or knocking-down miRNAs in vitro. It has been found that specific miRNAs can regulate a restricted number of proteins, while changes in protein levels can sometimes be subtle [12]. Such effects are somehow expected, as protein levels are determined by a number of factors including mRNA transcription rate or protein degradation.

### ***Identifying miRNAs in Tissues, Bodily Fluids and Exosomes***

Isolation of miRNA species can be achieved by using readily available kits in the market developed by various companies. Specific applications require specific kits usually depending on the starting material, for example isolation of an enriched miRNA fraction from urine samples, serum or formalin-fixed paraffin-embedded (FFPE) tissues can be performed using appropriate commercial kit protocols. Alternatively, the TRIzol reagent can be used, although a biased loss of small RNAs with low GC content when processing a small number of cells with TRIzol has been reported [13]. Exosomes are small, 30–150 nm sized vesicles secreted by cells that contain miRNAs among other molecules; the isolation of miRNAs from exosomes can be achieved either by the use of Total exosome isolation reagents or by ultracentrifugation in sucrose gradients [14, 15]. Quantitation and quality assessment of enriched miRNA fractions can be performed using a microfluidics-based platform or an equivalent electrophoresis system, rather than a standard spectrophotometer due to their small size and reduced abundance compared to total RNA.

MicroRNAs can be detected using a number of methods, such as northern blots, real-time PCR or miRNA-specific probe hybridization. Although having limited sensitivity, northern blots are widely used for the identification of specific miRNAs and while their workflow is relatively simple, they require heavy optimization. A sample is let to run on an electrophoresis gel, which is consequently transferred

to a porous membrane and miRNA-specific probes are let to hybridize onto targets while emitting fluorescence or radioactivity [16]. Relative quantification of cell-extracted miRNAs can be easily performed by real-time quantitative PCR (qRT-PCR). Following extraction, reverse transcription of miRNA species is performed in a two-step procedure: miRNA molecules are extended (3' end) and single strand synthesis is completed with a universal primer [17, 18]. With the use of appropriate primers, miRNA sequences are detected in qRT-PCR and can be quantified by being compared to a number of small RNAs used as reference. Alternatively, reverse transcription can be performed using a stem-loop approach and miRNAs can be accurately detected via highly specific TaqMan probes [19]. In samples originating from exosomes or biofluids, quantitative analysis of miRNA expression can be difficult, as there is usually a lack of a stably-expressed small RNA to be used as a reference. For this purpose, a synthetic miRNA is usually spiked-in at a predetermined concentration to assist in downstream analyses [20–22].

In fresh or preserved tissue sections, miRNAs can be easily detected by in situ hybridization (ISH), where labelled probes with complementary sequences to target miRNAs are left to hybridize and reveal miRNA localization and differential expression [23]. Despite its wide use, ISH requires optimization and can sometimes be a laborious process. Nevertheless, when the probe affinity for its target is high, ISH can be used as a semiquantitative method of determining miRNA abundance in tissues and single cells. High resolution analysis of miRNA expression at single-cell level can be also performed by pairing fluorescent ISH with flow cytometry (flow-FISH), a technique able to give additional useful information on mRNAs or proteins of interest simultaneously [24, 25].

### ***High-Throughput Methods in miRNA Research***

The special nature of miRNA molecules makes their study a cumbersome matter; miRNAs are quite small in size and, unlike mRNAs, they do not share any common sequence features that ease their simultaneous isolation [26]. Tissue and cell miRNA profiling or disease biomarker discovery can be performed with the use of high-throughput methods such as commercially available microarray platforms or next-generation sequencing (NGS) of isolated miRNA fractions [27, 28]. Both techniques are considered acceptable, albeit approaching miRNA identification in a different manner. Microarrays identify fluorescently labelled miRNA cDNAs as they hybridize in complementary glass-immobilized probes, while NGS detects miRNAs by sequencing them; hence through NGS previously unidentified or novel miRNAs can be identified, while miRNA chip microarrays work with a predetermined range of miRNAs depending on the chip of choice. Differential expression of miRNAs on the other hand can be efficiently performed by both techniques, while small-scale study of specific miRNAs can be also performed using qRT-PCR [29]. Results are quite simple to read after they are further validated with qRT-PCR: certain miRNAs are expected to be either up- or down-regulated in a pathogenic tissue or cell type compared to controls, thus giving a sense of a pattern to characterize a disease.

Nevertheless, the elucidation of the exact biological meaning of any findings can at times be problematical and unfortunately in studies available, further functional investigation of findings is rarely performed.

Furthermore, microRNA targets can be also detected using high-throughput methods. Integration of high-throughput sequencing methods and protein immunoprecipitation, led to HITS-CLIP (High-throughput sequencing of RNA isolated with crosslinking immunoprecipitation) a robust method established by Chi et al. [10], which is exploited for the simultaneous isolation of miRNA–mRNA couples.

Antibodies raised against AGO are used for the immunoprecipitation of RNA-binding protein complexes from 254 nm UV-crosslinked samples, and at the same time the mRNAs bound on the miRNAs which these complexes accompany. An improved version of HITS-CLIP was developed by Hafner et al. [30] in an attempt to overcome technical limitations emerging from inefficient UV-crosslinking, named as Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP). In PAR-CLIP, cells are treated prior to crosslinking with 4-thiuridine, which is incorporated into targeted mRNAs to facilitate the precise binding position of the riboprotein complex by detecting thymidine to cytidine transitions. By PAR-CLIP, crosslinking efficiency was severely enhanced by irradiating cells with UV light and RNA recovery was dramatically improved. Moreover, Cross-linking ligation and sequencing of hybrids, or CLASH is a recently developed technique used to map miRNA–mRNA interactions by NGS [31]. In CLASH, cells that keep a stable expression a tagged AGO1 protein (PTG-AGO) are UV irradiated and lysed. PTH-AGO is then purified and samples are treated with RNases that trim RNA–RNA duplexes, which are in turn ligated together and form chimeric miRNA–mRNA molecules that are eventually sequenced with NGS.

## MicroRNAs Triggering Diseases

### *miRNA-Related Mutations as the Primary Cause of a Disease*

A *miRSNP* (Fig. 2.1) can either be a single nucleotide change affecting the target region of a miRNA or its sequence at maturity. Such SNPs can effectively eliminate or weaken the binding of a miRNA to its target mRNA 3' UTR (Fig. 2.1b) site and/or create a new binding site for a different miRNA (Fig. 2.1c); thus miRNAs can be considered as both primary or secondary players in disease development. In both cases, the protein levels of a targeted mRNA can be altered; at times, such changes can be phenotypically evident. Mutations in genes coding for miRNAs are considered as being quite rare; up to date only a small number of publications report such mutations that run in families in a Mendelian manner. Following the one-to-many mode of action, miRNAs with mutated seed regions lose the ability to target the range of mRNAs they usually aim at but inevitably gain novel targets. In some cases, mutations in the seed or other vital miRNA gene regions affect the abundance of the mature miRNA.

The frequency of SNPs in miRNA genes was thoroughly investigated by Gong et al. in 2011, who gathered all known SNPs from dbSNP v.132 that fell into such sequences. Notably, only 757 polymorphisms (SNPs and indels) out of 30 million in total were found to be located in 440 pre-miRNA regions, with 50 of them to be positioned in the seed regions of 41 miRNAs [32]. Such small numbers imply that there is a great possibility of a seed region sequence change to be a disease causing mutation rather than have no effect. Moreover, Gong et al. report that evolutionary conserved miRNAs and clustered miRNA genes tend to have less SNPs, a fact that can be attributed to the functional importance of certain miRNAs. The potential role for each SNP was calculated and recorded in the miRNASNP database ([www.bioguo.org/miRNASNP](http://www.bioguo.org/miRNASNP)).

The first work reporting seed region mutations was by Mencia et al., who identified two variants in miR-96, at positions 4 (+13G>A) and 5 (+14C>A) of its seed region, in a Spanish family with autosomal dominant non-syndromic hearing loss (ADNSHL) [33]. These mutations are responsible for the defected action of miR-96, as it fails to target and regulate five genes expressed in the inner ear; *AQP5*, *CELSR2*, *MYRIP*, *ODF2* and *RYK*. MiR-96 is expressed together with miR-182 and miR-183 as a multicistronic transcript in the mouse retina as well as in the inner ear. However, miR-96 seed region mutation carriers did not have an ocular phenotype, hence miR-96 is thought to target genes expressed in the ear rather than in the retina. In a different study, 882 ADNSHL patients from Italy were screened for miR-96 seed region mutations [34]. Interestingly, a novel mutation that segregated with the disease in one family was successfully identified, but was located outside the mature miRNA sequence. Being part of the pre-miRNA hairpin sequence, this mutation was found to effectively alter both mature miR-96 and miR-96\* passenger miRNA biogenesis. Furthermore, Dorn et al. in 2012 described a similar mutation at the 3' end (u17c) of miR-499 sequence that fell outside its seed region [35]. This mutation was identified while investigating a cohort of 2,606 individuals in search of genetic factors contributing in cardiomyopathy. By using luciferase reporter constructs and a mouse model, the authors identified a series of mRNAs that escaped miR-499 regulation possibly due to the c17 mutation.

Mutations in the miR-184 seed region have also been reported. MiR-184 was found to be abundantly expressed in the corneal and lens epithelia [36]. A single mutation at the fourth seed region nucleotide (c.57 C>U) of miR-184 was found to be associated with autosomal dominant familial keratoconus with early-onset anterior polar cataract in a Northern Irish family [37]. This mutation was identified after deep sequencing of a genomic locus indicated by linkage analysis in three generations of the family. The same mutation was also identified in a Spanish family with early onset cataract paired with various ocular abnormalities, while it has also been found in patients with EDICT syndrome (endothelial dystrophy, iris hypoplasia, congenital cataract and stromal thinning) [38, 39]. A different study identified two other mutations (+8C>A and +3A>G) in miR-184 in patients with isolated keratoconus that significantly repressed the expression of miR-184 [40]. Nevertheless, the exact mechanism explaining the pathogenesis of miR-184 seed region mutations remains elusive.

## ***Mutations in miRNA Genes or Target Sites Contributing to Disease***

Currently, there has been great interest in the discovery and functional characterization of miRSNPs located both on miRNA genes and miRNA target sites, as being contributors to a pathological phenotype. Such polymorphisms can act as phenotype modifiers by improving or exacerbating disease manifestation, or contributing to the risk of developing primary or secondary clinical states. Validated and predicted miRSNPs in human and mouse genes are recorded into four databases, Patrocles, dbSMR, PolymiRTS and MiRSNP [41–44]. Published data implicate miRSNPs in diseases in more ways than one; they can have a significant contribution to the pathogenesis of a disease, they can modify well characterized phenotypes of patients bearing a single mutation in a specific gene, they can regulate drug responses, or they can have no effect at all. Whatever the case may be, miRSNPs cannot be overlooked by modern geneticists. The role of miRSNPs will be analyzed below using some good examples from the current bibliography.

In diseases with monogenic inheritance, miRSNPs can have a significant effect. Evidence for such mechanism was shown for miR-24 when a point mutation that altered its binding to *SLITRK1* gene was identified in patients with Tourette syndrome [45]. Similarly, point mutations on *REEPI* which is a candidate gene for hereditary spastic paraplegia were found on the binding sites of two miRNAs (miR-140 and miR-691) [46, 47].

The role of miRSNPs as phenotype modifiers was demonstrated in CFHR5 nephropathy, where all patients found as far share an identical duplication of exons 2 and 3 in the *CFHR5* gene [48]. Although related, certain patients are clinically distinguishable with a portion of them rapidly progressing to mid-life end-stage renal failure requiring renal transplantation, and the rest having only some episodes of microscopic or macroscopic hematuria but with uncompromised renal function [49]. During the progression of the disease a genetic trigger channels the clinical fate of each patient towards a certain direction. The SNP rs13385 located on the 3' UTR of the *HBEGF* gene and the target region of miR-1207-5p was reported to be associated with the severity of CFHR5 nephropathy in these patients, as the T-allele can eliminate the miRNA binding onto its target sequence [50].

Complex genetic traits are often the result of a joint action among a number of genetic and environmental factors that construct phenotypes, which are usually highly variable among patients. Impressively, a number of examples are available demonstrating the implication of the same common miRSNP in a number of different multifactorial phenotypes. For example, a miRSNP spotted on the miR-146a precursor (rs2910164 G>C) has been recently associated with susceptibility to leprosy [51]. When macrophage-like THP-1 cells were infected with live or irradiated strains of *Mycobacterium leprae*, live bacteria induced the expression of miR-146a, thus suggesting a pivotal role for this miRNA in disease progression. Consequently, C-allele carriers demonstrated a higher expression of miR-146a in nerves compared to patients with non-leprosy neuropathies and this result was



directly correlated with low levels of TNF recorded as a failure of the immune system to be effectively regulated. Impressively, the same SNP was also found to be associated with other multifactorial phenotypes, such as ischemic stroke, colorectal cancer survival [52], control of cell apoptosis, migration and growth in non-small cell lung cancer cells [53], or Hirschprung disease by eliminating *ROBO1* expression levels [54]. A different common SNP (rs11614913-C>T) on miR-162a2, was found to be related with increased susceptibility to esophageal squamous cell carcinomas [55], to the development of cardiovascular disease in patients with type 2 diabetes [56], but not Parkinson's disease [57]. Both SNPs on miR-146a and miR-162a2 are considered to be common polymorphisms, with Minor Allele Frequencies (MAF) 0.38 and 0.39 respectively, and together with rs71428439 (A/G, MAF 0.15) on miR-149, rs3746444 (A/G, MAF 0.18) on miR-499, rs895819 (T/C, MAF 0.36) on miR-27a stem loop, rs4938723 (T/C, MAF 0.31) on the Pri-miR-34b/c promoter form a team of miRSNPs that have been weakly or strongly associated with all kinds of diseases. However, only a small number of published works extend their findings in characterizing a functional relationship between the miRSNPs and relevant target genes to bridge gaps between genotypes and clinical phenotypes.

In a large number of publications, the role of miRSNPs in cancer susceptibility has been thoroughly evaluated [58]. Unfortunately, the functional characterization of such polymorphisms, as well as their comprehensive association to specific clinical features, is not the norm in most of them. Nevertheless, miRSNPs are proven to have an emerging role in cancer prognosis. A polymorphism on the 3' UTR of *IGF-1R* gene limited the binding of miR-515-5p and increased the risk of developing breast cancer in subjects with *BRCA1* mutation [59]. In addition, increased susceptibility to breast cancer was attributed to two more SNPs identified on *TGFBI* and *XRCC1* which alter their expression levels as the target site of miR-187 and miR-183 is respectively interrupted [60]. In a Chinese lung cancer cohort, a SNP on the 3' UTR of *CD133* was found to be significantly associated with a decreased risk in developing the disease, evidently by enhancing the binding of miR-135a/b to reduce CD133 levels [61].

### ***Non-canonical miRNA Targeting Properties in Disease***

As previously mentioned, miRNAs recognize and bind target sequences on the 3' UTR of mRNAs waiting to be translated. In some rare cases, miRNAs were found to target mRNAs in other regions as well: the coding sequence and the 5' UTR. Non-canonical miRNA targeting has been established by CLASH experiments, with 60 % of miRNAs to bind onto mRNAs with an irregular manner; mismatched nucleotides in the seed region, non-seed targeting and some of them binding to 5' UTRs [31]. In addition, miRNAs were found to have similar efficiency in binding onto 3' UTRs as they have when targeting 5' UTRs in vitro to regulate the expression of mRNAs [62]. In some instances, miRNAs acting on the 5' UTR of target

mRNAs induce rather than repress their translation [63, 64]. Contrastingly, coding sequence targets are thought to be recognized by miRNAs less effectively compared to 3' UTR target sites [65].

Akhtar et al. [66] presented evidence using luciferase reporter constructs that miR-602 and miR-608 target the sonic hedgehog (SHH) mRNA not on its 3' UTR but on predicted target sites of the coding region. In osteoarthritis, *SHH* expression levels are elevated and eventually promote cartilage degradation. A potential mechanism explaining SHH upregulation involves the induction of SHH expression indirectly by IL-1 $\beta$ , through the direct suppression on the expression of miR-602 and miR-608 that repress SHH expression levels. In another study, the p53 inactivator MDM4 was found to have a miR-34a target site on its last 11th exon [67]. Moreover, a previously reported polymorphism rs79824231 located on this target site appeared to have the ability to disrupt miR-34a binding.

Functional miRNA target sites on the 5' UTR are rarely found. Recently, miR-103a-3p was found to target and suppress the cancer related gene GPRC5A through two target sites on the gene's 5' UTR in pancreatic cells [68]. Kim et al. [69] demonstrated a relationship between miR-34 family members, induced by p53, and the *Axin2* mRNA in colorectal cancer. *Axin2* bears functional miR-34 target sites in both 5' and 3' UTRs that presumably act as "sponges" to negatively modulate miR-34 levels in cancer cells, which present elevated *Axin2* and low miR-34 levels. Furthermore, a good example of non-canonical 5' UTR miRNA binding is the ability of miR-122, a liver specific miRNA, to bind onto two 5' UTR target sites of the Hepatitis C virus (HCV) genome and protects the UTR from host nucleolytic degradation to eventually promote its autonomous replication [70].

### ***Emerging Pharmacogenomics Due to miRNA-Linked Genetic Variation***

As expected, genomic variation related to miRNAs has also been implicated in patient response to administrated pharmaceutical therapy. In addition, certain drugs were found to formulate unique responses by interfering with miRNA expression levels. Such microRNAs have been described by a number of researchers and are frequently teamed under the term "Pharmaco-miRs". One can assume that mutated miRNA target sites on mRNAs engaged in pathways related to drug metabolism or absorption, can inevitably implicate miRNAs in drug responses as well [71]. In 2007 Mishra et al. presented evidence that miRSNPs can actually regulate drug responses. A polymorphism at the 3' UTR of the dihydrofolate reductase (*DHFR*) gene, which was previously associated with upregulation of *DFHR* expression, was found to interrupt the conserved target site of miR-24 and caused the reported overexpression of *DHFR* [72]. As a result, elevated *DHFR* led to methotrexate resistance, a widely used chemotherapeutic agent. Polymorphisms in miRNA 3' UTR target sites and/or miRNA genes have been associated with resistance to chemotherapy in patients suffering from various subtypes of cancer. The CREAM (Chemotherapy ResistancE-Associated MiRSNP) repository lists 150 such SNPs

predicted to interfere with 1164 chemotherapy response compounds [73]. In breast cancer patients with estrogen receptor alpha expression, the acquired resistance to tamoxifen treatment has been associated with an elevated expression of miR-519a, which in turn targets a number of tumor-suppressor mRNAs to decrease the life expectancy of patients [74].

## MicroRNAs in Developing Disease

Over the last few years many studies were designed to capture the effects of miRNA action in complex disease entities. Multifactorial diseases not only depend on the genetic load of an individual but are assisted towards overcoming a triggering threshold by lifestyle as well. MicroRNAs are considered as key regulators in disease development as they are implicated in all kinds of cellular processes and most importantly cell and tissue development and differentiation. Hence, the elucidation of miRNA signatures in such diseases, is postulated to unveil basic and advanced understanding of their pathology and progression and at the same time assist the development of tailored therapies per different patient. For this purpose, DICER knockout animal models, in vitro studies and expression assays have been recruited, as well as next generation high-throughput technologies.

MicroRNA signature of complex disease is a trending topic in scientific literature. Biomarkers are molecular signatures of a pathological state and can either be substances or molecules, which can be detected and measured in an objective way. MiRNAs have the specifications of being ideal and powerful biomarkers for non-invasive assays, as they can be measured in more than one ways, are abundantly expressed in all tissues and bodily fluids, are stable molecules and belong to a diverse and multitudinous family of non-coding RNAs.

### *miRNAs and Cancer*

MicroRNA implication in cancer has been extensively studied in the past years and they have been found to be differentially expressed in a wide spectrum of malignant states. Being characterized as being both tumorigenic or tumor suppressive, miRNAs associated with cancer have been named as *Oncomirs* [75]; however, this characterization is only valid when a given miRNA is targeting an oncogene or a tumor suppressor gene. Deregulated miRNA expression under variable circumstances can alter their targeting potential against an mRNA, which in turn is associated with a specific type of cancer. In general, induction and progression of cancer is the orchestrated interaction and balance between tumor enhancers and suppressors and miRNAs seem to play a pivotal role in cancer development as more than half of miRNA encoding genes are found in genomic cancer hot-spots or at fragile chromosomal regions associated with translocations [76]. Tumor progression is marked by abnormal changes in cellular function and metabolism that lead to uncontrollable

proliferation, resistance to apoptosis, escape from tumor suppressor action, induction of angiogenesis and eventually, invasion and metastasis; inevitably, miRNAs were found to be involved in all stages [77].

The first study implicating miRNAs in cancer by Calin et al. in 2002 demonstrated the involvement of miR-15a and miR-16-1 in B cell chronic lymphocytic leukemia. Their expression was found to be compromised by frequently observed deletions of the genes that encode for them in the 13q14 locus, which in turn is associated with the disease [78]. The miR-15a/miR-16 cluster of miRNA genes, encodes for miRNAs that are thought to be tumor suppressors under physiological states, by targeting *BCL2* among other well described oncogenes [79]. The complexity of miRNA involvement in cancerous states has been widely observed. In some cases a specific miRNA has been given the properties of an oncomiR, while the same miRNA was found to act suppressively in other types of cancer. A good example is miR-125b; its levels drop in thyroid, ovarian and oral squamous cell carcinomas to halt cell proliferation and interfere with the progression of the cell-cycle, while in prostate cancer it was found to inhibit p53-dependent apoptosis of cancerous cells [80]. The p53 transcription factor is a direct regulator of miR-34a and miR-34b/c, while these miRNAs target and regulate p53 expression [81]. In chronic lymphocytic leukemia, miR-34a, miR-34b/c and *DAPK1* were found to be epigenetically inactivated by hypermethylation of their promoter to disrupt the tumor suppressive p53 pathway [82]. Hypermethylation of miR-34b/c is also considered as a potential diagnostic factor in Stage I non-small cell lung carcinoma [83]. In breast tumors, downregulation of miR-34a was significantly associated with metastasis [84].

In certain types of cancer miRNAs have a proven diagnostic and prognostic value, which has become of great significance in clinical practice. MicroRNA expression is evidently fluctuating in cancerous cells; affected tissues are distinguished by their expression potential of miRNAs. MiRNAs can be easily and efficiently isolated from formalin-fixed paraffin-embedded tissues, which is the starting material in most cases. Identification of miRNAs in biofluids, such as blood serum, saliva or urine, is also supporting the need of establishing non-invasive diagnostic and prognostic tests, as well as tumor classification tests.

The prognostic value of miRNAs has been investigated in many types of cancer, such as lung cancer, liver cancer, melanoma or prostate cancer. Inevitably, a number of specific miRNAs are recurrently found to be elevated or diminished in tissues or cell types studied. This fact can be explained by the potential role such miRNAs have in cancer development and their role as biomarkers cannot be overlooked. For example, in prostate cancer, miR-141 was found to be considerably elevated in the serum of patients with prostate cancer compared to controls and is considered as a prognostic marker with a 100 % specificity [85]. The same miRNA was also found to be increased in patients with ovarian cancer, while in metastatic colon cancer it was correlated with the levels of the carcinoembryogenic antigen (CEA) and poor prognosis [86, 87].

In a cohort of colon carcinoma patients, miR-21 was found to have a higher expression in adenomas as well as in patients with advance malignancy classification stage tumors. Survival of patients in the same study was also correlated with high miR-21 expression, as well as their response to therapy [88]. MiR-21 has also

been found to be overly expressed in tumorous tissue from both breast and lungs [89, 90]. It is considered as a very important oncomiR and apoptosis suppressor and the therapeutic value of its inhibition was examined in breast cancer cells and mice with positive results [91]. In non-small cell lung carcinoma let-7 is not only considered as a primal tumor suppressor but also as a putative therapeutic agent. Poor prognosis of lung cancer in patients was directly correlated with compromised let-7 expression [92].

### *miRNAs and Diabetes*

Diabetes mellitus (DM) is a complex disease affecting 347 million people of all ages worldwide. It is mainly characterized by elevated blood glucose levels and can be found in two forms depending on insulin availability or usage; pancreatic  $\beta$ -cells fail to produce insulin in Type 1 (T1D) patients, while Type 2 (T2D) patients are insulin resistant [93]. The role of miRNAs in diabetes starts with the control over pancreatic islet  $\beta$ -cell proliferation and function. In islet-specific Dicer1 mice knockouts,  $\beta$ -cells were completely absent and animals die soon after birth at P3 [94]. Beta-cell Dicer1 knockdown mice presented with a dramatic reduction in insulin production rates in isolated  $\beta$ -cells compared to wild-type animals [95]. Differentiation of insulin producing cells is a process mediated by a cross-talk between Neurogenin3 produced by endocrine progenitor cells and Hes1. Pancreatectomized mice presented a failure in  $\beta$ -cell regeneration from pancreatic pro-endocrine cells and defected post-transcriptional regulation of Neurogenin3 protein expression by miRNAs was found to be the cause; miR-15a, miR-15b, miR-16 and miR-195 were found to be highly expressed in treated mice and have predicted target sites on the Neurogenin3 transcript [96]. Beta-cell development and function is thought to be guarded specifically by miR-375. In vitro studies demonstrated an increase of insulin secretion after glucose stimulation in cells lacking miR-375, while miR-375 knockout mice presented with fasting hyperglycemia at the 12th week of life and  $\beta$ -cell mass was reduced responding to limited levels of proliferation [97, 98]. Using luciferase reporter constructs, miR-375 was found to have a conserved functional target site on the 3' UTR of 3'-phosphoinositide-dependent protein kinase-1 (*PKD1*), an actively involved protein in insulin signaling and  $\beta$ -cell response in insulin demand through the phosphatidylinositol 3 kinase (PI3-K) pathway [99]. Islet  $\beta$ -cell function is also regulated by miR-7a, which is regarded as a negative regulator of insulin granule exocytosis. In  $\beta$ -cell miR-7a2 knockout mice, insulin secretion was increased in response to high glucose levels, suggesting a higher tolerance to glucose [100].

Pancreatic  $\beta$ -cells sense glucose levels and respond by releasing insulin. Prolonged exposure of the pancreatic  $\beta$ -cell line MIN6 to high glucose levels initially induced miR-15a levels and eventually reduced them, in accordance with insulin production levels [101]. MiR-15a regulates insulin synthesis in an indirect manner, by targeting the mRNA of the uncoupling protein-2 gene (*UCP-2*), which codes for an important protein that monitors ATP generation triggered by glucose.

Insulin protein stability requires the polypyrimidine tract binding protein (PTB), which is in turn targeted by miR-133a. In glucose-treated human islets, miR-133 was found to be elevated and PTB biosynthesis was effectively reduced accompanied by a reduction in insulin synthesis as well [102]. In addition, insulin release is also regulated by miRNAs, with miR-124a targeting directly the exocytosis regulator Ras-related protein Rab27A, miR-96 and miR-9 increasing the expression levels of the Rab GTPase effector granuphilin, and miR-34a targeting the vesicle-associated membrane protein 2 (VAMP2) [103–105].

Tissue resistance to insulin leads to T2D and a number of studies demonstrate the involvement of miRNAs in this poorly understood process. A number of signaling pathways were found to be regulated by specific miRNAs in the liver, the adipose tissue and skeletal muscle, which uptake blood glucose in response to stimulation by endogenous or administrated insulin. Adipocyte development is facilitated by miR-143. Inhibition of miR-143 in pre-adipocytes reduced triglyceride accumulation by 75 %, while halted the expression of important genes such as *GLUT4*, *aP2*, *HSL* and *PPAR- $\gamma$ 2* [106]. The influence of miR-143 on adipogenesis was recently found to have a stage-specific role during their development, possibly by the direct regulation of MAP2K5 and consequently the MAPK signaling pathway [107]. In addition, insulin resistance in adipocytes is thought to be regulated by miR-320 via the direct regulation of the p85 PI3-K subunit, which in turn modulates the phosphorylation levels of Akt and Glut4 to assist downstream signaling pathways [108]. In obesity models, the pattern of miRNA expression in developing adipocytes appears to be paradoxically inversed [109].

The use of miRNAs as biomarkers in DM is also currently examined. In T1D non-obese mice, elevated miR-375 levels preceded the onset of diabetes by 2 weeks suggesting the use of this miRNA as a valid biomarker to indicate  $\beta$ -cell death and initiation of diabetes [110]. In T2D, a comprehensive evaluation of miRNAs in the plasma of patients, revealed the downregulation of miR-21, miR-24, miR-15a, miR-125, miR-191, miR-197, miR-223, miR-320 and miR-486 and the upregulation of miR-28-3p compared to healthy controls [111]. MiR-21 in particular, was found to be significantly upregulated in diabetic mice and correlated with the development of microalbuminuria and renal fibrosis and inflammation; soon after knocking down miR-21 in the same animals, renal symptoms ameliorated thus suggesting a potential role for miR-21 as a therapeutic agent for diabetic nephropathy [112]. Ethnic origin of T2D patients also appeared to play a role in circulating miRNA signatures. For instance, miR-144 was found to be significantly associated with T2D in Swedish patients, but not Iraqis, while miR-24 and miR-29b appeared to be consistently marking the disease in both populations [113].

### ***miRNAs and Neurodegeneration***

In neurodegenerative disorders, the physiological function or structure of neurons is gradually compromised leading to degeneration and inevitably cell death. Patients in most cases present with motor and/or cognitive decline depending on

the impairment of specific brain regions, while clinicopathological symptoms occasionally overlap among different disease entities. Defected development of the central nervous system has been a common finding in Dicer knock-out animals, suggesting a pivotal role for miRNAs in neuronal differentiation and brain cortex size, while neurogenesis was found to be induced by a number of miRNAs, such as miR-9, miR-24, miR-125b and miR-128 [114, 115].

In sporadic Parkinson's disease (PD), mutations in *LRRK2* are considered as causative factors. *LRRK2* protein levels are thought to be effectively regulated by miR-205 through a conserved binding site, while patients with sporadic PD demonstrated significantly reduced levels of miR-205, resulting in elevated *LRRK2* levels and neurite outgrowth [116]. The role of *LRRK2* in disease pathogenesis is still unclear, although its function as a kinase and GTPase implicates this protein in a number of molecular pathways possibly implicated with PD [117]. Nevertheless, in a drosophila model mutated *LRRK2* was found to interact with microRNA biogenesis through an RNA-independent association with the RISC complex [118]. Additionally, miR-133b was found to be reduced in the substantia nigra of PD patients and regulates the maturation and function of midbrain dopaminergic neurons possibly via *Pitx3*, which in turn modulates the expression level of miR-133b in a proposed negative feedback loop [119]. More genes involved in PD are also targeted by miRNAs, such as  $\alpha$ -synuclein which was found to be regulated post-transcriptionally by miR-7 and miR-153 [120].

In developing Alzheimer's Disease (AD), specific miRNAs were found to regulate relevant genes. The  $\beta$ -amyloid precursor protein (APP) was found to be targeted by miR-106a and miR-502c [121], the tau protein is bound and regulated by miR-34a [122], and miR-98 targets IGF-1 [123]. Furthermore, exons 7 and 8 of APP are abnormally spliced in post-mitotic neurons of dicer conditionally knocked-out mice and miR-124 was found to be responsible for this effect assisted by its target gene *PTBP1* [124]. It appears that the abundance of miR-124 in neurons is concomitant with the occurrence of the neuronal APP isoform which lacks exons 7 and 8; hence, its absence promotes AD through the accumulation of non-neuronal APP. Furthermore, miR-9 was also found to be decreased in response to amyloid beta ( $A\beta$ ) accumulation in primary neurons and is a direct regulator of *BACE1* expression, which in turn regulates APP cleavage [125, 126]. *BACE1* mRNA is also targeted by miR-29 and miR-107, with the latter found to be downregulated in both AD and PD patients [119, 127, 128]. Moreover, in AD neuronal aging is promoted by an increase in miR-34 levels prior to the accumulation of  $A\beta$  in mice, possibly through its target gene *Bcl-2* [129].

The role of miR-146a in neurodegeneration has been explored by a number of studies. This miRNA is upregulated in brain regions affected in AD, while it is also induced by IL-1 $\beta$ , TNF $\alpha$  and  $A\beta$ 42 peptides which in turn are pro-inflammatory cytokines triggering AD (reviewed in [130]). The same miRNA was also found to mark prion induced neurodegeneration [131]. The expression of miR-146a was found to be mainly induced by NF- $\kappa$ B in Toll/IL-1 receptors and represses the release of chemokines and IL-8 via a negative regulation of IL-1 $\beta$  as a part of a feedback loop to eventually regulate innate immune responses [132].



Identification of solid biomarkers in neurodegenerative disorders is still under great consideration by the scientific community. Examples of biomarkers in AD include miR-125b that has been proposed as a circulating biomarker with 68.3 % specificity and 80.8 % sensitivity when patients are compared to healthy controls [133]. Moreover, miR-384 which targets APP and BACE-1 was isolated from the cerebrospinal fluid of patients with mild cognitive impairment and Alzheimer's type dementia and was found to be significantly lower compared to controls [134]. In a recent meta-analysis of eight studies in search of the diagnostic validity of biomarkers in neurodegeneration, authors found contrasting results between different studies and suggest the usage of assays taking into account multiple miRNAs instead of a single species assay [135]. In other disorders such as multiple sclerosis, miR-21, miR-142-3p, miR-146a/b, miR-155 and miR-326 were found to be elevated in mononuclear blood cells and white matter brain regions in MS patients (reviewed in [136]). Also, in amyotrophic lateral sclerosis mice and patients the muscle-enriched miR-206 was found to be elevated in circulation and is proposed as a potential disease prognostic marker [137].

### ***miRNAs and Cardiovascular Disease***

The cardiovascular disease (CVD) group consists of a number of highly prevalent disorders that, together with cancer, are the leading causes of death in the western world [138]. Coronary artery disease presenting with acute myocardial infarction (MI), as well as essential hypertension, cardiac hypertrophy and atherosclerosis are the main categories of CVDs. Cardiac hypertrophy and failure are responses triggered by stress factors such as MI or hypertension, which in turn alter the hemodynamic environment and lead heart cells to undergo reprogramming in order to adapt. Consequently, heart cells regress to an expression potential that resembles a fetal type and cardiac cell specifically expressed miRNAs are thought to play a pivotal role in this procedure [139]. Dicer inactivation in cardiac cells of mice caused progressive dilated cardiomyopathy, accompanied by heart failure and eventually death after birth, while impaired expression of the dicer endonuclease was also identified in patients with dilated cardiomyopathy [140].

In the developing heart, miR-1, miR-133a/b and miR-208 are considered as basic players for regulating the expression of genes in cardiac muscles and therefore the heart's growth and function [141]. It has been demonstrated that at the onset of pressure-overload cardiac hypertrophy, miR-1 expression is repressed to cause downstream global changes in gene expression [142]. In Dahl hypertensive rats, silencing of miR-208a in the heart, prevented cardiac remodeling and myosin switching while increased rat survival [143].

MicroRNAs contribute in the development and stabilization of atherosclerotic plaques, with miR-92a to modulate plaque angiogenesis in mice [144], miR-21 to be stress-induced in the endothelium and modulates apoptosis [145], and miR-155 to regulate pro-inflammatory macrophages, act repressively on Bcl6 and enhance



plaque formation [146], among others. In addition, miR-155, miR-145 and miR-126 are thought as potential candidates for atherosclerosis treatment [147]. Other factors conferring risk in atherosclerosis development, such as the high levels of low-density lipoprotein (LDL) and low levels of the high-density lipoprotein are regulated by miRNAs, with miR-122 to be responsible for increasing LDL levels and miR-33 upregulation to be associated with low HDL levels [148, 149]. Elevated LDL due to miR-122 upregulation is used as a control feature in miR-122 administered antagomir therapy against Hepatitis C virus [148]. In an atherosclerotic mouse model, administration of miR-33 antagomirs increased HDL levels and plaque size appeared to be reduced and their stability increased [150].

In mice undergoing acute MI, members of the miR-29 family were found to be downregulated in the fibrotic heart region being adjacent to the infarct and to regulate the expression of fibrotic genes such as *COL1A1*, *COL1A2*, *COL3A1* and *FN1* [151]. Cardiac fibrosis due to miR-29 might consequently lead to hypertension and it has been shown that adjustment of aerobic training habits in rats individuals helped in increasing miR-29c levels [152]. Moreover, miR-15 family members are found to be regulated in infarcted heart regions responding to ischemia-reperfusion injury in MI mice and pig models [153]. Therapeutic administration of miR-15 antagomirs, succeeded in sequestering miR-15 tissue levels and reduced infarct size, while induced tissue remodeling.

In essential hypertension, human cytomegalovirus (HCMV)-encoded miRNA, hvmn-miR-UL112 was found to be differentially expressed between hypertensive patients and controls and it direct regulator of interferon regulatory factor 1 (IRF-1) mRNA, which in turn upregulates angiotensin II type3 receptor to deregulate blood pressure [154]. In pulmonary arterial hypertension (PAH), miR-204 was found to be significantly downregulated in vitro as a response to the aberrant expression of STAT3, which normally induces the transcription of miR-204 host gene [155]. Intratracheal delivery of miR-204 mimics in PAH affected rats presented with reduced arterial pulmonary pressure. Additionally, pulmonary vascular remodeling due to hypoxia in PAH has been associated with elevated miR-21 in mice [156].

The use of miRNAs as biomarkers in CVDs has also been extensively explored (reviewed in [157]). Myocardial damage is characterized by a release of miR-208b and miR-499 into circulation and the prognostic value of both miRNAs is under study [158]. In addition, the use of miR-133a as a biomarker for cardiomyocyte death in CVD patients has also been established [159]. Circulating levels of let-7b, miR-30a and miR-195 were identified in patients with developing MI and found to have up to 90 % sensitivity and 90 % specificity in discriminating patients from controls [160].

## ***miRNAs and Renal Disease***

Kidney development and growth is dependent on miRNAs. In mouse podocytes, the highly differentiated epithelial cells of the glomerulus, Dicer inactivation depleted foot processes and induced apoptosis, while animals developed albuminuria

followed by glomerular sclerosis, tubulo-interstitial fibrosis, an abnormal appearance of the glomerular basement membrane, mesangial expansion, acute renal disease progression and eventually died after 6–8 weeks [161–163]. Impressively, proteins involved in podocyte function, such as nephrin and podocin, were found to be significantly decreased, while the major transcription factor that drives podocyte differentiation WT1 was found to be unaffected; hence miRNAs are believed to have limited influence in triggering podocyte differentiation but are essential in preserving podocyte function [163]. Like Dicer knockouts, clinical features of end-stage renal disease (ESRD) were recorded in Droscha-ablated mouse podocytes and animals presented with collapsing glomerulopathy, thus suggesting a pivotal role for miRNAs in podocyte function [164]. Microarray experiments revealed the specific enrichment of miR-192, miR-194, miR-204, miR-215 and miR-216 in the kidney, with miR-192 having a proven role in diabetic nephropathy (DN) [165, 166].

Diabetic patients developing nephropathy present proteinuria, thickening of the glomerular basement membrane (GBM), expansion of the mesangium and accumulation of the extracellular matrix, where laminin, fibronectin and collagens type I and IV fail to preserve GBM's normal structure [167]. In diabetic mice, miR-192 was found to be elevated in their glomeruli [168]. This miRNA can regulate E-box repressors expression, which in turn regulate *Colla1* and *Colla2* gene expression through TGF- $\beta$ , leading to their accumulation. In addition, hyperglycemia seems to induce the abnormally high expression of miR-377 in mesangial cells, causing the targeted PAK1 and mnSOD mRNA downregulation and finally enhanced production of fibronectin [169]. Furthermore, in early diabetic nephropathy miRNA-21 was found to have reduced levels in db/db mice, while its over-expression paused mesangial cell proliferation and decreased the albumin excretion rate [170]. In podocytes cultured in high glucose levels, as well as in diabetic mice manifesting proteinuria, miR-195 expression was found to be elevated [171]. Furthermore, in Hypertensive Nephrosclerosis (HN), a disease where hypertension leads to arterial sclerosis which in turn causes glomerular sclerosis and hypertrophy, atrophy of the tubules and interstitial fibrosis, miR-200a and b, miR-141, miR-429, miR-205 and miR-192 were found in abundance and their levels correlated with the presence of proteinuria [172].

A frequent clinicopathological finding in glomerular disease is focal segmental glomerulosclerosis (FSGS), and urine miR-196a, miR-30a-5p and miR-490 were found to characterize patients with active FSGS compared to patients with FSGS in remission [173]. In mesangial glomerulonephritis, miR-21 and miR-124 were found to be upregulated in WKY rats [174]. Lupus nephritis (LN) manifests with mesangial glomerulonephritis, and in LN patients, miR-146a of glomerular origin was also found to be upregulated in both B6.MRLc1 mice and humans [175, 176]. In patients with IgA nephropathy, miR-200c was found to be downregulated with its levels of expression correlating with proteinuria, while miR-192, miR-141, miR-205 were upregulated, with miR-192 demonstrating an association with glomerulosclerosis and a decrease in glomerular filtration rate [177]. Furthermore, in a group of ESRD patients having received a kidney transplant, serum miR-181a, miR-483-5p and miR-557 were differentially expressed during the first 7 days following transplantation surgery, hence acting as factors predicting graft rejection [178].

In polycystic kidney disease (PKD), miRNAs were found to be important players in disease pathogenesis. Cyst development mechanisms in PKD are still poorly understood [179]. MiR-15a expression levels were found to be downregulated in both autosomal dominant and recessive PKD, which subsequently caused the upregulation of its target *Cdc25A*, a cell cycle regulator [180]. In a PKD rat model, differential expression of miRNAs demonstrated an increased expression of miR-21 and downregulation of miR-31, miR-164a and miR-125 [181]. Furthermore, the overexpression of the oncogenic miRNA cluster miR-17-92 in mice was found to be directly regulated with cyst growth, while its inactivation in a PKD mouse model delayed cyst development, as *Pkd1*, *Pkd2* and *Hnf-1b* gene transcripts have functional binding sites for both miR-17 and miR-92a [182].

## MicroRNAs as Therapeutic Agents

The use of artificial miRNA molecules as therapeutic agents is currently under progressive development. Such molecules are modified oligonucleotides that can either sequester a specific miRNA to enhance the expression of its targets (antagomirs), or boost its levels to target disease promoting mRNAs (mimics). Based on their one-to-many way of function miRNA-like molecules can be used to treat diseases having more than one pathways affected, but on the other hand limit the specificity of a putative therapeutic approach [183]. Hence, good therapeutic candidates can be miRNAs that have a well-documented way of action and are ideally tissue specific. Moreover, drug delivery can be problematic and challenging in some cases (reviewed in [184]).

As mentioned earlier, in a work by Joplin et al. [70] miR-122 was found to target two specific sites at the 5' UTR of the HCV genome to stabilize and protect it from nucleolytic degradation, thus assisting in hepatitis C propagation [185]. HCV infection is the primary cause of liver disease and affects more than 170 million people worldwide; hence the development of effectual therapeutic approaches is more than necessary and the exploitation of miR-122 properties over HCV will hopefully give rise to the first approved miRNA-related drug [186]. To develop this *miravirsin* drug, a series of anti-miR-122 oligonucleotides targeting the miRNA's 5' end sequence were tested in order to block its seed region, and finally a highly stable sequence specific 15-mer LNA-modified oligo (SPC3649) demonstrated strong inhibitory effects on miR-122 function in hepatocytes of both mice and African green monkeys, even at low concentrations [148, 187]. When this *miravirsin* was administered in HCV infected chimpanzees, they presented a significant reduction in traceable HCV, while HCV levels continued to drop even 2 weeks after the drug administration was concluded, thus rendering the *miravirsin* a more comprehensive therapeutic agent compared to other approaches [188]. Currently, this *miravirsin* is in Phase 2a clinical trials with very encouraging results. Clinical trials exhibit a dose-dependent and persistent reduction of HCV levels, while no side-effects were recorded [189]. Impressively, 14 weeks after concluding the drug administration four out of nine patients that received a high dose (7 mg/kg) of

miravirsin, the viral RNA was undetectable, depicting a lack of resistance of the virus against the drug.

Besides miR-122, a small number of other miRNAs are also thought as potential therapeutic candidates. For example, miR-92a was found to control angiogenesis and its inhibition through an antagomir in mouse models presenting with limb ischemia and myocardial infarction, accelerated the recovery of affected tissues and promoted angiogenesis as it naturally targets relevant genes [144]. Additionally, the members of miR-15 family are found to be implicated in cell survival and regulate cell cycle progression and are upregulated in infarcted regions of mice and pigs in response to injury caused by ischemia [153]. Administration of modified LNAs in animals sequestered the expression levels of miR-15 family members and increased the viability of cardiomyocytes after hypoxia, while cardiac function was enhanced.

Another important miRNA drug under development is MRX34, a double stranded RNA molecule that mimics miR-34 and is currently in Phase 1 clinical trials. As mentioned above, miR-34 family expression is promoted by p53 and is found to be downregulated in various types of cancer, as it is considered an important tumor suppressor (reviewed in [183]). The miR-34 family consists of three miRNAs that share the same seed-region, namely miR-34a, b and c, with miR-34a to be the most abundant. Tumor suppression *in vivo* was succeeded in animals with xenografts that promoted prostate cancer [190], lymphoma [191], non-small cell lung cancer [192] and others, which had miR-34 delivered intravenously or directly with intratumoral injections. Phase 1 trials of miR-34 replacement are ongoing and started with a debate on the drug delivery approach to be used. After exploring a number of available solutions, an ionizable liposome (NOV340—SMARTICLES) was selected to carry the miR-34 oligonucleotide based on its use in mouse models, the miRNA bio-distribution and the vehicle's safety [193]. When this liposome is released in biofluids with neutral pH it gains a slightly anionic character that prevents it from having non-specific interactions with negatively charged cellular membranes, while it becomes cationic in tumor regions where the environment has lower pH and becomes active. This specific vehicle is preferably delivered to the liver, thus liver cancer was proposed as the disease model in this case.

## Conclusions

Without doubt, miRNA involvement in diseases is a trending matter in the literature. From causing a disease to modifying complex genotypes, miRNAs seem to be implicated in every aspect of a cell's effort to develop, differentiate, and lead a healthy living or program its death. Although a number of different approaches both *in vitro* and *in vivo* have been developed as a response to the growing needs of the scientific community to study miRNAs, the need to discover robust prognostic and diagnostic miRNA markers is essential. In addition, miRNA bioinformatics have been greatly used as the starting point in deciphering miRNA–mRNA target pairings and have become more and more efficient through time. Hopefully the

development of novel high-throughput technique in the years to come will facilitate the comprehensive functional characterization of miRNAs in disease; the knowledge emerging from endless lists of differentially expressed miRNAs in cells or tissues is of little help in understanding their true biological meaning.

Conclusively, miRNAs have what it takes to become the next generation of therapeutic agents. Taking miRNA research from the bench to the bedside, it is indeed exciting that the first two miRNA drugs, miravirsen and miR-34 replacement, are already in clinical trials. What remains is the development of more drugs and why not, tailored therapies for patients.

## References

1. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116(2): 281–297
2. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* 136(2): 215–233
3. Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 19(1):92–105
4. Dweep H, Gretz N, Sticht C (2014) miRWalk database for miRNA-target interactions. *Methods Mol Biol* 1182:289–305
5. Kumar A, Wong AK, Tizard ML, Moore RJ, Lefevre C (2012) miRNA\_Targets: a database for miRNA target predictions in coding and non-coding regions of mRNAs. *Genomics* 100(6):352–356
6. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120(1):15–20
7. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS (2003) MicroRNA targets in *Drosophila*. *Genome Biol* 5(1):R1
8. Wang X (2008) miRDB: a microRNA target prediction and functional annotation database with a wiki interface. *RNA* 14(6):1012–1017
9. Miranda KC, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM et al (2006) A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. *Cell* 126(6):1203–1217
10. Chi SW, Zang JB, Mele A, Darnell RB (2009) Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. *Nature* 460(7254):479–486
11. Zhang B, Pan X, Cobb GP, Anderson TA (2007) microRNAs as oncogenes and tumor suppressors. *Dev Biol* 302(1):1–12
12. Vinther J, Hedegaard MM, Gardner PP, Andersen JS, Arctander P (2006) Identification of miRNA targets with stable isotope labeling by amino acids in cell culture. *Nucleic Acids Res* 34(16):e107
13. Kim YK, Yeo J, Kim B, Ha M, Kim VN (2012) Short structured RNAs with low GC content are selectively lost during extraction from a small number of cells. *Mol Cell* 46(6):893–895
14. Zeringer E, Li M, Barta T, Schageman J, Pedersen KW, Neurauter A et al (2013) Methods for the extraction and RNA profiling of exosomes. *World J Methodol* 3(1):11–18
15. Schageman J, Zeringer E, Li M, Barta T, Lea K, Gu J et al (2013) The complete exosome workflow solution: from isolation to characterization of RNA cargo. *Biomed Res Int* 2013:253957
16. Rio DC (2014) Northern blots for small RNAs and microRNAs. *Cold Spring Harb Protoc* 2014(7):793–797

17. Raymond CK, Roberts BS, Garrett-Engele P, Lim LP, Johnson JM (2005) Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. *RNA* 11(11):1737–1744
18. Ro S, Park C, Jin J, Sanders KM, Yan W (2006) A PCR-based method for detection and quantification of small RNAs. *Biochem Biophys Res Commun* 351(3):756–763
19. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT et al (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 33(20):e179
20. Li Y, Kowdley KV (2012) Method for microRNA isolation from clinical serum samples. *Anal Biochem* 431(1):69–75
21. McAlexander MA, Phillips MJ, Witwer KW (2013) Comparison of methods for miRNA extraction from plasma and quantitative recovery of RNA from cerebrospinal fluid. *Front Genet* 4:83
22. Turchinovich A, Weiz L, Langheinz A, Burwinkel B (2011) Characterization of extracellular circulating microRNA. *Nucleic Acids Res* 39(16):7223–7233
23. Nuovo GJ (2008) In situ detection of precursor and mature microRNAs in paraffin embedded, formalin fixed tissues and cell preparations. *Methods* 44(1):39–46
24. Wu M, Piccini M, Koh CY, Lam KS, Singh AK (2013) Single cell microRNA analysis using microfluidic flow cytometry. *PLoS One* 8(1):e55044
25. Porichis F, Hart MG, Griesbeck M, Everett HL, Hassan M, Baxter AE et al (2014) High-throughput detection of miRNAs and gene-specific mRNA at the single-cell level by flow cytometry. *Nat Commun* 5:5641
26. de Planell-Saguer M, Rodicio MC (2011) Analytical aspects of microRNA in diagnostics: a review. *Anal Chim Acta* 699(2):134–152
27. Yin JQ, Zhao RC, Morris KV (2008) Profiling microRNA expression with microarrays. *Trends Biotechnol* 26(2):70–76
28. Hafner M, Landgraf P, Ludwig J, Rice A, Ojo T, Lin C et al (2008) Identification of microRNAs and other small regulatory RNAs using cDNA library sequencing. *Methods* 44(1):3–12
29. Zollner H, Hahn SA, Maghnoij A (2014) Quantitative RT-PCR specific for precursor and mature miRNAs. *Methods Mol Biol* 1095:121–134
30. Hafner M, Landthaler M, Burger L, Khorshid M, Hausser J, Berninger P et al (2010) Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell* 141(1):129–141
31. Helwak A, Kudla G, Dudnakova T, Tollervey D (2013) Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. *Cell* 153(3):654–665
32. Gong J, Tong Y, Zhang HM, Wang K, Hu T, Shan G et al (2012) Genome-wide identification of SNPs in microRNA genes and the SNP effects on microRNA target binding and biogenesis. *Hum Mutat* 33(1):254–263
33. Mencia A, Modamio-Hoybjør S, Redshaw N, Morin M, Mayo-Merino F, Olavarrieta L et al (2009) Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss. *Nat Genet* 41(5):609–613
34. Solda G, Robusto M, Primignani P, Castorina P, Benzoni E, Cesarani A et al (2012) A novel mutation within the MIR96 gene causes non-syndromic inherited hearing loss in an Italian family by altering pre-miRNA processing. *Hum Mol Genet* 21(3):577–585
35. Dorn GW 2nd, Matkovich SJ, Eschenbacher WH, Zhang Y (2012) A human 3' miR-499 mutation alters cardiac mRNA targeting and function. *Circ Res* 110(7):958–967
36. Ryan DG, Oliveira-Fernandes M, Lavker RM (2006) MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity. *Mol Vis* 12:1175–1184
37. Hughes AE, Bradley DT, Campbell M, Lechner J, Dash DP, Simpson DA et al (2011) Mutation altering the miR-184 seed region causes familial keratoconus with cataract. *Am J Hum Genet* 89(5):628–633
38. Iliff BW, Riazuddin SA, Gottsch JD (2012) A single-base substitution in the seed region of miR-184 causes EDICT syndrome. *Invest Ophthalmol Vis Sci* 53(1):348–353

39. Bykhovskaya Y, Caiado Canedo AL, Wright KW, Rabinowitz YS (2013) C.57 C > T Mutation in MIR 184 is Responsible for Congenital Cataracts and Corneal Abnormalities in a Five-generation Family from Galicia, Spain. *Ophthalmic Genet* DOI:10.3109/13816810.2013.848908
40. Lechner J, Bae HA, Guduric-Fuchs J, Rice A, Govindarajan G, Siddiqui S et al (2013) Mutational analysis of MIR184 in sporadic keratoconus and myopia. *Invest Ophthalmol Vis Sci* 54(8):5266–5272
41. Georges M, Clop A, Marcq F, Takeda H, Pirottin D, Hiard S et al (2006) Polymorphic microRNA-target interactions: a novel source of phenotypic variation. *Cold Spring Harb Symp Quant Biol* 71:343–350
42. Hariharan M, Scaria V, Brahmachari SK (2009) dbSMR: a novel resource of genome-wide SNPs affecting microRNA mediated regulation. *BMC Bioinformatics* 10:108
43. Bao L, Zhou M, Wu L, Lu L, Goldowitz D, Williams RW et al (2007) PolymiRTS Database: linking polymorphisms in microRNA target sites with complex traits. *Nucleic Acids Res* 35(Database issue):D51–D54
44. Liu C, Zhang F, Li T, Lu M, Wang L, Yue W et al (2012) MirSNP, a database of polymorphisms altering miRNA target sites, identifies miRNA-related SNPs in GWAS SNPs and eQTLs. *BMC Genomics* 13:661
45. Abelson JF, Kwan KY, O’Roak BJ, Baek DY, Stillman AA, Morgan TM et al (2005) Sequence variants in SLITRK1 are associated with Tourette’s syndrome. *Science* 310(5746):317–320
46. Beetz C, Schule R, Deconinck T, Tran-Viet KN, Zhu H, Kremer BP et al (2008) REEP1 mutation spectrum and genotype/phenotype correlation in hereditary spastic paraplegia type 31. *Brain* 131(Pt 4):1078–1086
47. Zuchner S, Wang G, Tran-Viet KN, Nance MA, Gaskell PC, Vance JM et al (2006) Mutations in the novel mitochondrial protein REEP1 cause hereditary spastic paraplegia type 31. *Am J Hum Genet* 79(2):365–369
48. Gale DP, de Jorge EG, Cook HT, Martinez-Barricarte R, Hadjisavvas A, McLean AG et al (2010) Identification of a mutation in complement factor H-related protein 5 in patients of Cypriot origin with glomerulonephritis. *Lancet* 376(9743):794–801
49. Athanasiou Y, Voskarides K, Gale DP, Damianou L, Patsias C, Zavros M et al (2011) Familial C3 glomerulopathy associated with CFHR5 mutations: clinical characteristics of 91 patients in 16 pedigrees. *Clin J Am Soc Nephrol* 6(6):1436–1446
50. Papagregoriou G, Erguler K, Dweep H, Voskarides K, Koupepidou P, Athanasiou Y et al (2012) A miR-1207-5p binding site polymorphism abolishes regulation of HBEGF and is associated with disease severity in CFHR5 nephropathy. *PLoS One* 7(2):e31021
51. Cezar-de-Mello PF, Toledo-Pinto TG, Marques CS, Arnez LE, Cardoso CC, Guerreiro LT et al (2014) Pre-miR-146a (rs2910164 G>C) single nucleotide polymorphism is genetically and functionally associated with leprosy. *PLoS Negl Trop Dis* 8(9):e3099
52. Chae YS, Kim JG, Lee SJ, Kang BW, Lee YJ, Park JY et al (2013) A miR-146a polymorphism (rs2910164) predicts risk of and survival from colorectal cancer. *Anticancer Res* 33(8):3233–3239
53. Chen G, Umelo IA, Lv S, Teugels E, Fostier K, Kronenberger P et al (2013) miR-146a inhibits cell growth, cell migration and induces apoptosis in non-small cell lung cancer cells. *PLoS One* 8(3):e60317
54. Zhu H, Cai P, Zhu D, Xu C, Li H, Tang J et al (2014) A common polymorphism in pre-miR-146a underlies Hirschsprung disease risk in Han Chinese. *Exp Mol Pathol* 97(3):511–514
55. Wang N, Li Y, Zhou RM, Wang GY, Wang CM, Chen ZF et al (2014) Hsa-miR-196a2 functional SNP is associated with the risk of ESCC in individuals under 60 years old. *Biomarkers* 19(1):43–48
56. Buraczynska M, Zukowski P, Wacinski P, Ksiazek K, Zaluska W (2014) Polymorphism in microRNA-196a2 contributes to the risk of cardiovascular disease in type 2 diabetes patients. *J Diabetes Complications* 28(5):617–620
57. Haixia D, Hairong D, Weixian C, Min Y, Qiang W, Hang X (2012) Lack of association of polymorphism in miRNA-196a2 with Parkinson’s disease risk in a Chinese population. *Neurosci Lett* 514(2):194–197

58. Ryan BM, Robles AI, Harris CC (2010) Genetic variation in microRNA networks: the implications for cancer research. *Nat Rev Cancer* 10(6):389–402
59. Gilam A, Edry L, Mamluk-Morag E, Bar-Ilan D, Avivi C, Golan D et al (2013) Involvement of IGF-1R regulation by miR-515-5p modifies breast cancer risk among BRCA1 carriers. *Breast Cancer Res Treat* 138(3):753–760
60. Nicoloso MS, Sun H, Spizzo R, Kim H, Wickramasinghe P, Shimizu M et al (2010) Single-nucleotide polymorphisms inside microRNA target sites influence tumor susceptibility. *Cancer Res* 70(7):2789–2798
61. Cheng M, Yang L, Yang R, Yang X, Deng J, Yu B et al (2013) A microRNA-135a/b binding polymorphism in CD133 confers decreased risk and favorable prognosis of lung cancer in Chinese by reducing CD133 expression. *Carcinogenesis* 34(10):2292–2299
62. Lytle JR, Yario TA, Steitz JA (2007) Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc Natl Acad Sci U S A* 104(23):9667–9672
63. Tsai NP, Lin YL, Wei LN (2009) MicroRNA mir-346 targets the 5'-untranslated region of receptor-interacting protein 140 (RIP140) mRNA and up-regulates its protein expression. *Biochem J* 424(3):411–418
64. Orom UA, Nielsen FC, Lund AH (2008) MicroRNA-10a binds the 5' UTR of ribosomal protein mRNAs and enhances their translation. *Mol Cell* 30(4):460–471
65. Forman JJ, Collier HA (2010) The code within the code: microRNAs target coding regions. *Cell Cycle* 9(8):1533–1541
66. Akhtar N, Makki MS, Haqqi TM (2015) MicroRNA-602 and microRNA-608 regulate sonic hedgehog expression via target sites in the coding region in human chondrocytes. *Arthritis Rheumatol* 67(2):423–434
67. Mandke P, Wyatt N, Fraser J, Bates B, Berberich SJ, Markey MP (2012) MicroRNA-34a modulates MDM4 expression via a target site in the open reading frame. *PLoS One* 7(8):e42034
68. Zhou H, Rigoutsos I (2014) MiR-103a-3p targets the 5' UTR of GPRC5A in pancreatic cells. *RNA* 20(9):1431–1439
69. Kim NH, Cha YH, Kang SE, Lee Y, Lee I, Cha SY et al (2013) p53 regulates nuclear GSK-3 levels through miR-34-mediated Axin2 suppression in colorectal cancer cells. *Cell Cycle* 12(10):1578–1587
70. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P (2005) Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 309(5740):1577–1581
71. Mishra PJ, Banerjee D, Bertino JR (2008) MiRSNPs or MiR-polymorphisms, new players in microRNA mediated regulation of the cell: Introducing microRNA pharmacogenomics. *Cell Cycle* 7(7):853–858
72. Mishra PJ, Humeniuk R, Longo-Sorbello GS, Banerjee D, Bertino JR (2007) A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. *Proc Natl Acad Sci U S A* 104(33):13513–13518
73. Dai E, Lv Y, Meng F, Yu X, Zhang Y, Wang S et al (2014) CREAM: a database for chemotherapy resistance-associated miRSNP. *Cell Death Dis* 5:e1272
74. Ward A, Shukla K, Balwierc A, Soons Z, Konig R, Sahin O et al (2014) MicroRNA-519a is a novel oncomir conferring tamoxifen resistance by targeting a network of tumour-suppressor genes in ER+ breast cancer. *J Pathol* 233(4):368–379
75. Esquela-Kerscher A, Slack FJ (2006) Oncomirs – microRNAs with a role in cancer. *Nat Rev Cancer* 6(4):259–269
76. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S et al (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A* 101(9):2999–3004
77. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144(5):646–674
78. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E et al (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* 99(24):15524–15529



79. Aqeilan RI, Calin GA, Croce CM (2010) miR-15a and miR-16-1 in cancer: discovery, function and future perspectives. *Cell Death Differ* 17(2):215–220
80. Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin GA (2011) MicroRNAs in body fluids--the mix of hormones and biomarkers. *Nat Rev Clin Oncol* 8(8):467–477
81. Hermeking H (2010) The miR-34 family in cancer and apoptosis. *Cell Death Differ* 17(2):193–199
82. Wang LQ, Kwong YL, Wong KF, Kho CS, Jin DY, Tse E et al (2014) Epigenetic inactivation of mir-34b/c in addition to mir-34a and DAPK1 in chronic lymphocytic leukemia. *J Transl Med* 12:52
83. Wang Z, Chen Z, Gao Y, Li N, Li B, Tan F et al (2011) DNA hypermethylation of microRNA-34b/c has prognostic value for stage non-small cell lung cancer. *Cancer Biol Ther* 11(5):490–496
84. Javeri A, Ghaffarpour M, Taha MF, Houshmand M (2013) Downregulation of miR-34a in breast tumors is not associated with either p53 mutations or promoter hypermethylation while it correlates with metastasis. *Med Oncol* 30(1):413
85. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL et al (2008) Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 105(30):10513–10518
86. Taylor DD, Gercel-Taylor C (2008) MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol Oncol* 110(1):13–21
87. Cheng H, Zhang L, Cogdell DE, Zheng H, Schetter AJ, Nykter M et al (2011) Circulating plasma MiR-141 is a novel biomarker for metastatic colon cancer and predicts poor prognosis. *PLoS One* 6(3):e17745
88. Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N et al (2008) MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 299(4):425–436
89. Iorio MV, Casalini P, Tagliabue E, Menard S, Croce CM (2008) MicroRNA profiling as a tool to understand prognosis, therapy response and resistance in breast cancer. *Eur J Cancer* 44(18):2753–2759
90. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M et al (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* 9(3):189–198
91. Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY (2007) miR-21-mediated tumor growth. *Oncogene* 26(19):2799–2803
92. Markou A, Liang Y, Lianidou E (2011) Prognostic, therapeutic and diagnostic potential of microRNAs in non-small cell lung cancer. *Clin Chem Lab Med* 49(10):1591–1603
93. Chen H, Lan HY, Roukos DH, Cho WC (2014) Application of microRNAs in diabetes mellitus. *J Endocrinol* 222(1):R1–R10
94. Lynn FC, Skewes-Cox P, Kosaka Y, McManus MT, Harfe BD, German MS (2007) MicroRNA expression is required for pancreatic islet cell genesis in the mouse. *Diabetes* 56(12):2938–2945
95. Kalis M, Bolmeson C, Esguerra JL, Gupta S, Edlund A, Tormo-Badia N et al (2011) Beta-cell specific deletion of Dicer1 leads to defective insulin secretion and diabetes mellitus. *PLoS One* 6(12):e29166
96. Joglekar MV, Parekh VS, Mehta S, Bhonde RR, Hardikar AA (2007) MicroRNA profiling of developing and regenerating pancreas reveal post-transcriptional regulation of neurogenin3. *Dev Biol* 311(2):603–612
97. Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE et al (2004) A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* 432(7014):226–230
98. Poy MN, Hausser J, Trajkovski M, Braun M, Collins S, Rorsman P et al (2009) miR-375 maintains normal pancreatic alpha- and beta-cell mass. *Proc Natl Acad Sci U S A* 106(14):5813–5818

99. El Ouamari A, Baroukh N, Martens GA, Lebrun P, Pipeleers D, van Obberghen E (2008) miR-375 targets 3'-phosphoinositide-dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic beta-cells. *Diabetes* 57(10):2708–2717
100. Latreille M, Hausser J, Stutzer I, Zhang Q, Hastoy B, Gargani S et al (2014) MicroRNA-7a regulates pancreatic beta cell function. *J Clin Invest* 124(6):2722–2735
101. Sun LL, Jiang BG, Li WT, Zou JJ, Shi YQ, Liu ZM (2011) MicroRNA-15a positively regulates insulin synthesis by inhibiting uncoupling protein-2 expression. *Diabetes Res Clin Pract* 91(1):94–100
102. Fred RG, Bang-Berthelsen CH, Mandrup-Poulsen T, Grunnet LG, Welsh N (2010) High glucose suppresses human islet insulin biosynthesis by inducing miR-133a leading to decreased polypyrimidine tract binding protein-expression. *PLoS One* 5(5):e10843
103. Lovis P, Gattesco S, Regazzi R (2008) Regulation of the expression of components of the exocytotic machinery of insulin-secreting cells by microRNAs. *Biol Chem* 389(3):305–312
104. Plaisance V, Abderrahmani A, Perret-Menoud V, Jacquemin P, Lemaigre F, Regazzi R (2006) MicroRNA-9 controls the expression of Granuphilin/Slp4 and the secretory response of insulin-producing cells. *J Biol Chem* 281(37):26932–26942
105. Lovis P, Roggli E, Laybutt DR, Gattesco S, Yang JY, Widmann C et al (2008) Alterations in microRNA expression contribute to fatty acid-induced pancreatic beta-cell dysfunction. *Diabetes* 57(10):2728–2736
106. Esau C, Kang X, Peralta E, Hanson E, Marcusson EG, Ravichandran LV et al (2004) MicroRNA-143 regulates adipocyte differentiation. *J Biol Chem* 279(50):52361–52365
107. Chen L, Hou J, Ye L, Chen Y, Cui J, Tian W et al (2014) MicroRNA-143 regulates adipogenesis by modulating the MAP2K5-ERK5 signaling. *Sci Rep* 4:3819
108. Ling HY, Ou HS, Feng SD, Zhang XY, Tuo QH, Chen LX et al (2009) CHANGES IN microRNA (miR) profile and effects of miR-320 in insulin-resistant 3T3-L1 adipocytes. *Clin Exp Pharmacol Physiol* 36(9):e32–e39
109. Xie H, Lim B, Lodish HF (2009) MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. *Diabetes* 58(5):1050–1057
110. Erener S, Mojibian M, Fox JK, Denroche HC, Kieffer TJ (2013) Circulating miR-375 as a biomarker of beta-cell death and diabetes in mice. *Endocrinology* 154(2):603–608
111. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M et al (2010) Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circ Res* 107(6):810–817
112. Zhong X, Chung AC, Chen HY, Dong Y, Meng XM, Li R et al (2013) miR-21 is a key therapeutic target for renal injury in a mouse model of type 2 diabetes. *Diabetologia* 56(3):663–674
113. Wang X, Sundquist J, Zoller B, Memon AA, Palmer K, Sundquist K et al (2014) Determination of 14 circulating microRNAs in Swedes and Iraqis with and without diabetes mellitus type 2. *PLoS One* 9(1):e86792
114. Krichevsky AM, Sonntag KC, Isacson O, Kosik KS (2006) Specific microRNAs modulate embryonic stem cell-derived neurogenesis. *Stem Cells* 24(4):857–864
115. Kawase-Koga Y, Low R, Otaegi G, Pollock A, Deng H, Eisenhaber F et al (2010) RNAase-III enzyme Dicer maintains signaling pathways for differentiation and survival in mouse cortical neural stem cells. *J Cell Sci* 123(Pt 4):586–594
116. Cho HJ, Liu G, Jin SM, Parisiadou L, Xie C, Yu J et al (2013) MicroRNA-205 regulates the expression of Parkinson's disease-related leucine-rich repeat kinase 2 protein. *Hum Mol Genet* 22(3):608–620
117. Esteves AR, Swerdlow RH, Cardoso SM (2014) LRRK2, a puzzling protein: insights into Parkinson's disease pathogenesis. *Exp Neurol* 261:206–216
118. Gehrke S, Imai Y, Sokol N, Lu B (2010) Pathogenic LRRK2 negatively regulates microRNA-mediated translational repression. *Nature* 466(7306):637–641
119. Kim J, Inoue K, Ishii J, Vanti WB, Voronov SV, Murchison E et al (2007) A microRNA feedback circuit in midbrain dopamine neurons. *Science* 317(5842):1220–1224

120. Doxakis E (2010) Post-transcriptional regulation of alpha-synuclein expression by mir-7 and mir-153. *J Biol Chem* 285(17):12726–12734
121. Patel N, Hoang D, Miller N, Ansaloni S, Huang Q, Rogers JT et al (2008) MicroRNAs can regulate human APP levels. *Mol Neurodegener* 3:10
122. Dickson JR, Kruse C, Montagna DR, Finsen B, Wolfe MS (2013) Alternative polyadenylation and miR-34 family members regulate tau expression. *J Neurochem* 127(6):739–749
123. Hu YK, Wang X, Li L, Du YH, Ye HT, Li CY (2013) MicroRNA-98 induces an Alzheimer's disease-like disturbance by targeting insulin-like growth factor 1. *Neurosci Bull* 29(6):745–751
124. Smith P, Al Hashimi A, Girard J, Delay C, Hebert SS (2011) In vivo regulation of amyloid precursor protein neuronal splicing by microRNAs. *J Neurochem* 116(2):240–247
125. Schonrock N, Ke YD, Humphreys D, Staufenbiel M, Ittner LM, Preiss T et al (2010) Neuronal microRNA deregulation in response to Alzheimer's disease amyloid-beta. *PLoS One* 5(6):e11070
126. Yan R, Vassar R (2014) Targeting the beta secretase BACE1 for Alzheimer's disease therapy. *Lancet Neurol* 13(3):319–329
127. Hebert SS, Horre K, Nicolai L, Papadopoulou AS, Mandemakers W, Silahtaroglu AN et al (2008) Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. *Proc Natl Acad Sci U S A* 105(17):6415–6420
128. Wang WX, Rajeev BW, Stromberg AJ, Ren N, Tang G, Huang Q et al (2008) The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. *J Neurosci* 28(5):1213–1223
129. Wang X, Liu P, Zhu H, Xu Y, Ma C, Dai X et al (2009) miR-34a, a microRNA up-regulated in a double transgenic mouse model of Alzheimer's disease, inhibits bcl2 translation. *Brain Res Bull* 80(4–5):268–273
130. Alexandrov PN, Dua P, Lukiw WJ (2014) Up-regulation of miRNA-146a in progressive, age-related inflammatory neurodegenerative disorders of the human CNS. *Front Neurol* 5:181
131. Saba R, Gushue S, Huzarewich RL, Manguiat K, Medina S, Robertson C et al (2012) MicroRNA 146a (miR-146a) is over-expressed during prion disease and modulates the innate immune response and the microglial activation state. *PLoS One* 7(2):e30832
132. Williams AE, Perry MM, Moschos SA, Lerner-Svensson HM, Lindsay MA (2008) Role of miRNA-146a in the regulation of the innate immune response and cancer. *Biochem Soc Trans* 36(Pt 6):1211–1215
133. Tan L, Yu JT, Liu QY, Tan MS, Zhang W, Hu N et al (2014) Circulating miR-125b as a biomarker of Alzheimer's disease. *J Neurol Sci* 336(1–2):52–56
134. Liu CG, Wang JL, Li L, Wang PC (2014) MicroRNA-384 regulates both amyloid precursor protein and beta-secretase expression and is a potential biomarker for Alzheimer's disease. *Int J Mol Med* 34(1):160–166
135. Zi Y, Yin Z, Xiao W, Liu X, Gao Z, Jiao L et al (2014) Circulating microRNA as potential source for neurodegenerative diseases biomarkers. *Mol Neurobiol* DOI:10.1007/s12035-014-8944-x
136. Ma X, Zhou J, Zhong Y, Jiang L, Mu P, Li Y et al (2014) Expression, regulation and function of microRNAs in multiple sclerosis. *Int J Med Sci* 11(8):810–818
137. Toivonen JM, Manzano R, Olivan S, Zaragoza P, Garcia-Redondo A, Osta R (2014) MicroRNA-206: a potential circulating biomarker candidate for amyotrophic lateral sclerosis. *PLoS One* 9(2):e89065
138. Bronze-da-Rocha E (2014) MicroRNAs expression profiles in cardiovascular diseases. *Biomed Res Int* 2014:985408
139. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW et al (2007) MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. *Circulation* 116(3):258–267

140. Chen JF, Murchison EP, Tang R, Callis TE, Tatsuguchi M, Deng Z et al (2008) Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. *Proc Natl Acad Sci U S A* 105(6):2111–2116
141. Bostjancic E, Zidar N, Stajer D, Glavac D (2010) MicroRNAs miR-1, miR-133a, miR-133b and miR-208 are dysregulated in human myocardial infarction. *Cardiology* 115(3):163–169
142. Sayed D, Hong C, Chen IY, Lypowy J, Abdellatif M (2007) MicroRNAs play an essential role in the development of cardiac hypertrophy. *Circ Res* 100(3):416–424
143. Montgomery RL, Hullinger TG, Semus HM, Dickinson BA, Seto AG, Lynch JM et al (2011) Therapeutic inhibition of miR-208a improves cardiac function and survival during heart failure. *Circulation* 124(14):1537–1547
144. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A et al (2009) MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science* 324(5935):1710–1713
145. Weber M, Baker MB, Moore JP, Searles CD (2010) MiR-21 is induced in endothelial cells by shear stress and modulates apoptosis and eNOS activity. *Biochem Biophys Res Commun* 393(4):643–648
146. Nazari-Jahantigh M, Wei Y, Noels H, Akhtar S, Zhou Z, Koenen RR et al (2012) MicroRNA-155 promotes atherosclerosis by repressing Bcl6 in macrophages. *J Clin Invest* 122(11):4190–4202
147. Wei Y, Nazari-Jahantigh M, Neth P, Weber C, Schober A (2013) MicroRNA-126, -145, and -155: a therapeutic triad in atherosclerosis? *Arterioscler Thromb Vasc Biol* 33(3):449–454
148. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M et al (2005) Silencing of microRNAs in vivo with ‘antagomirs’. *Nature* 438(7068):685–689
149. Rayner KJ, Suarez Y, Davalos A, Parathath S, Fitzgerald ML, Tamehiro N et al (2010) MiR-33 contributes to the regulation of cholesterol homeostasis. *Science* 328(5985):1570–1573
150. Rotllan N, Ramirez CM, Aryal B, Esau CC, Fernandez-Hernando C (2013) Therapeutic silencing of microRNA-33 inhibits the progression of atherosclerosis in Ldlr<sup>-/-</sup> mice--brief report. *Arterioscler Thromb Vasc Biol* 33(8):1973–1977
151. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS et al (2008) Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci U S A* 105(35):13027–13032
152. Soci UP, Fernandes T, Hashimoto NY, Mota GF, Amadeu MA, Rosa KT et al (2011) MicroRNAs 29 are involved in the improvement of ventricular compliance promoted by aerobic exercise training in rats. *Physiol Genomics* 43(11):665–673
153. Hullinger TG, Montgomery RL, Seto AG, Dickinson BA, Semus HM, Lynch JM et al (2012) Inhibition of miR-15 protects against cardiac ischemic injury. *Circ Res* 110(1):71–81
154. Li S, Zhu J, Zhang W, Chen Y, Zhang K, Popescu LM et al (2011) Signature microRNA expression profile of essential hypertension and its novel link to human cytomegalovirus infection. *Circulation* 124(2):175–184
155. Courboulain A, Paulin R, Giguere NJ, Saksouk N, Perreault T, Meloche J et al (2011) Role for miR-204 in human pulmonary arterial hypertension. *J Exp Med* 208(3):535–548
156. Yang S, Banerjee S, Freitas A, Cui H, Xie N, Abraham E et al (2012) miR-21 regulates chronic hypoxia-induced pulmonary vascular remodeling. *Am J Physiol Lung Cell Mol Physiol* 302(6):L521–L529
157. Bostjancic E, Glavac D (2014) miRNome in myocardial infarction: future directions and perspective. *World J Cardiol* 6(9):939–958
158. Corsten MF, Dennert R, Jochems S, Kuznetsova T, Devaux Y, Hofstra L et al (2010) Circulating microRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. *Circ Cardiovasc Genet* 3(6):499–506
159. Kuwabara Y, Ono K, Horie T, Nishi H, Nagao K, Kinoshita M et al (2011) Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage. *Circ Cardiovasc Genet* 4(4):446–454
160. Long G, Wang F, Duan Q, Yang S, Chen F, Gong W et al (2012) Circulating miR-30a, miR-195 and let-7b associated with acute myocardial infarction. *PLoS One* 7(12):e50926

161. Harvey SJ, Jarad G, Cunningham J, Goldberg S, Schermer B, Harfe BD et al (2008) Podocyte-specific deletion of *dicer* alters cytoskeletal dynamics and causes glomerular disease. *J Am Soc Nephrol* 19(11):2150–2158
162. Shi S, Yu L, Chiu C, Sun Y, Chen J, Khitrov G et al (2008) Podocyte-selective deletion of *dicer* induces proteinuria and glomerulosclerosis. *J Am Soc Nephrol* 19(11):2159–2169
163. Ho J, Ng KH, Rosen S, Dostal A, Gregory RI, Kreidberg JA (2008) Podocyte-specific loss of functional microRNAs leads to rapid glomerular and tubular injury. *J Am Soc Nephrol* 19(11):2069–2075
164. Zhdanova O, Srivastava S, Di L, Li Z, Tchelebi L, Dworkin S et al (2011) The inducible deletion of *Drosha* and microRNAs in mature podocytes results in a collapsing glomerulopathy. *Kidney Int* 80(7):719–730
165. Sun Y, Koo S, White N, Peralta E, Esau C, Dean NM et al (2004) Development of a microarray to detect human and mouse microRNAs and characterization of expression in human organs. *Nucleic Acids Res* 32(22):e188
166. Tian Z, Greene AS, Pietrusz JL, Matus IR, Liang M (2008) MicroRNA-target pairs in the rat kidney identified by microRNA microarray, proteomic, and bioinformatic analysis. *Genome Res* 18(3):404–411
167. Dalla Vestra M, Arboit M, Bruseghin M, Fioretto P (2009) The kidney in type 2 diabetes: focus on renal structure. *Endocrinol Nutr* 56(Suppl 4):18–20
168. Kato M, Zhang J, Wang M, Lanting L, Yuan H, Rossi JJ et al (2007) MicroRNA-192 in diabetic kidney glomeruli and its function in TGF- $\beta$ -induced collagen expression via inhibition of E-box repressors. *Proc Natl Acad Sci U S A* 104(9):3432–3437
169. Wang Q, Wang Y, Minto AW, Wang J, Shi Q, Li X et al (2008) MicroRNA-377 is up-regulated and can lead to increased fibronectin production in diabetic nephropathy. *FASEB J* 22(12):4126–4135
170. Zhang Z, Peng H, Chen J, Chen X, Han F, Xu X et al (2009) MicroRNA-21 protects from mesangial cell proliferation induced by diabetic nephropathy in db/db mice. *FEBS Lett* 583(12):2009–2014
171. Stitt-Cavanagh E, MacLeod L, Kennedy C (2009) The podocyte in diabetic kidney disease. *ScientificWorldJournal* 9:1127–1139
172. Wang G, Kwan BC, Lai FM, Choi PC, Chow KM, Li PK et al (2010) Intrarenal expression of miRNAs in patients with hypertensive nephrosclerosis. *Am J Hypertens* 23(1):78–84
173. Zhang W, Zhang C, Chen H, Li L, Tu Y, Liu C et al (2014) Evaluation of microRNAs miR-196a, miR-30a-5P, and miR-490 as biomarkers of disease activity among patients with FSGS. *Clin J Am Soc Nephrol* 9(9):1545–1552
174. Denby L, Ramdas V, McBride MW, Wang J, Robinson H, McClure J et al (2011) miR-21 and miR-214 are consistently modulated during renal injury in rodent models. *Am J Pathol* 179(2):661–672
175. Ichii O, Otsuka S, Sasaki N, Namiki Y, Hashimoto Y, Kon Y (2012) Altered expression of microRNA miR-146a correlates with the development of chronic renal inflammation. *Kidney Int* 81(3):280–292
176. Lu J, Kwan BC, Lai FM, Tam LS, Li EK, Chow KM et al (2012) Glomerular and tubulointerstitial miR-638, miR-198 and miR-146a expression in lupus nephritis. *Nephrology (Carlton)* 17(4):346–351
177. Wang G, Kwan BC, Lai FM, Choi PC, Chow KM, Li PK et al (2010) Intrarenal expression of microRNAs in patients with IgA nephropathy. *Lab Invest* 90(1):98–103
178. Sui W, Yang M, Li F, Chen H, Chen J, Ou M et al (2014) Serum microRNAs as new diagnostic biomarkers for pre- and post-kidney transplantation. *Transplant Proc* 46(10):3358–3362
179. Deltas C, Papagregoriou G (2010) Cystic diseases of the kidney: molecular biology and genetics. *Arch Pathol Lab Med* 134(4):569–582
180. Lee SO, Masyuk T, Splinter P, Banales JM, Masyuk A, Stroope A et al (2008) MicroRNA15a modulates expression of the cell-cycle regulator *Cdc25A* and affects hepatic cystogenesis in a rat model of polycystic kidney disease. *J Clin Invest* 118(11):3714–3724

181. Pandey P, Brors B, Srivastava PK, Bott A, Boehn SN, Groene HJ et al (2008) Microarray-based approach identifies microRNAs and their target functional patterns in polycystic kidney disease. *BMC Genomics* 9:624
182. Patel V, Williams D, Hajarnis S, Hunter R, Pontoglio M, Somlo S et al (2013) miR-17~92 miRNA cluster promotes kidney cyst growth in polycystic kidney disease. *Proc Natl Acad Sci U S A* 110(26):10765–10770
183. Agostini M, Knight RA (2014) miR-34: from bench to bedside. *Oncotarget* 5(4):872–881
184. Zhao X, Pan F, Holt CM, Lewis AL, Lu JR (2009) Controlled delivery of antisense oligonucleotides: a brief review of current strategies. *Expert Opin Drug Deliv* 6(7):673–686
185. Shimakami T, Yamane D, Welsch C, Hensley L, Jangra RK, Lemon SM (2012) Base pairing between hepatitis C virus RNA and microRNA 122 3' of its seed sequence is essential for genome stabilization and production of infectious virus. *J Virol* 86(13):7372–7383
186. Lindow M, Kauppinen S (2012) Discovering the first microRNA-targeted drug. *J Cell Biol* 199(3):407–412
187. Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S et al (2008) LNA-mediated microRNA silencing in non-human primates. *Nature* 452(7189):896–899
188. Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME et al (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 327(5962):198–201
189. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K et al (2013) Treatment of HCV infection by targeting microRNA. *N Engl J Med* 368(18):1685–1694
190. Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H et al (2011) The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nat Med* 17(2):211–215
191. Craig VJ, Tzankov A, Flori M, Schmid CA, Bader AG, Muller A (2012) Systemic microRNA-34a delivery induces apoptosis and abrogates growth of diffuse large B-cell lymphoma in vivo. *Leukemia* 26(11):2421–2424
192. Wiggins JF, Ruffino L, Kelnar K, Omotola M, Patrawala L, Brown D et al (2010) Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34. *Cancer Res* 70(14):5923–5930
193. Bader AG (2012) miR-34 – a microRNA replacement therapy is headed to the clinic. *Front Genet* 3:120

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