CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF BIOCHEMICAL SCIENCES

Mgr. Barbora Hanousková

The role of microRNA in physiology and pathology

Doctoral thesis

Supervisor: doc. Ing. Petra Matoušková, Ph.D.

Hradec Králové 2021

I hereby declare that this thesis is my original work which I solely composed by myself under the supervision of doc. Ing. Petra Matoušková, Ph.D. All used literature and other sources are summarized in the list of references and properly cited. This work has not been submitted for any different or equal degree.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně pod vedením své školitelky doc. Ing. Petry Matouškové, Ph.D. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

……..……..

Mgr. Barbora Hanousková

Acknowledgements

First of all, my big thank goes to my supervisor doc. Ing. Petra Matoušková, Ph.D. for her professional guidance since my diploma thesis, help in writing publications and the dissertation itself, and especially for her friendly approach and great patience.

I would also like to thank the people at the Department of Biochemical Sciences for the friendly working environment that has always prevailed there, and for their support and willingness to help.

I am grateful to David Kunke for accepting and leading my internship at the Department of Immunology, Norwegian Center for Stem Cell Research, and for helping with in the initial adaptation in Oslo.

With deep sense of gratitude, I would like to express my thanks to Dr. Jan Brinchmann for his trust in me, valuable advice and professional leadership, and for the opportunity to extend my stay and spend an amazing almost 2 years in Norway and in his research group. During that time, I not only gained a lot of new knowledge, experiences and unforgettable moments, but I also met great friends that I will never forget. I am grateful to all of you for your support, understanding, willingness, patience and for fun every day at work and beyond.

My special thanks go to my parents for their unfailing love, support, confidence, patience, and for the chance to become who I am.

I would also like to thank my sister, my newly baked husband, and my friends for their support and distraction during this not always easy journey.

Thank you all for being here for me.

Abstract

Charles University

Faculty of Pharmacy in Hradec Králové

Department of Biochemical Sciences

Candidate: Mgr. Barbora Hanousková

Supervisor: doc. Ing. Petra Matoušková, Ph.D.

Title of Doctoral Thesis: The role of microRNA in physiology and pathology

MicroRNAs (miRNAs) are small single-stranded non-coding RNA molecules that play an important role in the regulation of gene expression. They are evolutionarily highly conserved and are present in the genome of all eukaryotic organisms, suggesting their importance in physiological processes. Due to the tissue specificity and their involvement in the pathogenesis of various diseases, miRNAs have been widely studied in connection with their potential use as specific and early biomarkers, or as a therapeutic target.

The doctoral thesis, written in the form of an annotated set of publications, dealt with the study of miRNAs in various systems *in vitro* and *in vivo*, focusing on their role in adipogenesis and their use as biomarkers of pathological conditions. The introduction summarizes the theoretical information on epigenetic regulation with a more detailed focus on miRNAs, followed by comments on the author´s individual publications and brief conclusions.

In two studies using mouse models, several miRNAs were identified to have their expression profile altered due to the pathological condition, and which would be interesting to be studied in more depth. For instance, in a study of the effect of a high-fat diet and fructose, miR-335 and miR-221 were overexpressed in obese individuals in the liver as well as in all three types of adipose tissue. In a study of cardiotoxicity induced by the

administration of drugs doxorubicin and imatinib, altered expression of some miRNAs, which are considered specific for cardiac tissue, was demonstrated in the plasma of treated mice. These miRNAs could serve as early biomarkers of heart damage at a time when the level of classically used markers (e.g. Troponin) is not yet affected. Furthermore, decreased expression of miR-205 was observed in the cardiac tissue of these mice. This miRNA may play a role in the pathogenesis of heart damage or may aim to protect the heart from drug-induced toxicity.

Next part of the thesis is focused on microRNAs that could be involved in the regulation of glutathione peroxidases (GPxs). GPxs are important antioxidant enzymes whose altered expression/activity is associated with a number of pathological conditions, including obesity. After a thorough bioinformatics analysis and the search for potential miRNAs that could regulate all GPx isoforms, the binding of miRNAs itself and the effect of certain miRNAs on the selected GPx7 enzyme were investigated. Bioinformatically predicted direct binding of miR-29b-3p and miR-137 to 3´UTR GPx7 was confirmed in various cell lines, while in the case of miR-335-5p this theory was refuted. Although the regulatory effect of miR-29b-3p and miR-137 on the GPx7 expression has been demonstrated, the importance of these miRNAs in relation to adipogenesis and obesity is not yet fully understood.

Abstrakt

Univerzita Karlova

Farmaceutická fakulta v Hradci Králové

Katedra biochemických věd

Kandidát: Mgr. Barbora Hanousková

Školitel: doc. Ing. Petra Matoušková, Ph.D.

Název disertační práce: Úloha mikroRNA ve fyziologii a patologii

MikroRNA (miRNA), malé jednořetězcové nekódující molekuly RNA, se významně podílejí na regulaci genové. Jsou evolučně vysoce konzervované a vyskytují se v genomu všech eukaryotických organismů, což naznačuje jejich důležitost ve fyziologických procesech. Vzhledem k jejich tkáňové specifitě a zapojení do patogeneze různých onemocnění jsou miRNA hojně studované v souvislosti s jejich potenciálním využitím jako specifických a časných biomarkerů, případně jako terapeutický cíl.

Disertační práce, sepsána formou komentovaného souboru publikací se zabývala studiem miRNA v různých systémech *in vitro* a *in vivo* se zaměřením na jejich roli v adipogenezi i jejich využití jako biomarkerů patologických stavů. Práce v úvodu shrnuje teoretické informace o epigenetické regulaci s detailnějším zaměřením na miRNA. Pak následují komentáře k jednotlivým publikacím autorky a stručné závěry.

Ve dvou studiích využívajících myší modely bylo odhaleno několik miRNA, jejichž expresní profil byl důsledkem patologického stavu změněn, a které by bylo zajímavé studovat hlouběji. Například ve studii zaměřené na vliv vysokotučné stravy a fruktózy byly u obézních myší zvýšeně exprimované miR-335 a miR-221 a to jak v játrech, tak i ve všech třech typech tukové tkáně. Ve studiu kardiotoxicity vyvolané podáváním léčiv doxorubicinu a imatinibu byla v plazmě léčených myší prokázána změněná exprese některých miRNA,

které jsou považované za specifické pro srdeční tkáň. Tyto miRNA by mohly sloužit jako časné markery srdečního poškození, kdy hladina klasicky používaných markerů (například troponinu), ještě není ovlivněna. V srdeční tkáni těchto myší byla dále zaznamenána snížená exprese miR-205, která by mohla hrát roli v patogenezi srdečního poškození, případně se stát cílem k ochraně srdce před toxicitou léčiv.

V další části práce jsme se věnovali mikroRNA, které by se mohly podílet na regulaci glutathionperoxidas (GPx), významných antioxidačních enzymů, jejichž změněná exprese/aktivita souvisí s řadou patologických stavů včetně obezity. Po důkladné bioinformatické analýze a hledání potenciálních miRNA, které by mohly regulovat všechny isoformy GPx, byla zkoumána i samotná vazba a vliv určitých miRNA na vybraný enzym GPx7. Bioinformaticky predikovaná přímá vazba miR-29b-3p a miR-137 na 3´UTR GPx7 byla potvrzena v různých buněčných liniích, naopak u miR-335-5p byla tato teorie vyvrácena. I když se nám podařilo prokázat, že miR-29b-3p a miR-137 regulují expresi GPx7, význam těchto miRNA v souvislosti s adipogenezí a obezitou není zcela zřejmý.

Content

1. Introduction

Since 1993, when the first miRNA was discovered in *C. elegans*, the interest in these small non-coding RNAs has grown at an exponential rate. Currently, the main topic of thousands of publications each year are just miRNAs. With the advent of new technologies and extensive data analysis, these regulatory molecules, first considered “junk” or by-products of transcription, changed the dogma of gene expression regulation, and generally revolutionized the field of molecular biology.

To date, thousands of human miRNAs have been identified, and many have been shown to be highly evolutionarily conserved across species. In addition, a large number of human miRNAs show highly conserved interactions with human mRNA, suggesting their importance in crucial developmental and biological processes. Although a miRNA usually uses a very short sequence to fulfill its regulatory function, one miRNA can target up to 100 different genes, as well as one gene can be regulated by several miRNAs.

Due to their properties, miRNAs are considered to be very sensitive, specific and early released circulating biomarkers of pathological conditions. As clearly shown, human diseases are complex and have multiple genes and pathways dysregulated. miRNA regulation is usually based on fine-tuning of biological processes and seems to be a promising target for treatment with a great potential for future medicine. Some miRNA-based drugs have already reached the stage of clinical trials, and although this field is essentially still in its infancy, there is no doubt that this direction has a huge perspective and a chance to succeed.

Since miRNAs are part of a large chapter of epigenetics, other types of epigenetic regulation are briefly mentioned in the theoretical part. In the present work, we tried to identify miRNAs as biomarkers of pathological conditions. On the other hand, we also looked at the mechanism of action of these molecules at molecular level.

2. Theoretical part

2.1. Epigenetics

Multicellular organisms are composed of genetically homogeneous cells, carrying the same DNA, and originating from one single cell, fertilized zygote, but which are structurally and functionally distinct and show a different expression of various genes. In other words, cells of a multicellular organism have the same genome with different transcriptome, proteome, and function. Activation and repression of specific sets of genes arise and are required during embryonic development and are maintained through the mitosis, heritable from cell to cell. These heritable alterations are essential for normal organism development, genomic imprinting, and genome stability [1, 2]. In summary, cells with the same genotype have hugely different phenotypes resulting also from differences in the epigenome.

The term epigenetics, as such very old, has changed its meaning dramatically over time, especially since 2000. In 1942, embryologist Conrad Waddington described the concept of epigenetics as a set of inheritable developmental changes between the genotype and phenotype, which are independent of any changes in the DNA sequence. However, the research in the field of epigenetics did not grow strongly until the 21st century [3]. In the last decades, ever-improving knowledge of epigenetics is changing our view of both the naturally occurring processes in the body and the disease pathogenesis. Epigenetics may be defined and manifest itself in many forms. According to the latest knowledge, epigenetic mechanisms can be collectively defined as mitotically and meiotically inherited modifications in phenotype, independent of any alterations of DNA sequence, which affects how the cells read the genes and which genes manifest their function [4]. These modifications are usually reversible and either allow or repress the expression of target genes. However, they are not simply on and off mechanisms. In most cases, it is rather about a fine regulation of gene expression, where these do not work independently. On the

contrary, they operate in a coordinated manner as a complex, within which one enhances the effect of the other [5]. Information transmitted by epigenetic variations plays a crucial role in the regulation of gene transcription, translation, DNA replication and reparation. Therefore, the abnormal expression and genomic alteration of these processes can give the initiation of diverse types of diseases and cancers [6]. Epigenetics can also be considered as a mechanism through which environmental factors may have medium-term effects on gene expression without any change in DNA sequence [7].

As mentioned above, epigenetic modifications are mitotically transferred from cell to cell within a tissue, and the states of gene activity are maintained for the necessary rounds of cell division [8] [9]. Epigenetic marks were thought to be removed and then newly established with the onset of a new generation. But there is evidence about the inheritance of epigenetics also among generations, supported by several studies [10-13].

It is well known that chromatin is greatly reshaped during the development of germ cells and cell differentiation after fertilization. However, some loci seem to escape epigenetic reprogramming. Long-lived RNA molecules have been shown to be less affected by these changes and therefore, these may carry the epigenetic information on to future generations [2]. However, the mechanism of transmission remains unknown, and it is not clear if some epigenetic features are shared because they are inherited, or if these are rearranged based on shared genes and/or environment. Therefore, most studies already published require independent validation and repetition in order to draw certain conclusions.

As well as genetic information in the form of DNA can be a direct target of several adverse and destructive effects, epigenetic mechanisms are also affected by the factors of environment, lifestyle, diet, stress, drugs, age, radiation, pathogens, or toxins, which may enter the cell in some form and have a direct or indirect impact [4]. From the point of view of epigenetic inheritance, here is a certain predisposition for the development of various diseases and malignancies arising from epigenetics, which can also be passed on to

offspring. The best-known epigenetic mechanisms include histone modifications, DNA methylation, and non-coding RNA.

2.2. Histone modifications

Chromatin, a complex of DNA and proteins forming chromosomes within the nucleus, is a dynamic structure that must respond to a huge number of stimuli to regulate access to DNA. Chromatin basically occurs in two different states. Heterochromatin, a highly compacted inactive form of chromatin, where DNA is inaccessible to transcriptional machinery, and euchromatin, an active state rich in genes that are meant to be transcribed.

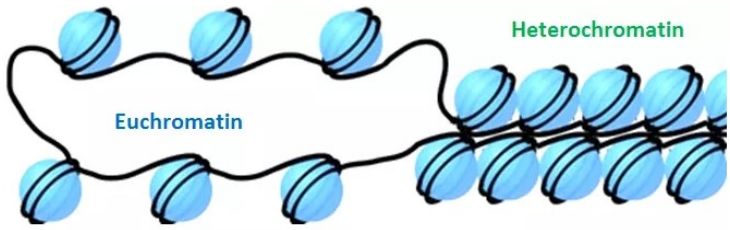

Figure 1. Active vs inactive state of chromatin structure

The nucleosome is the basic functional unit of chromatin, which is composed of about 146 base pairs long DNA wrapped nearly twice around an octamer of histones, a protein component of the nucleosome. Histone octamer consists of a central part of histones H3 and H4, with histones H2A and H2B, flanked around [14]. Nucleosomes in general prevent transcription of DNA either by physical obstruction, by bending the DNA and thus reducing its accessibility to transcription, or by carrying many post-translational modifications (PTMs).

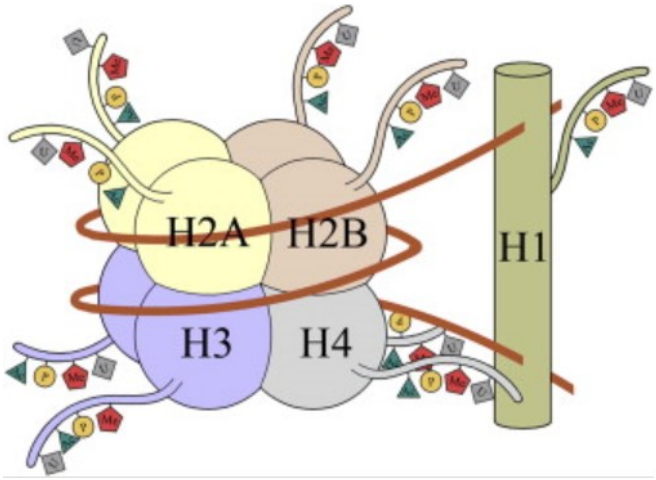

Figure 2. Illustration of nucleosome representing both linker and core histones wrapped by DNA and sites of individual modifications - Ac (acetylation); Me (methylation); P (phosphorylation); U (ubiquitination) [15]

Post-translational histone modifications are one of the major mechanisms of epigenetic regulation, influencing chromatin compaction and accessibility, influencing histone-histone and histone-DNA interactions [16]. PTMs are in principle associated with every cellular process requiring DNA transcription, replication, and repair. These can include phosphorylation, methylation, acetylation, sumoylation, ubiquitylation, ribosylation, and recently discovered glycosylation of histones at their C- and N-terminal regions. Among the most commonly occurring amino acid residues that are amenable to these modifications are lysine (K), arginine (R), serine (S), tyrosine (Y), threonine (T), histidine (H), and glutamic acid (E) [17]. Enzymes responsible for the addition of, relatively small but chemically and structurally distinct, chemical groups are usually termed “writers”, whereas enzymes involved in their removal are called “erasers”. These marks, either added or removed from specific histone residues, play a signalling role for chromatin-modifying or remodelling proteins [18]. Histone PTMs are sequestrated at distinct regions in the genome and play their role as enhancers, or they maintain a robustly repressed status. PTMs are generally implicated in transcription activation or repression, but have also an effect on DNA

replication, mitosis, meiosis, chromatin assembly, telomeric silencing, or DNA repair. PTMs are highly associated with chromosome X inactivation, genomic imprinting, or with other massively inactive areas in the genome. There is an ever-growing list of histone PTMs, their combinations and functional consequences [19]. Individual modifications, but even rather their mutual combinations, are highly conserved to ensure the proper embryonic development and cell differentiation. In addition, PTMs are also essential in response to external stimuli and DNA damage, where they can protect against DNA disruptors by stopping the cell cycle at one of the control points, while waiting for DNA repair or signal to trigger the controlled cell death (apoptosis). However, in the event of deregulation due to internal or external adverse effects, or when one amino acid is replaced with another, alter of PTMs of histones may lead to impaired genomic stability and increases the risk of developing a subset of diseases [20]. There are many different parts on histones, allowing post-translational modifications. Until recently, the most frequently studied modifications were those at the N-terminal histone tail, a moiety that is structurally readily accessible for the respective enzymes and factors [21]. However, the central globular domains of histones have been also shown to contain a large number of PTMs. Especially their lateral side surface, which is in direct contact with DNA, seems to be an attractive area of interest [22].

The most studied and probably most commonly occurring modifications include acetylation, phosphorylation, and methylation. The first described modification was histone acetylation. Acetylation of the lysine residues neutralizes their positive charge and causes a weakening of the interaction between histones or between histone and DNA, thereby increasing the structural accessibility of DNA [19]. Acetylation occurs almost exclusively at lysine residues, is recognized by chromatin-associated protein bromodomain, and is generally associated with active transcription. Acetylation-caused relaxing of histone-DNA contact has also been shown to be important for efficient DNA replication and to allow access of repair factors to double-stranded breaks of DNA [23] [24]. Phosphorylation also results in a negative charge of its modified histone residues, usually serine and threonine, weakening the bond between

nucleotides and DNA. This suggests a similar role in the regulation of nucleosome dynamics as acetylation [25]. Histone phosphorylation is involved in the regulation of various biological processes in the cell. However, instead of having a direct effect, this modification probably works rather by establishing mutual cooperation with other histone modifications, leading to a subsequent cascade of events [26]. Methylation may occur in the form of mono-, di-, or trimethylated lysines, mono- or dimethylated arginines, and rarely appearing monomethylated histidines. Compared to acetylation and phosphorylation, methylation does not cause the chain neutralization. On the contrary, methyl groups increase the positive charge and hydrophobicity of corresponding moieties [27]. Methylation is highly context dependent. It very much depends on the degree of methylation (me1, me2, or me3), the location of the methyl residue on the histone, and also on whether the corresponding marks occur together or in isolation (H3K4me3 and H3K27me3) [28]. Some modifications correspond to active transcription (H3K4me3), next associate with repressed chromatin (H3K4me2, H3K27me3), others are implicated in different cellular fates, whereas differing slightly from each other [25].

Moreover, many modifications of the same amino acid residue within the same histone domain may have a dual role and correlate with diverse consequences for the cell, depending on the type and number of moieties added. There is also some form of antagonism based on a specific preference and rate of the catalytic reaction, since some amino acid residues, such as lysine, are the target for many and various modifications. Histone PTMs are extremely abundant, and some form of dynamic and complex crosstalk has been shown among them. While some marks occur together, others are mutually exclusive, leading to either a positive or negative effect on their recognition proteins [29]. There has been a huge amount of histone PTMs, their combinations, signalling pathways, or direct effect on chromatin identified. Certainly, there will be more to discover and identify.

2.3. DNA methylation

DNA methylation is based on the enzymatic attachment of a methyl group (-CH3) to nucleotides by several types of DNA methyltransferases (DNMTs) and other cooperating enzymes. While the function of DNMT1, is to maintain pre-existing DNA methylation pattern after each cycle of DNA replication, the DNMT3a / DNMT3b complex is responsible for *de novo* DNA methylation and is essential during gametogenesis and early embryogenesis. Another type of DNMTs is type 2, which carries enzymatic modifications in DNA and RNA methylation [30] [31]. DNA demethylation is usually performed by a group of enzymes called ten-eleven translocation family (TET) enzymes, through oxidation of 5-methylcytosine [32].

DNA methylation is a covalent modification of DNA, and simultaneously, it is a powerful way of epigenetic regulation, associated with gene silencing. DNA methylation plays a crucial role in setting up the gene expression infrastructure of the whole organism. It generally represses genes that are not meant to be expressed at a given stage of development and cell type and form the expression pattern of differentiating cells during the development [33]. Another way to interpret DNA methylation is as a host defense mechanism that represses endogenous retroviral sequences and transposons. In this way, it contributes to the stability of the genome by preventing translocations and mobility of these and repetitive sequences by keeping them silent [34].

DNA methylation occurs almost exclusively at position 5 of cytosine bases preceding guanine base, in CpG dinucleotides. CpG dinucleotides occur at different densities and distribution across the genome, depending on the respective function they perform at a given site. Regions, which are rich in CpG dinucleotides are called CpG islands [31]. Abundantly and densely occurring CpG islands are mostly present in areas, that are meant to be highly repressed, and therefore they are exclusively in a methylated state. Examples are inactivated X chromosome, repetitive genomic regions, or imprinted alleles [35]. On the other hand, CpG islands are often located at the 5´end of gene at the position of their

promoters. About 75% of all promoters contain CpG islands within their sequence, which facilitates the regulation of relevant genes by DNA methylation. Approximately 94% of all CpG islands in somatic cells remain unmethylated, accessible to the transcription machinery. It has later been shown that DNA methylation occurs also intragenic or intergenic, where CpG dinucleotides appear in low density. DNA methylation within the gene body may affect alternative splicing, can stimulate transcription elongation, prevent false initiation of transcription, and indicate the transcription start site of non-coding RNAs [36].

DNA methylation plays a crucial role in controlling several cellular processes, including embryonic development. Almost all methylated regions, originating from germ cells, are erased after fertilization and in the early embryo state to induce pluripotency. Except for sex-specific methylation and CpG-rich transposons, that escape this demethylation. The methylation pattern is then re-established *de novo* throughout the genome apart from a large group of specifically recognized CpG islands in promoter sequences, these remain unmethylated. Likewise, promoters of many housekeeping-like genes remain unmethylated and open for constitutive expression in various cells at any stage of development [37].

There is also another phase in resetting the methylation pattern and de novo methylation. The changes in methylation are necessary for cell-lineage differentiation and organogenesis, and these modifications take place in a precisely programmed manner. A number of unmethylated genes occurring in the phase of implantation and early development, responsible, for instance, for cell pluripotency, need to be suppressed in the specific cell type or in the particular stage of development [38]. Similarly, some of the genes which are purposefully methylated at a certain stage of development need to be expressed afterwards. Their promoters must be demethylated to allow their expression and appropriate cell differentiation.

Methylation of CpG island at the transcription start site is usually associated with long-term silencing. Once, usually after birth, this new methylation formula is destined, is preserved

for the rest of the organism´s life. That makes the structural basis for tissue-specific gene expression and prevents possible reactivation of genes in the subsequent generation of cells [1]. Taken together, DNA methylation itself does not work only as a repressor of gene expression. Methylation does not turn genes on or off, but more likely prevents their activation and may serve as long-term memory of gene expression decisions [3]. In addition, it also cooperates with histone modification machinery.

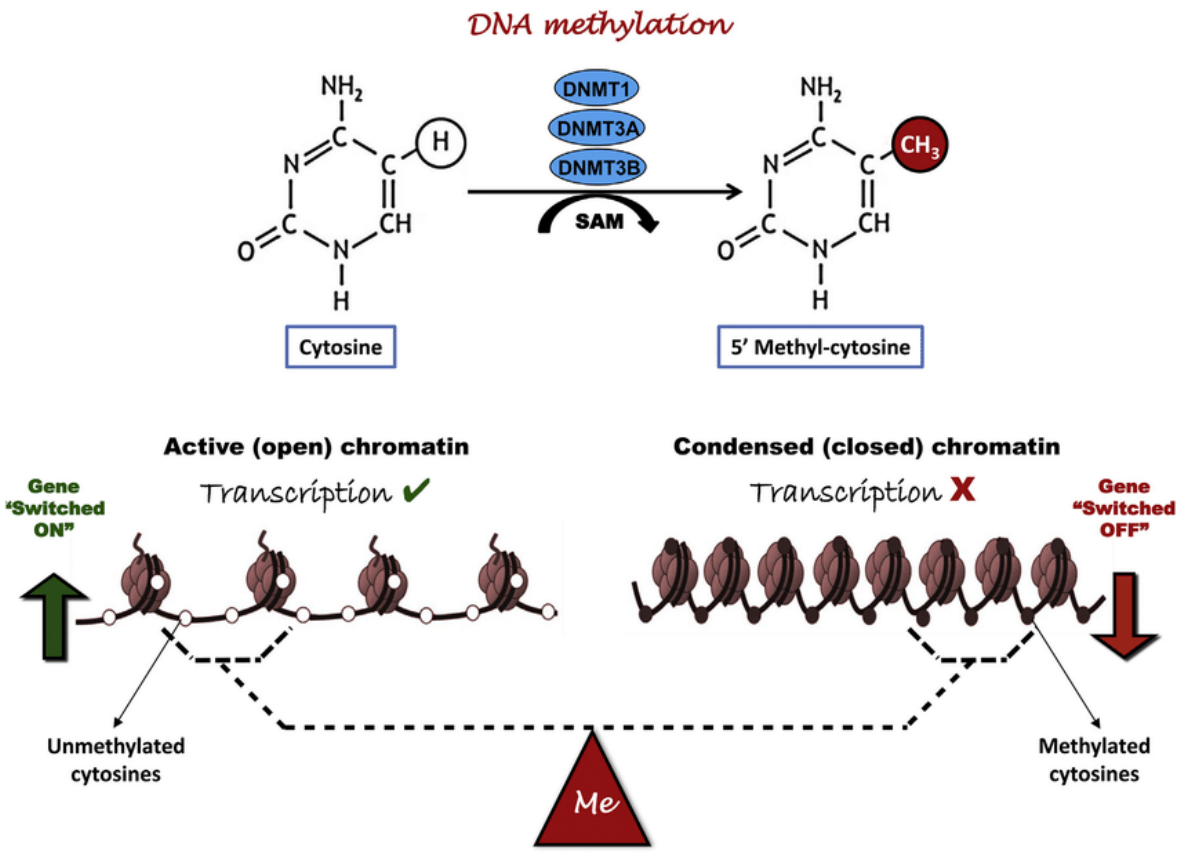

Figure 3. DNA methylation [39]

2.4. Non-coding RNA

At the beginning of the 21st century, with the advent of new technologies and extensive data analysis, it was clearly shown that although the human genome is broadly transcribed, only about 2% of these transcripts are translated into protein. The term non-coding RNA (ncRNA) is commonly used for all RNA transcripts that do not encode a protein. The main goal of RNA research has been focused on messenger RNA (mRNA), as a template for protein synthesis, on components of the ribosome in the form of ribosomal RNA (rRNA), and on translators of codon sequence in the form of transfer RNA (tRNA), for a long time. The remaining non-protein-coding transcripts were at first regarded as “junk” or by-products of transcription with less biological importance. It has later become apparent that ncRNAs are not only fully functional but are of crucial importance for normal development, physiology, and disease. As they perform their regulatory function independent of any changes in the DNA sequence, they have become a new attractive field of epigenetic regulating mechanisms. They modulate complex molecular and cellular processes, and play a key role in gene regulatory networks, where they are involved in the regulation of various genes at all different molecular levels. Therefore, their aberrant expression can be associated with many diverse pathologies [40, 41]. This fact also makes them a new class of biomarkers and targets for drug development.

ncRNAs can be transcribed from various parts of DNA, from protein-coding genes, through introns and enhancer regions, to transposon elements. In addition, many ncRNAs can subsequently be spliced and/or processed into shorter products [42].

Based on their regulatory function, ncRNA can be divided into two major groups, housekeeping and regulatory ncRNAs. Housekeeping ncRNAs, like rRNA, tRNA, snRNA, snoRNA, tiRNA, or telomerase RNA, regulate primarily generic cellular functions and are essential for cell viability. They are ubiquitously and constitutively expressed in all cell types. tRNAs and rRNAs play an essential role in protein synthesis, snRNAs, together with other

cooperating components, are involved in RNA splicing, and snoRNAs participate in RNA modification [43]. Besides these essential roles, some of the housekeeping ncRNAs may have the regulatory function as well, for example in cleavage. It has been shown that tiRNAs, a new class of small regulatory ncRNAs, derived either from tRNA or pre-tRNA, could inhibit translation under stress situations [44].

Unlike housekeeping ncRNAs, regulatory ncRNAs, such as miRNA, siRNA, piRNA, eRNA, lncRNA, or circRNA, are cell and/or tissue specific. They regulate gene expression at the different molecular levels such as transcriptional, post-transcriptional, or epigenetic level [42]. Regulatory ncRNAs can be further divided into two groups based on their length, with each group comprising several RNA subtypes that differ in their function, biogenic processes, or genomic origins. Transcripts with less than 200 nucleotides in length are classified as short ncRNA, while all larger products belong to long ncRNA (lncRNA) [45]. Here are several examples.

2.4.1. siRNA

Small interfering RNAs (siRNAs), part of short ncRNAs, are double-stranded RNA molecules with 2 nucleotide overhangs at the 3´end and about 21-24 nucleotides in length. They repress transcription mainly in mammal germ cells by targeting their RNA through the mRNA interference (RNAi) pathway based on complementary base-pairing. They are directly processed by endoribonuclease Dicer, which takes place in the cytoplasm, without the previous maturation in the nucleus. siRNAs are cleaved from long double-stranded precursor RNA, usually from transposable elements. siRNAs formed from repeat elements in the nucleus may guide chromatin modification and silencing through RNAi-induced transcriptional silencing (RITS) [46, 47].

2.4.2. piRNA

In contrast to miRNAs and siRNAs, which are cleaved by the ribonuclease Dicer from double-stranded RNA precursors into 20-24nt length species, piRNAs are processed via a

Dicer-independent way and these slightly longer products, ranging from 23 to 32 nt in length, originating from single-stranded precursors [48]. The piRNA biogenesis usually takes place in the cytoplasm, where individual piRNA molecules are cleaved from so called piRNA clusters. It is further loaded into PIWI proteins, the subfamily of AGO proteins, to form the functional piRNA-induced silencing complex. However, some piRNAs also form in the nucleus, where they scan nascent transcripts, guide transcriptional silencing and the deployment of repressive chromatin tags. Their role in antiviral defense has also been described [49]. piRNA pathway is crucial for genome defense in gonadal cells, where its main function is silencing of transposable elements expression and mobilization through transcriptional and posttranscriptional gene silencing. There are two basic mechanisms of gene repression, base-pairing recognition and heterochromatin-mediated gene silencing. So-called “ping-pong cycle” is an interesting mechanism of silencing, where piRNAs are capable to repress the transcript of their origin [50] [51].

2.4.3. lncRNA

lncRNAs are proposed to be the class of the largest transcripts and comprise more than 80% of the total ncRNAs. lncRNAs form a heterogeneous group of RNAs that differ in their structure and function and can be further divided into several subgroups based on their origin, biogenesis, and function. Unlike short ncRNAs, lncRNAs are supposedly poorly conserved. Their sequence length, usually more than 200 nucleotides, and the fact that they are not translated into the protein structure are probably the only common features of lncRNAs. In many instances, lncRNAs are processed similarly to mRNA and often transcribed from protein-coding sequences [52]. These are RNA polymerase II transcripts lacking an open reading frame.

lncRNAs may serve as a precursor for the production of shorter ncRNAs. Conversely, they can act as a “sponge” and sequester miRNAs, based on their sequence complementarity, and thus regulate the ability of miRNAs to regulate gene expression. They are involved in a range of biological functions, such as developmental processes, transcriptional and post-

transcriptional modulation of gene expression, the recruitment of chromatin-modifying enzymes, or imprinting genomic loci. Nevertheless, although the roles of some lncRNAs are well understood, the exact functional mechanism of the majority of lncRNAs remains not fully known, since many lncRNAs may not have apparent functions[53, 54].

2.4.4. microRNA

microRNAs (miRNAs) are small single-stranded non-coding RNAs of about 22 nucleotides in length, playing an essential role as epigenetic regulators. Through the RNA-induced silencing complex (RISC), they mediate post-transcriptional gene repression through a mechanism known as RNA interference (RNAi). miRNAs are among the best-known and probably the most studied class of ncRNA, and their presence has been demonstrated in almost all eukaryotes. Besides, they have been found also in some viruses, which points to the necessity and power of their existence [55]. miRNAs were first discovered in the early 1990s and the first miRNA was identified in *Caenorhabditis elegans* as a product of the lin-4 gene, repressing the lin-14 mRNA. The time of greatest expansion in their cognition came up to 10 years since their discovery. Gradually, it became apparent that their role in organism development and disease is complex and convoluted and that they are involved probably in all cellular processes [56].

miRNAs have been shown to exhibit tissue-specific patterns and their expression profile is often altered in various disorders and diseases, which is usually regulated transcriptionally. As well as they could serve as specific markers of some pathologies, they are also proving to be potential targets for novel treatment therapies and drug development. Due to these facts, they have become an attractive and increasingly studied subject of scientific research [57].

miRNAs are produced either by canonical or non-canonical biogenesis pathway and their biogenesis is a rapid process, one of the fastest among transcripts.

2.4.4.1. Canonical biogenesis pathway

The canonical pathway of miRNA biogenesis starts with the transcription of miRNA-coding loci by RNA polymerase II into 5´-capped and 3´-polyadenylated imperfect hairpin structure,

primary miRNA transcript (pri-miRNA). MiRNAs can be transcribed from the genome either as parts of individual genes (monocistronic), from clusters containing several miRNAs that are transcribed together (polycistronic), might be also transcribed as parts of host gene introns (intronic), or from other ncRNAs, such as lnc-RNAs [58]. Microprocessor-guided nuclear processing of pri-miRNA is a crucial initial step of miRNA maturation and results in the production of a single hairpin stem-loop precursor miRNA (pre-miRNA) of approximately 70nt in length. This complex comprising the RNase III type enzyme Drosha, DiGeorge syndrome critical region 8 gene (DGCR8), the double-stranded RNA-binding protein (RBP), and associated proteins [59]. Pri-miRNA hairpin needs to be recognized and distinguish from other hairpin-containing transcripts by microprocessor. In this step, additional sequence features of the hairpin or nearby are required. Among the important is the UG motif, recognized by Drosha, apical UGUG motif, required for precise and effective cleavage and bound by DGCR8, or basal CNNC motif identified by Serine/arginine-rich splicing factor 3 (SRSF3), promoting cleavage. Further stem features, such as mismatched GHG motif (H represents A, C, or U), the presence of N6-methyladenosine mark around the pri-miRNA stem-loop, or ideal stem length of 35nt, may help the processing [60]. DGCR8 acts as a cofactor and stabilizer for Drosha and interacts simultaneously with the apical motif of pri-miRNA. It performs its function as a dimer and its active conformation is stimulated by heme binding. Drosha, consisting of two catalytic RNase III domains, plays the important role of a catalytic subunit of the microprocessor complex. In cooperation with the so-called Bump helix, which is a part of the enzyme, Drosha cleaves both strands of pri-miRNA at the base of the stem. The Bump helix works here as a molecular ruler measuring the distance of 11 base pairs from the junction of basal single-stranded RNA to double-stranded RNA (ssRNA-dsRNA) and defines the Drosha cleavage site, that is recognized and bound by Dicer [61] [60]. The microprocessor-produced precursor miRNA (pre-miRNA) has typical features after Drosha cleavage, such as 2-nucleotide overlap at the 3´end, 3´hydroxyl, and 5´phosphate.

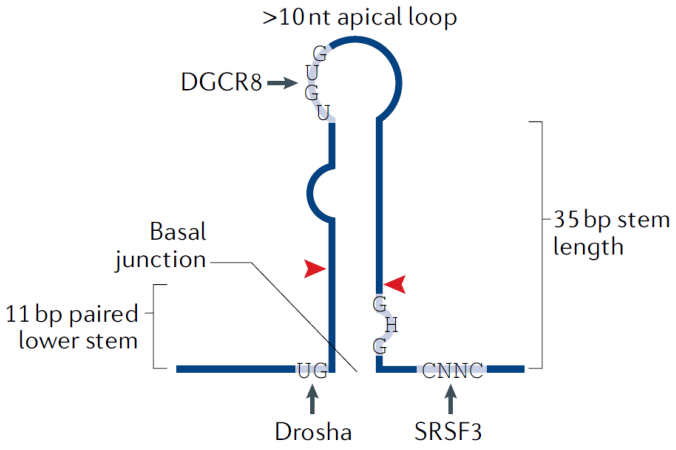

Figure 4. pri-miRNA stem loop structure indicating sequence parts required for efficient cleavage. The red arrows represent the cleavage site of the Microprocessor. [62]

Pre-miRNA is then transported from the nucleus to the cytoplasm via the export receptor Exportin 5 (Exp-5) in a RAS-related nuclear protein-guanosine-5'-triphosphate-ase (Ran-GTPase) dependent manner. Exp-5 recognizes both the double-stranded stem structure of pre-miRNA and the 2-nucleotide overhang at its 3´end, typical of RNase III products. After the pre-miRNA is released in the cytoplasm, the receptor is recycled back to the nucleus [63]. Exp-5 was thought to be necessary for the nuclear export of miRNA. However, it has recently been published that only a moderate decrease in miRNAs production was observed in Exp-5 knockout cells, suggesting the importance for the nuclear transmission, but also the presence of an alternative export mechanism. While Drosha and Dicer have been shown to be essential for canonical miRNA pathway [64].

In the cytoplasm, pre-miRNA is processed by the RNase III enzyme Dicer into the mature-miRNA duplex of about 20-25 nucleotides in length. Dicer specifically recognizes the structure of the stem-loop, 5´phosphate, and 3´overhang of pre-miRNA, which are then bound in a pocket within the associated domains. Dicer consists of several domains and is bound to a trans-activation-responsive RNA-binding protein (TRBP), responsible for pre-

miRNA binding. Its catalytical unit is in the form of a dimer with two catalytical RNase III domains (RIIIDs), where each RIIID cleaves one strand of the dsRNA. The cleavage site is located close to the terminal loop of the hairpin, on the opposite side where the Drosha cleavage was performed [65] [62].

The mature-miRNA duplex with 2-nucleotides 3´overhangs on each end, resulting from both Drosha and Dicer cleavage, is then loaded onto AGO protein in a process called RISC loading. RISC is a multiprotein complex that together with the bound miRNA forms the active catalytical unit named miRISC. TRBP, the part of RISC, identifies the “guide” and the “passenger” strands of miRNA duplex based on their thermodynamic properties and determines the correct orientation of the ds-miRNA for AGO loading. The process of the selection of either the 5p or 3p miRNA arm to be bound to the RISC depends mainly on the mismatches in positions 2-7 of the duplex and stability of the 5´end of miRNA. When loaded, the miRNA duplex is first destabilized by the slicer activity of AGO2, then unwonted by RNA helicases (RNA helicases H and P68), and the passenger strand is finally degraded by an endoribonuclease complex component 3 of the promoter of RISC (C3PO). Based on which strand is chosen to be the active strand, the mature miRNA is then named either miRNA-5p if the miRNA´s guide strand originates from the 5´-end, or miRNA-3p if it comes from the 3´-end of the pre-miRNA. miRNA-5p and miRNA-3p have different target specificity [66] [67].

The mature miRNAs in binding with AGO are four times more stable compared to mRNAs. The guide miRNA strand recognizes the target mRNA and drives the complex for the interaction with its 3´untranslated region (3´UTR) in a complementary-nucleotides-based manner. Depending on the degree of complementarity, miRISC causes either the degradation of the mRNA or, in the case of only partial complementarity, the translational suppression of the target gene.

The most important region of miRNA is the seed sequence (or 5´ region) between the nucleotides 2 to 8 from the 5´end, that is crucial for determining target specificity and is

highly conserved among species. Based on the complementarity between the miRNA seed sequence and 3´UTR region of mRNA, algorithms and subsequently bioinformatics programs for the prediction of functional targets such as TargetScan, Diana tools, miRDB and others have been developed [68] [69]. In addition to the complementarity of seed sequence, the presence of adenine nucleotide (A) cross from miRNA position 1 and the supplementary pairing at the position of nucleotides 13-16 is beneficial for binding. This model is called the canonical model and is typical for seed-based targets which are perfectly paired to the 5´region. Other sequences present on the miRNA strand are the proximal region (9-12) and the 3´region (nucleotide 16 to the end). These regions are dispensable for canonical target pairing, but in the case of imperfect seed sequence binding, they may allow miRNA-mediated silencing by binding non-canonical targets. The fact that miRNAs share the same seed sequence within the family, but their targets differ from each other, supports the idea of the importance of these additional sequences [70] [71].

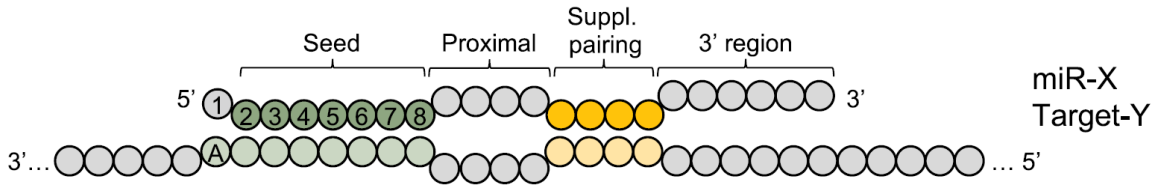

Figure 5. Canonical model for miRNA:target recognition. [72]

miRNAs loaded into AGO are stable from hours to days and their turnover appears to be actively regulated, especially during development, but with little known about the exact mechanism. It has been shown that single-stranded miRNA can be unloaded from the RISC by binding mRNA transcripts with a near-perfect complementary to miRNA containing center mismatches. These may destabilize miRNA and cause the release of miRNA from AGO [73]. In addition, 3´end destabilization and conformational changes in the hinge region of AGO promote the release of miRNA. This type of decay is called target-directed miRNA

degradation. The structural analysis also revealed that the shape of the central cleft of the AGO2 and centered mismatches in the miRNA targets allow modifications by unknown enzymes leading to the remodelling and miRNA destabilization [74].

In addition to cytoplasmic miRNAs, low miRISC activity has also been found in mammalian cell nuclei. In this pathway, the miRNA is in the cytoplasm bound only to AGO2, and in this form is subsequently transported into the nucleus by importin 8 [75] [76]. These miRNAs then regulate both coding and non-coding RNA transcriptome in the nucleus, where are also implicated in alternative splicing and regulation of the stability of mRNA in nucleoli. Moreover, they are involved in chromatin structure remodelling or regulation of pre-miRNA maturation, and thus the regulation of itself [77].

As can be seen, miRISC is involved in multiple steps of protein synthesis, and therefore miRNAs are not only considered as post-transcriptional gene regulators, as has been the case so far, but have been shown to affect gene expression also at the transcription level. Moreover, just as miRNAs act as epigenetic regulators, miRNA itself is also an entity that is subject to epigenetic regulation and post-translational modifications (PTMs) affecting miRNA processing and loading into RISC.

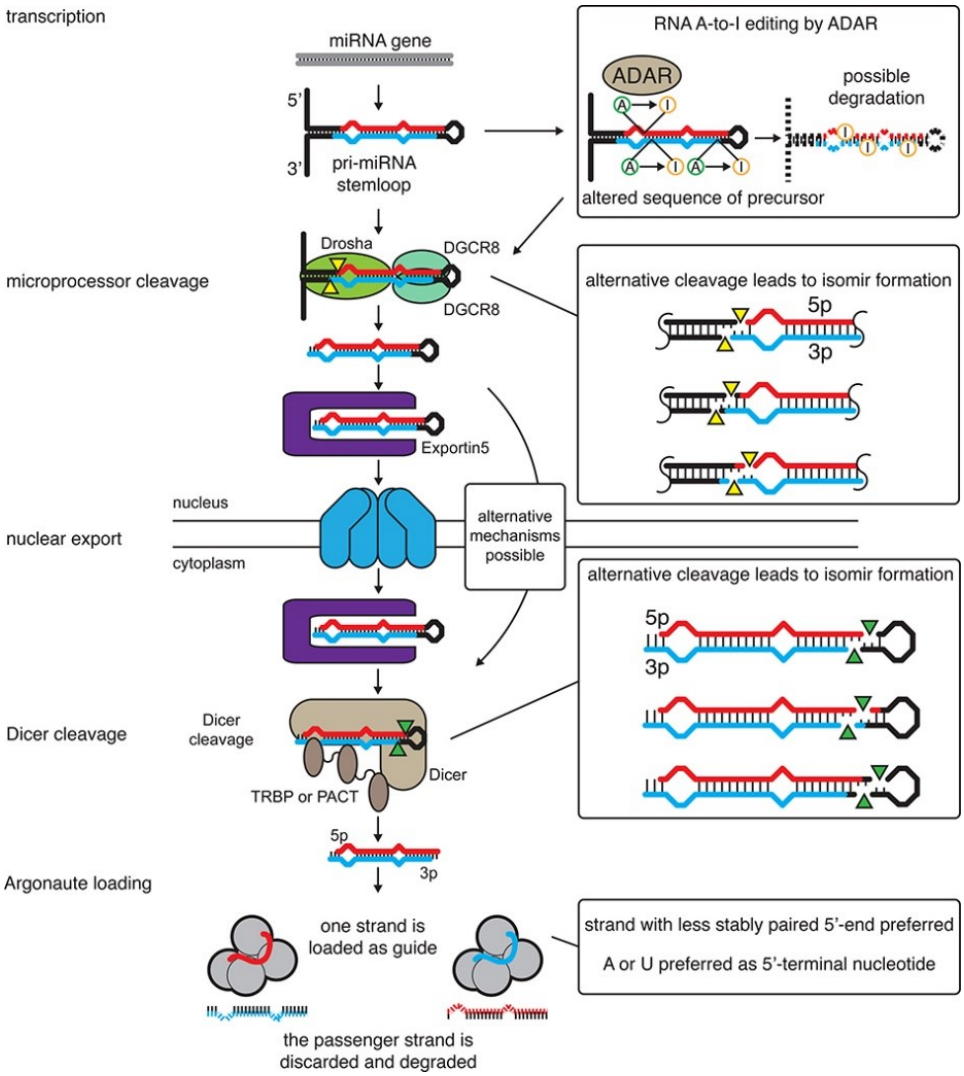

Figure 6. Illustration of individual steps of canonical miRNA processing and isomiR generation. [78]

2.4.4.2. IsomiRs

With the introduction of high-throughput deep sequencing, many miRNA variants, so-called isomiRs, have been revealed. These mature miRNA isoforms differ in length, sequence composition, or both, compared to the canonical miRNA and appear to be derived from a single miRNA locus [79]. They were first described by Morin *et al.* in 2008 [80].

isomiRs can be distinguished into several classes. 5´-isomiRs, with a change at the 5´-end, 3´-isomiRs, with 3´-end altered, polymorphic isomiRs, having a different nucleotide sequence, or the combination of each. Primarily 5´- and 3´-isomiRs are provided by variations in Drosha and/or Dicer cleavage during miRNA biogenesis. A further mechanism to generate the mentioned isomiRs is nucleotide trimming, tailing and their combination, which is less common, and occurs rather at the 3´-end. In this process, one or more nucleotides are removed from either the 3´- or 5´-end by exoribonucleases or, on the contrary, one or more nucleotides can be added to one of the ends by nucleotidyltransferases, most commonly by the uridyltransferase and adenyltransferase. The polymorphic isomiRs have usually the same length as the canonical miRNA, but their sequence is mutated by RNA-editing enzymes. In this way, any nucleotide within the sequence occurring at the 5´-end, 3´-end, seed region, or in any internal region, can be altered. The most prevalent RNA-editing enzyme in the isomiRs production is the double-stranded RNA-specific adenosine deaminase, converting adenosine (A) to inosine (I). The last class of isomiR generation can be considered various combinations of all the mentioned factors from Drosha/Dicer imprecise cleavage through nucleotide trimming to RNA editing [81] [82] [83].

While 3´-isomiRs are generally more abundant than 5´- isomiRs, some miRNAs do have predominant 5´- isomiRs. As the 5´-end of the miRNA determines the seed sequence, 5´-isomiRs are characterized by having a shifted seed region compared to the canonical miRNA. Therefore, they have an altered targetome and are functionally different. In contrast, 3´-isomiRs retain the same seed sequence with canonical miRNA but differ in the length or sequence of the 3´-end, which may alter their stability, turnover, half-life, or targeting strength [84] [85]. However, due to the non-canonical target base pairing, apparently any modification in the miRNA sequence may cause a variation in the target specificity and biological function, as well as different loading efficiency onto AGO proteins. Like canonical miRNAs, isomiRs are evolutionarily highly conserved and their biogenesis is

tightly regulated. Their abundance is also related to cell types, may be tissue specific, or typical of a particular pathological condition [86] [87]. Moreover, it has been shown that some isomiRs are gender specific, associated with population origin and race [88]. It turns out that isomiRs and their certain patterns could be used as more beneficial, effective, and sensitive prognostic and diagnostic biomarkers than the cognate miRNAs alone.

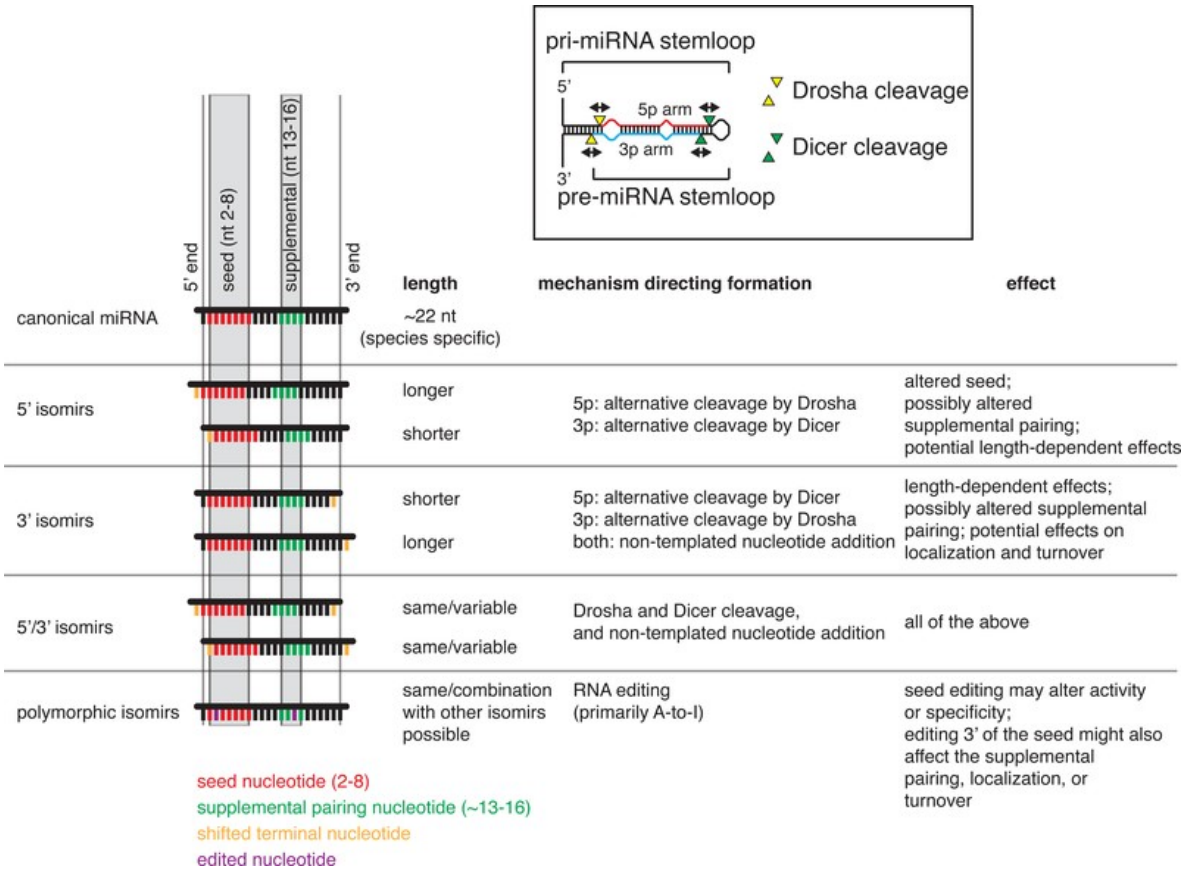

Figure 7. isomiRs differ in sequence, length, and function. [78]

Up to now, several different tools for miRNAs and isomiRs detection and analysis have been created. Some of the databases are for instance MiRGator v3.0 [89], IsomiRex [90], CPSS [91], or SeqBuster [92]. Moreover, a number of platforms or bioanalytical software to study miRNA, isomiRs and their interactions with target mRNAs, such as isomiR-SEA [93],

SRNAbench [94] or Prost [95] have been developed. Databases, algorithms, or bioinformatics tools have been created and designed for accurate mapping, expression analysis, or studying a downstream of isomiR analysis [96]. In addition, an analytical project called miRNA Transcriptomic Open Project (miRTOP), has recently been proposed, which allows to develop the downstream isomiR analysis tool compatible with available detection and quantification tools [97].

2.4.4.3. Non-canonical biogenesis pathway

As mentioned above, in addition to the canonical pathway, there is a non-canonical biogenesis pathway for miRNA production. In this way, miRNAs are generated from unexpected non-coding RNAs as precursors and processed by mechanisms bypassing either Drosha/DGCR8- or Dicer-catalyzed cleavage [98]. In Drosha/DGCR8-independent pathway, pre-miRNAs usually resemble Dicer substrates.

An example is a prominent class of intron-derived miRNAs, so-called miRtons. Pri-miRNAs are encoded in introns of coding genes, wherefrom are processed by the nuclear splicing and debranching into the stable hairpin structures. Since these hairpins are shorter compared to canonical pri-miRNAs, they are therefore insufficient to be recognized and cleaved by Drosha/DGCR8. MiRtons are then, like canonical pre-miRNAs, transported from the nucleus into the cytoplasm by Xpo-5 for processing by Dicer. Many miRNAs, independent of Drosha, but requiring Dicer cleavage, originate from non-coding RNAs such as snoRNAs) or tRNAs, generating many small RNAs due to their clover shape [99] [100].

So far, the only identified miRNA generated in Dicer-independent pathway is miR-451, indicating an indispensable role of Dicer in both canonical and non-canonical miRNA production. Pre-miRNA-451, which is cleaved from endogenous short hairpin RNA (shRNA) by Drosha, is too short to be processed by Dicer and therefore the AGO2-mediated/dependent slicing is required to complete the miR-451 maturation [101]. Unlike

canonical miRNAs and isomiRs, non-canonical miRNAs do not appear to be evolutionarily conserved to such an extent.

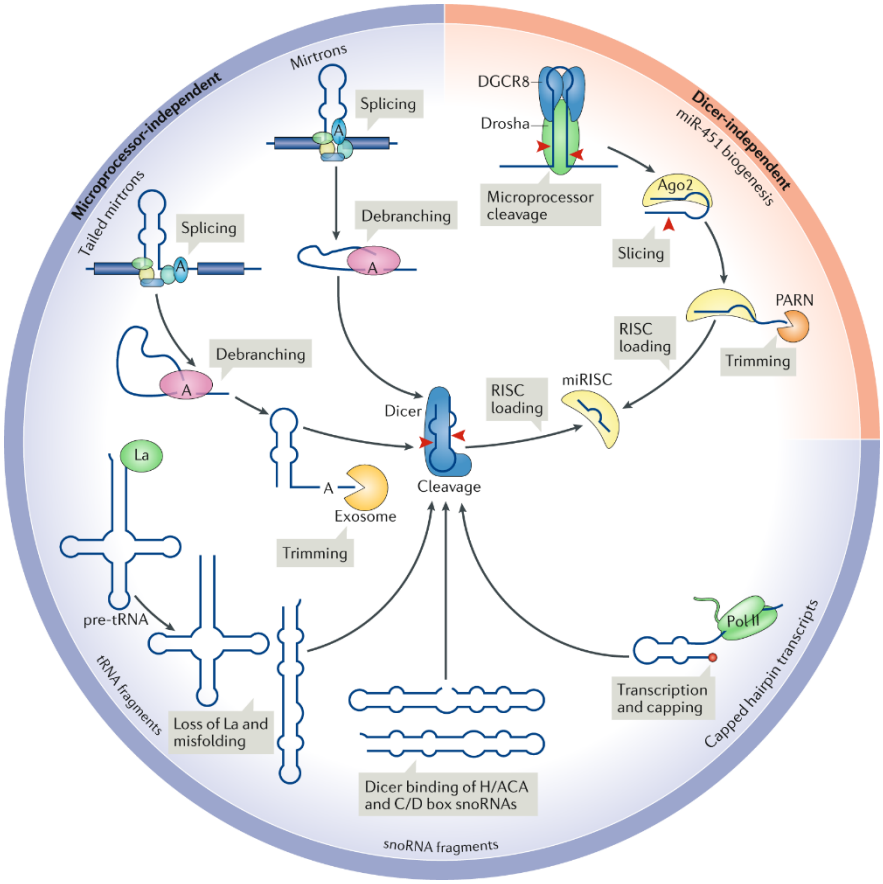

Figure 8. illustration of non-canonical microRNA biogenesis pathways. [62]

2.4.4.4. miRNAs distribution and turnover

Some miRNAs are located in the cell nucleus, where they perform their function, while others are released from cells in a pool of circulating miRNAs. Circulating miRNAs can be said to function as hormones and are implicated in intercellular communication. Cells constantly produce miRNAs that are released into the extracellular space to affect both surrounding and distant cells. To fulfil their regulatory role at the appropriate destinations, miRNAs can be transported either bound to protein carriers or in the form of transport vesicles, commonly called “exosomes” [102]. In these forms, they are simultaneously

protected from degradation. Exosomes are formed in the cytoplasm and are secreted from the cell by process of exocytosis, followed by recognition and attachment to the appropriate receptors on the target cell. The Exosome can then either enter the cell by any type of endocytosis, release its contents into the cell by fusion, or simply activate intracellular signalling pathways by binding to the membrane receptor [103, 104]. The exact mechanisms may vary depending on the type of exosome or host cell; however, this area is still poorly understood.

In addition, miRNAs can be distributed from cell to cell in complexes with proteins that can also enter the cells. Most miRNAs are transported bound to ribonucleoproteins, usually AGO2. miRNAs can also be distributed in the blood by both high-density lipoproteins (HDL) and low-density lipoproteins (LDL) [105, 106]. The presence of circulating miRNAs in blood or other body fluids offers their use as biomarkers of diseases, but also as a new branch of cell signalling and intercellular and intertissue communication.

miRNA degradation is a highly regulated process depending on the stage of development or cell cycle of the cell, as well as on the miRNA sequence itself. Therefore, miRNA turnover can range from a few minutes to several days [107]. Some miRNA degrading enzymes, predominantly exonucleases, have been identified. Another way of miRNA degradation is associated with miRNA released from the RISC complex by the mechanism called "Target RNA-directed miRNA degradation" (TDMD), where highly complementary mRNA with both 3´and 5´ends of miRNA can destabilize AGO2-miRNA binding [74, 108].

2.4.4.5. miRNAs in diagnosis and treatment

miRNAs can be isolated from all body fluids, cells, and tissues. The term biomarker refers to a molecule that is highly associated with the disease and allows for early diagnosis or prognosis of the disease. A promising biomarker should have certain properties, such as specificity, sensitivity, and stability. Ideally, the molecule should be determined by the least

invasive method and detected at a time when the ongoing disease is still at an early stage. miRNAs appear to be very promising molecules useful both as biomarkers of diseases or as therapeutic targets for treatment.

As already mentioned, many miRNAs are tissue-specific, they reflect very early the presence of an ongoing disease by altering their expression profile, which is relatively stable and well detectable in various body fluids. miRNAs can be readily determined by methods of molecular biology such as RT-qPCR, microarrays, or RNAseq, with each method having its advantages and disadvantages. The main problem with miRNA determination probably still remains at the beginning of the analysis, namely the correct and efficient isolation of the miRNA fraction. Another pitfall of using miRNAs as biomarkers of diseases may be the fact that a huge number of different miRNAs have already been detected and this number is very likely to increase. It is very important to correctly determine the origin of the relevant circulating miRNAs and to pay attention to the reality that the expression profile of miRNAs changes even under physiological conditions and is affected by, for instance, lifestyle, stress, or exercise.

Regarding miRNAs as a therapeutic target, we also encounter possible pitfalls here; for example, the availability/delivery of a miRNA or anti-miRNA in a particular tissue or the fact that one miRNA may regulate up to 100 different genes and may have a different effect in each tissue.

Despite all these pitfalls, miRNAs appear to be promising molecules and an exciting area for research. However, it is more than clear that we currently see only the tip of the iceberg.

3. Objectives

This doctoral thesis is presented in the form of an annotated set of scientific publications with a main focus on microRNAs, their role in physiology and pathology.

Specific aims of experimental parts of the thesis were:

- Literary review based on thorough bioinformatics analysis of individual GPx isoforms regulation by miRNAs and their association with obesity
- Confirmation of the bioinformatics analysis, exploring the effects of selected miRNAs on glutathione peroxidase 7 (GPx7) expression *in vitro*
- Monitoring of selected miRNAs and GPx7 in individual types of adipose tissue cells from human donors, as well as their expression during *in vitro* adipogenesis
- Observation of the effect of high fat and fructose diet on the expression of selected miRNAs in mice
- Literary review of known information on the role of miRNAs in drug-induced cardiotoxicity
- Determination of the effect of imatinib and doxorubicin administration on mouse cardiac tissue with the main focus on miRNAs as biomarkers of drug-induced cardiotoxicity

4. Autor's contribution in publications included in the dissertation thesis

I. Matoušková, P., **Hanousková, B**., & Skálová, L. (2018). MicroRNAs as potential regulators of glutathione peroxidases expression and their role in obesity and related pathologies. *International journal of molecular sciences*, *19*(4), 1199. Review. (IF 2018: **4,355**)

- Literature review
- Participation in the writing of the manuscript

II. **Hanousková, B.**, Vávrová, G., Ambrož, M., Boušová, I., Karlsen, TA., Skálová, L., & Matoušková, P. (2021) MicroRNAs mediated regulation of glutathione peroxidase 7 expression and its changes during adipogenesis. *BBA – gene regulatory mechanisms*. Accepted (IF: **4,49**)

- Construction of plasmids and corresponding mutants for human GPx7 gene reporter assay
- Adipose tissue derived stem cells (AT-MSCs) and adipocytes isolation from adipose tissue
- AT-MSCs cultivation and differentiation into adipocytes *in vitro* and Oil Red cell staining
- miRNA overexpression in AT-MSCs and two different cell lines by electroporation and by lipofectamine
- Luciferase reporter gene assay
- Determination of gene expression and miRNA expression by RT-qPCR and protein levels by Western blotting
- Data processing
- Drafting the manuscript

III. **Hanousková, B.**, Neprašová, B., Skálová, L., Maletínská, L., Zemanová, K., Ambrož, M., & Matoušková, P. (2019). High-fructose drinks affect microRNAs expression differently in lean and obese mice. *The Journal of nutritional biochemistry*, *68*, 42-50. (IF 2019: **4,873**)

- Samples processing
- Measurement of miRNA expression by RT-qPCR and data processing
- Participation in the writing of the manuscript

IV. Skála, M., **Hanousková, B.**, Skálová, L., & Matoušková, P. (2019). MicroRNAs in the diagnosis and prevention of drug-induced cardiotoxicity. *Archives of toxicology*, *93*(1), 1-9. Review (IF: **5,741**)

- Literature review
- Participation in the writing of the manuscript

V. **Hanousková, B.**, Skála, M., Brynychová, V., Zárybnický, T., Skarková, V., Kazimírová, P., ... & Matoušková, P. (2019). Imatinib-induced changes in the expression profile of microRNA in the plasma and heart of mice - A comparison with doxorubicin. *Biomedicine & Pharmacotherapy*, *115*, 108883. (IF: **4,545**)

- Determination of miRNA expression by RT-qPCR in plasma and heart and data processing
- Participation in the writing of the manuscript

5. Articles published in journals with impact factor associated with a topic of doctoral dissertation accompanied by candidate´s commentary

5.1. Publication I

Matoušková, P., **Hanousková, B**., & Skálová, L. (2018). MicroRNAs as potential regulators of glutathione peroxidases expression and their role in obesity and related pathologies. *International journal of molecular sciences*, *19*(4), 1199. Review. (IF 2018: **4,355**)

As microRNAs are involved in almost all cellular processes and their expression profile is often changed in some pathologies, including obesity and obesity related pathologies, they can be used as biomarkers of diseases or as a therapeutic target for the treatment. MicroRNAs perform their function by binding usually to the 3´UTR region of genes and thus suppress the expression of the respective genes. Prediction of microRNAs targets is challenging and relies on the mutual complementarity with 3´UTR, sequence conservation across the species, thermodynamic stability of the mRNA:miRNA duplex etc.

Since it is well known that obesity is associated with elevated oxidative stress and inflammation, antioxidant enzymes could be involved in pathogenesis. Among others, glutathione peroxidases (GPxs) protect cells from oxidative damage.

The main outcome of this study is a list of miRNAs that could regulate the expression of GPxs with their potential role in the pathogenesis of obesity. Four different bioinformatic tools using different algorithms were used to identify miRNAs that could possibly interact with the 3´UTR of individual GPx genes. In addition, the cross-reference search was performed to select *in silico* identified miRNAs having the reported link to obesity or obesity-related pathologies. It has been shown that all 8 members of the GPx family can be post-

transcriptionally regulated by many miRNAs and that the length of their 3´UTR region correlates positively with the number of putative regulatory miRNAs. The study may thus serve as an important source of information for further miRNA research.

5.2. Publication II

Hanousková, B., Vávrová, G., Ambrož, M., Boušová, I., Karlsen, TA., Skálová, L., & Matoušková, P. MicroRNAs mediated regulation of glutathione peroxidase 7 expression and its changes during adipogenesis. *BBA – gene regulatory mechanisms*. Accepted (IF: **4,49**)

Glutathione peroxidase 7 (GPx7) is a member of one of the most important group of antioxidant enzymes which, in addition to maintaining ROS homeostasis, plays a role in adipogenesis. Moreover, GPx7 has been reported to be associated with obesity and excessive fat accumulation, probably as this process is accompanied by increased inflammation and ROS production. As miRNAs are involved in the regulation of almost all cellular processes including pathological processes and affect the expression of vast majority of genes, the present study aims to reveal a possible link between miRNAs, GPx7, adipogenesis and obesity, respectively.

miRNAs were selected on the basis of their bioinformatically predicted recognition sites within the 3´UTR region of GPx7 and simultaneously on the basis of their proven relation to obesity and adipogenesis. The inhibitory effect of selected miRNAs on GPx7 expression was tested by their overexpression in three cell lines and also by the luciferase reporter gene assay, which allows to demonstrate the direct binding of miRNAs to a target site in the selected gene 3´UTR.

The inhibitory effect of miR-29b and miR-137 on both mRNA and protein levels was proven, as well as their direct binding to the predicted recognition site in the GPx7 3´UTR. In addition, the expression levels of GPx7 and tested miRNAs were determined during 21 days of *in vitro* adipogenesis, in AT-MSCs and corresponding adipocytes from four adipose tissue donors. Although a negative correlation between GPx7 and miR-335-5p was observed in AT-MSCs and adipocytes in all three donors, as well as in adipogenesis, no direct regulatory

link was observed in this study. On the other hand, the inhibitory effect of miR-140-3p on GPx7 was demonstrated, however no recognition site on the 3´UTR was predicted.

5.3. Publication III

Hanousková, B., Neprašová, B., Skálová, L., Maletínská, L., Zemanová, K., Ambrož, M., & Matoušková, P. (2019). High-fructose drinks affect microRNAs expression differently in lean and obese mice. *The Journal of nutritional biochemistry*, *68*, 42-50. (IF 2019: **4,873**)

Excessive fructose consumption, especially in the form of soft drinks and sweets, has increased significantly in population. Due to its metabolism, fructose has a highly prooxidative and lipogenic effect in the body. When consumed excessively, it is considered as harmful and its consumption in the population increases rapidly over time. The excessive fructose intake can be associated with metabolic changes leading to the development of chronic diseases, including obesity and obesity-related pathologies.

This study is focused on finding the potential effect of excessive fructose consumption on miRNA expression in lean and obese mice. Four experimental groups of mice were used - standard lean mice as a control group, mice with obesity induced by high-fat diet, and both groups additionally with and without fructose administration.

The individual physiological and biochemical parameters were measured and total body weight, as well as the weight of the liver and various adipose tissues (brown, white, and subcutaneous) of each individual. As expected, a high-fat diet had a significant impact on the vast majority of measured parameters. Regarding the effect of fructose on these parameters, only liver hypercholesterolemia was observed in both lean and obese mice.

Furthermore, we have evaluated the effect on several miRNAs that were selected based on their established role in lipid metabolism, obesity, metabolic syndrome, or non-alcoholic fatty liver disease (NAFLD). Their expression levels were determined in brown, white, and subcutaneous adipose tissue, as well as in plasma and liver tissue. A different miRNA expression profile was observed in lean and obese mice. Moreover, several miRNAs were further elevated by fructose intake. Surprisingly, a higher number of altered

miRNAs was detected in lean mice compared to obese individuals, suggesting a high risk of excessive fructose consumption even for normal weight individuals.

5.4. Publication IV

Skála, M., **Hanousková, B.**, Skálová, L., & Matoušková, P. (2019). MicroRNAs in the diagnosis and prevention of drug-induced cardiotoxicity. *Archives of toxicology*, *93*(1), 1-9. (IF: **5,741**)

Cardiotoxicity is a serious adverse effect associated with the administration of many drugs and is among the most common causes of drug failure during their development and testing. In many cases, drugs can cause only partial subacute damage, which is difficult to detect by currently used methods, and which can later develop into severe heart issues. This remains a serious problem, especially for widely used drugs and chronic long-term treatment. The importance of identifying early and specific biomarkers is more than clear in this respect.

The use of miRNAs in this context is also offered. Compared to markers used to date, miRNAs have been shown to be more sensitive, tissue specific, and early released markers which appears when the damage is still reversible.

This review article aims to summarize the existing information about the role of microRNAs in drug-induced cardiotoxicity. Three main topics are discussed here. First, the existing information on miRNAs, which are at the same time cardiac specific and whose expression levels have shown some association with the toxic effect of drugs, is summarized. Likewise, their potential use in testing cardioprotective compounds or preclinical testing of drug-induced cardiotoxicity *in vitro* is discussed. A second aspect reflected in this review is the importance of circulating plasma/serum miRNAs as early biomarkers of incipient cardiac damage, which may help to reveal subacute complications or indicate the need to initiate prophylactic treatment. The last part summarizes the use of miRNAs for prevention and/or attenuation of drug-induced cardiotoxicity, either by targeted decrease of overexpressed miRNAs or, conversely, the targeted increase of silenced

miRNAs. In addition, the effect of natural compounds as cardio protectants, affecting the expression of certain miRNAs is discussed.

5.5. Publication V

Hanousková, B., Skála, M., Brynychová, V., Zárybnický, T., Skarková, V., Kazimírová, P., ... & Matoušková, P. (2019). Imatinib-induced changes in the expression profile of microRNA in the plasma and heart of mice - A comparison with doxorubicin. *Biomedicine & Pharmacotherapy*, *115*, 108883. (IF: **4,545**)

As mentioned before, cardiotoxicity is a serious side effect related to drug administration, especially in cancer chemotherapy. In this context, miRNAs appear to be suitable early release biomarkers for detecting the toxic effects of drugs even in small doses. Anthracycline cytostatic doxorubicin (DOX) has been widely used to treat various types of cancers. Since its toxic effect on heart tissue has later been shown, DOX was used for comparison and evaluation in this study. Imatinib mesylate (IMB), specific tyrosine kinase inhibitor, is commonly used for anti-cancer therapy but have also a certain possibility to cause heart tissue damage.

This study is designed to determine the effect of IMB and DOX treatment on selected miRNAs in an effort to reveal potential miRNAs associated with drug-induced cardiotoxicity that circulate in plasma and/or are present in cardiac tissue. Mice, chosen as an experimental model for this study, were divided into three groups and treated either with IMB, DOX, or only solvents for 9 weeks. Plasma levels of circulating miRNAs and troponin T, as well as expression profiles of selected cardio-specific miRNAs in heart tissue were determined in all individuals.

The developed heart damage was demonstrated by elevated troponin T levels in plasma samples of both treated groups in comparison to controls. Several circulating miRNAs affected by DOX treatment showed the same trends also after IMB treatment, but with higher interindividual variability. In heart tissue only miR-205 was significantly changed after both DOX and IMB treatment, and miR-34a was increased only in the DOX group.

6. Conclusions

The presented dissertation thesis was interested in small non-coding RNA molecules, microRNAs. It was focused on their role in physiology and pathology in terms of their use as suitable biomarkers, as well as the mechanism of their action at the molecular level.

- A comprehensive bioinformatics analysis was performed to identify hypothetical miRNAs that could regulate individual isoforms of GPx family by binding to their 3´UTR region. The available literature was cross-referenced to find a possible intersection with the results of our bioinformatics analysis allowing the selection of miRNAs suitable for our purposes. The main outcome is a list of miRNAs that could regulate the expression of GPxs with their potential role in the pathogenesis of obesity.
- Bioinformatically predicted hypothesis of GPx7 regulation by selected miRNAs was tested by various approaches *in vitro*. The luciferase reporter gene assay demonstrated direct binding of miR-29b-3p and miR-137 to 3´UTR GPx7, while refuting miR-335-5p binding. This was further supported by an experimental study of *in vitro* overexpression of individual miRNAs and their effect on GPx7 expression at the mRNA and protein levels. GPx7 levels showed a positive correlation with miR-29b and, conversely, a negative correlation with miR-335 in adipocytes and mesenchymal stem cells isolated from human adipose tissue.
- Several miRNAs, such as miR-335, miR-221, miR-21, and miR-34a, were found to be affected by a high-fat diet in the liver and all three types of adipose tissue in a mouse model. Some of them were further altered by the fructose administration. Interestingly, a higher number of miRNAs was affected in lean animals compared to obese individuals.
- The available literature on the involvement of miRNAs in drug-induced cardiotoxicity was summarized. MiRNAs have been viewed in various contexts. The use of

circulating miRNAs as promising early biomarkers of heart damage, the view of miRNAs altered in cardiac tissue due to drug treatment, as molecules involved in cardiac damage itself, or the role of miRNAs as a therapeutic target to prevent or attenuate toxic effects of drugs on the heart are discussed.

- Several circulating miRNAs affected by IMB treatment showed the same trends also after DOX treatment, but with higher interindividual variability in IMB treated mice. None of the miRNAs determined in the cardiac tissue of our mouse model have been shown to be a reliable biomarker of IMB or DOX-induced heart damage.

7. Active participation in scientific conferences

Hanousková B., Matoušková P., Skálová L. – MikroRNA regulace glutathion peroxidasy 7 – XXIX. Xenobiochemické symposium – Czech Republic, Telč 2017 – poster section (winner of 1st place in the poster section)

Hanousková B., Matoušková P., Skálová L. – GPxs, microRNAs and obesity association – 8th Postgraduate and 6th Postdoc Conference – Czech Republic, Hradec Králové 2018 – oral presentation

Hanousková B., Skálová L., Ambrož M., Matoušková P., Zemanová K. – The high-fructose diet differently affects microRNAs expression in lean and obese mice – 23rd Interdisciplinary Toxicological Conference – Slovakia, Stará Lesná 2018 – poster section

Hanousková B., Vávrová G., Matoušková P., Skálová L. – Regulation of Glutathione peroxidase 7 by microRNAs and the potential link to obesity – 25th Interdisciplinary Toxicological Conference – Czech Republic, Prague 2020 – poster section

8. Abbreviations

3´UTR – 3´untranslated region

AGO – Argonaute protein

circRNA – circular RNA

DGCR8 – DiGeorge syndrome critical region 8 gene

DNA - deoxyribonucleic acid

DNMT – DNA methyltransferase

dsRNA – double-stranded RNA

eRNA – enhancer RNA

Exp5 – Exportin 5

lncRNA – long non-coding RNA

miRNA – microRNA

miRTOP – miRNA Transcriptomic Open Project

ncRNA – non-coding RNA

nt – nucleotide

piRNA – Piwi-interacting RNA

PIWI – P-element-induced wimpy testis proteins

pre-miRNA – precursor miRNA

pre-tRNA – precursor tRNA

pri-miRNA – primary miRNA

PTMs – post-translational modifications

Ran-GTPase – RAS-related nuclear protein-guanosine-5´-triphosphate-ase

RBP – RNA-binding protein

RIIID – Ribonuclease III domain

RISC – RNA-induced silencing complex

RITS – RNAi-induced transcriptional silencing

RNA – ribonucleic acid

RNAi – mRNA interference

shRNA – short hairpin RNA

siRNA – small inhibitory RNA

snoRNA – small nucleolar RNA

snRNA – small nuclear RNA

SRSF3 – Serine/arginine-rich splicing factor 3

ssRNA – single-stranded RNA

TDMD - Target RNA-directed miRNA degradation

TET – ten-eleven translocation enzyme

tiRNA – tRNA-derived stress-induced RNA

TRBP – trans-activation-responsive RNA-binding protein

tRNA – transfer RNA

9. References

1. Jaenisch, R. and A. Bird, *Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals.* Nature genetics, 2003. **33**(3): p. 245-254.
2. Horsthemke, B., *A critical view on transgenerational epigenetic inheritance in humans.* Nature communications, 2018. **9**(1): p. 1-4.
3. Deichmann, U., *Epigenetics: The origins and evolution of a fashionable topic.* Developmental biology, 2016. **416**(1): p. 249-254.
4. Kumar, H., et al., *A review on epigenetics: Manifestations, modifications, methods & challenges.* Journal of Entomology and Zoology Studies, 2020. **8**(4): p. 01-06.
5. De Sario, A., *Clinical and molecular overview of inherited disorders resulting from epigenomic dysregulation.* European journal of medical genetics, 2009. **52**(6): p. 363-372.
6. Dawson, M.A. and T. Kouzarides, *Cancer epigenetics: from mechanism to therapy.* Cell, 2012. **150**(1): p. 12-27.
7. Handel, A.E., G.C. Ebers, and S.V. Ramagopalan, *Epigenetics: molecular mechanisms and implications for disease.* Trends in molecular medicine, 2010. **16**(1): p. 7-16.
8. Probst, A.V., E. Dunleavy, and G. Almouzni, *Epigenetic inheritance during the cell cycle.* Nature reviews Molecular cell biology, 2009. **10**(3): p. 192-206.
9. Sharif, J., et al., *The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA.* Nature, 2007. **450**(7171): p. 908-912.
10. Anway, M.D., et al., *Epigenetic transgenerational actions of endocrine disruptors and male fertility.* science, 2005. **308**(5727): p. 1466-1469.
11. Rassoulzadegan, M., et al., *RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse.* Nature, 2006. **441**(7092): p. 469-474.
12. Wagner, K.D., et al., *RNA induction and inheritance of epigenetic cardiac hypertrophy in the mouse.* Developmental cell, 2008. **14**(6): p. 962-969.
13. Vastenhouw, N.L., et al., *Long-term gene silencing by RNAi.* Nature, 2006. **442**(7105): p. 882-882.

14. Audia, J.E. and R.M. Campbell, *Histone modifications and cancer.* Cold Spring Harbor perspectives in biology, 2016. **8**(4): p. a019521.

15. Li, J., Y. Ding, and L. Zheng, *Histone-Mediated Transgenerational Epigenetics*, in *Transgenerational Epigenetics*. 2014, Elsevier. p. 87-103.

16. Andrews, F.H., B.D. Strahl, and T.G. Kutateladze, *Insights into newly discovered marks and readers of epigenetic information.* Nature chemical biology, 2016. **12**(9): p. 662-668.

17. Taverna, S.D., et al., *How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers.* Nature structural & molecular biology, 2007. **14**(11): p. 1025-1040.

18. Jezek, M. and E.M. Green, *Histone modifications and the maintenance of telomere integrity.* Cells, 2019. **8**(2): p. 199.

19. Bannister, A.J. and T. Kouzarides, *Regulation of chromatin by histone modifications.* Cell research, 2011. **21**(3): p. 381-395.

20. Zhao, Z. and A. Shilatifard, *Epigenetic modifications of histones in cancer.* Genome biology, 2019. **20**(1): p. 1-16.

21. Shogren-Knaak, M., et al., *Histone H4-K16 acetylation controls chromatin structure and protein interactions.* Science, 2006. **311**(5762): p. 844-847.

22. Lawrence, M., S. Daujat, and R. Schneider, *Lateral thinking: how histone modifications regulate gene expression.* Trends in Genetics, 2016. **32**(1): p. 42-56.

23. Unnikrishnan, A., P.R. Gafken, and T. Tsukiyama, *Dynamic changes in histone acetylation regulate origins of DNA replication.* Nature structural & molecular biology, 2010. **17**(4): p. 430.

24. Xu, Y. and B.D. Price, *Chromatin dynamics and the repair of DNA double strand breaks.* Cell cycle, 2011. **10**(2): p. 261-267.

25. Zentner, G.E. and S. Henikoff, *Regulation of nucleosome dynamics by histone modifications.* Nature structural & molecular biology, 2013. **20**(3): p. 259.

26. Banerjee, T. and D. Chakravarti, *A peek into the complex realm of histone phosphorylation.* Molecular and cellular biology, 2011. **31**(24): p. 4858-4873.

27. Greer, E.L. and Y. Shi, *Histone methylation: a dynamic mark in health, disease and inheritance.* Nature Reviews Genetics, 2012. **13**(5): p. 343-357.

28. Bernstein, B.E., et al., *A bivalent chromatin structure marks key developmental genes in embryonic stem cells.* Cell, 2006. **125**(2): p. 315-326.

29. Zhang, T., S. Cooper, and N. Brockdorff, *The interplay of histone modifications–writers that read.* EMBO reports, 2015. **16**(11): p. 1467-1481.

30. Schaefer, M., et al., *RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage.* Genes & development, 2010. **24**(15): p. 1590-1595.

31. Rottach, A., H. Leonhardt, and F. Spada, *DNA methylation-mediated epigenetic control.* Journal of cellular biochemistry, 2009. **108**(1): p. 43-51.

32. Kohli, R.M. and Y. Zhang, *TET enzymes, TDG and the dynamics of DNA demethylation.* Nature, 2013. **502**(7472): p. 472-479.

33. Kim, M. and J. Costello, *DNA methylation: an epigenetic mark of cellular memory.* Experimental & molecular medicine, 2017. **49**(4): p. e322-e322.

34. Rakyan, V.K., et al., *An integrated resource for genome-wide identification and analysis of human tissue-specific differentially methylated regions (tDMRs).* Genome research, 2008. **18**(9): p. 1518-1529.

35. Robertson, K.D., *DNA methylation and human disease.* Nature Reviews Genetics, 2005. **6**(8): p. 597-610.

36. Jones, P.A., *Functions of DNA methylation: islands, start sites, gene bodies and beyond.* Nature Reviews Genetics, 2012. **13**(7): p. 484-492.

37. Hirst, M. and M.A. Marra, *Epigenetics and human disease.* The international journal of biochemistry & cell biology, 2009. **41**(1): p. 136-146.

38. Deaton, A.M. and A. Bird, *CpG islands and the regulation of transcription.* Genes & development, 2011. **25**(10): p. 1010-1022.

39. Agrawal, K., et al., *Nucleosidic DNA demethylating epigenetic drugs–a comprehensive review from discovery to clinic.* Pharmacology & therapeutics, 2018. **188**: p. 45-79.

40. Panni, S., et al., *Non-coding RNA regulatory networks.* Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 2020. **1863**(6): p. 194417.

41. Zhang, P., et al., *Non-Coding RNAs and their Integrated Networks.* Journal of Integrative Bioinformatics, 2019. **16**(3).

42. Cech, T.R. and J.A. Steitz, *The noncoding RNA revolution—trashing old rules to forge new ones.* Cell, 2014. **157**(1): p. 77-94.

43. Chen, Q., et al., *Versatile interactions and bioinformatics analysis of noncoding RNAs.* Briefings in bioinformatics, 2019. **20**(5): p. 1781-1794.

44. Schimmel, P., *The emerging complexity of the tRNA world: mammalian tRNAs beyond protein synthesis.* Nature reviews Molecular cell biology, 2018. **19**(1): p. 45.

45. Peschansky, V.J. and C. Wahlestedt, *Non-coding RNAs as direct and indirect modulators of epigenetic regulation.* Epigenetics, 2014. **9**(1): p. 3-12.

46. Watanabe, T., et al., *Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes.* Nature, 2008. **453**(7194): p. 539-543.

47. Filipowicz, W., et al., *Post-transcriptional gene silencing by siRNAs and miRNAs.* Current opinion in structural biology, 2005. **15**(3): p. 331-341.

48. Esteller, M., *Non-coding RNAs in human disease.* Nature reviews genetics, 2011. **12**(12): p. 861-874.

49. Czech, B., et al., *piRNA-guided genome defense: from biogenesis to silencing.* Annual review of genetics, 2018. **52**: p. 131-157.

50. Siomi, M.C., et al., *PIWI-interacting small RNAs: the vanguard of genome defence.* Nature reviews Molecular cell biology, 2011. **12**(4): p. 246-258.

51. Luteijn, M.J. and R.F. Ketting, *PIWI-interacting RNAs: from generation to transgenerational epigenetics.* Nature Reviews Genetics, 2013. **14**(8): p. 523-534.

52. Quinn, J.J. and H.Y. Chang, *Unique features of long non-coding RNA biogenesis and function.* Nature Reviews Genetics, 2016. **17**(1): p. 47.

53. Dykes, I.M. and C. Emanueli, *Transcriptional and post-transcriptional gene regulation by long non-coding RNA.* Genomics, proteomics & bioinformatics, 2017. **15**(3): p. 177-186.

54. Kornienko, A.E., et al., *Gene regulation by the act of long non-coding RNA transcription.* BMC biology, 2013. **11**(1): p. 1-14.

55. Baumann, V. and J. Winkler, *miRNA-based therapies: strategies and delivery platforms for oligonucleotide and non-oligonucleotide agents.* Future medicinal chemistry, 2014. **6**(17): p. 1967-1984.

56. Wightman, B., I. Ha, and G. Ruvkun, *Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans.* Cell, 1993. **75**(5): p. 855-862.

57. Rupaimoole, R. and F.J. Slack, *MicroRNA therapeutics: towards a new era for the management of cancer and other diseases.* Nature reviews Drug discovery, 2017. **16**(3): p. 203.

58. Ha, M. and V.N. Kim, *Regulation of microRNA biogenesis.* Nature reviews Molecular cell biology, 2014. **15**(8): p. 509-524.

59. Gregory, R.I., et al., *The Microprocessor complex mediates the genesis of microRNAs.* Nature, 2004. **432**(7014): p. 235-240.

60. Fang, W. and D.P. Bartel, *The menu of features that define primary microRNAs and enable de novo design of microRNA genes.* Molecular cell, 2015. **60**(1): p. 131-145.

61. Nguyen, T.A., et al., *Functional anatomy of the human microprocessor.* Cell, 2015. **161**(6): p. 1374-1387.

62. Treiber, T., N. Treiber, and G. Meister, *Regulation of microRNA biogenesis and its crosstalk with other cellular pathways.* Nature Reviews Molecular Cell Biology, 2019. **20**(1): p. 5-20.

63. Wu, K., et al., *The role of exportin-5 in microRNA biogenesis and cancer. Genom Proteom Bioinf 16: 120–126*. 2018.

64. Kim, Y.-K., B. Kim, and V.N. Kim, *Re-evaluation of the roles of DROSHA, Exportin 5, and DICER in microRNA biogenesis.* Proceedings of the National Academy of Sciences, 2016. **113**(13): p. E1881-E1889.

65. Lau, P.-W., et al., *The molecular architecture of human Dicer.* Nature structural & molecular biology, 2012. **19**(4): p. 436.

66. Noland, C.L. and J.A. Doudna, *Multiple sensors ensure guide strand selection in human RNAi pathways.* Rna, 2013. **19**(5): p. 639-648.

67. Siomi, H. and M.C. Siomi, *On the road to reading the RNA-interference code.* Nature, 2009. **457**(7228): p. 396-404.

68. Schirle, N.T., J. Sheu-Gruttadauria, and I.J. MacRae, *Structural basis for microRNA targeting.* Science, 2014. **346**(6209): p. 608-613.

69. Fromm, B., et al., *A uniform system for the annotation of vertebrate microRNA genes and the evolution of the human microRNAome.* Annual review of genetics, 2015. **49**: p. 213-242.

70. E.Pasquinelli, J.P.B.M.T.L.J.L.H.G.W.Y.A., *Pairing beyond the seed supports microRNA targeting specificity.* Molecular Cell, 2016. **64**(2): p. 320-333.

71. Brancati, G. and H. Großhans, *An interplay of miRNA abundance and target site architecture determines miRNA activity and specificity.* Nucleic acids research, 2018. **46**(7): p. 3259-3269.

72. Bofill-De Ros, X., A. Yang, and S. Gu, *IsomiRs: expanding the miRNA repression toolbox beyond the seed.* Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 2020. **1863**(4): p. 194373.

73. De, N., et al., *Highly complementary target RNAs promote release of guide RNAs from human Argonaute2.* Molecular cell, 2013. **50**(3): p. 344-355.

74. Park, J.H., S.-Y. Shin, and C. Shin, *Non-canonical targets destabilize microRNAs in human Argonautes.* Nucleic acids research, 2017. **45**(4): p. 1569-1583.

75. Gagnon, K.T., et al., *RNAi factors are present and active in human cell nuclei.* Cell reports, 2014. **6**(1): p. 211-221.

76. Wei, Y., et al., *Importin 8 regulates the transport of mature microRNAs into the cell nucleus.* Journal of Biological Chemistry, 2014. **289**(15): p. 10270-10275.

77. Catalanotto, C., C. Cogoni, and G. Zardo, *MicroRNA in control of gene expression: an overview of nuclear functions.* International journal of molecular sciences, 2016. **17**(10): p. 1712.

78. Gebert, L.F. and I.J. MacRae, *Regulation of microRNA function in animals.* Nature reviews Molecular cell biology, 2019. **20**(1): p. 21-37.

79. Berezikov, E., *Evolution of microRNA diversity and regulation in animals.* Nature Reviews Genetics, 2011. **12**(12): p. 846-860.

80. Morin, R.D., et al., *Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells (Genome Research (2008) 18,(610-621)).* Genome research, 2009. **19**(5).

81. Heydarzadeh, S., et al., *Overview of host miRNA properties and their association with epigenetics, long non-coding RNAs, and Xeno-infectious factors.* Cell & Bioscience, 2021. **11**(1): p. 1-17.

82. Zhao, D., *Single nucleotide alterations in MicroRNAs and human cancer-A not fully explored field.* Non-coding RNA research, 2020. **5**(1): p. 27-31.

83. Telonis, A.G., et al., *Beyond the one-locus-one-miRNA paradigm: microRNA isoforms enable deeper insights into breast cancer heterogeneity.* Nucleic acids research, 2015. **43**(19): p. 9158-9175.

84. van der Kwast, R.V., P.H. Quax, and A.Y. Nossent, *An emerging role for isomiRs and the microRNA epitranscriptome in neovascularization.* Cells, 2020. **9**(1): p. 61.

85. Wu, C.W., et al., *A Comprehensive Approach to Sequence-oriented IsomiR annotation (CASMIR): demonstration with IsomiR profiling in colorectal neoplasia.* BMC genomics, 2018. **19**(1): p. 1-12.

86. Tan, G.C., et al., *5′ isomiR variation is of functional and evolutionary importance.* Nucleic acids research, 2014. **42**(14): p. 9424-9435.

87. Magee, R.G., et al., *Profiles of miRNA isoforms and tRNA fragments in prostate cancer.* Scientific reports, 2018. **8**(1): p. 1-13.

88. Loher, P., E.R. Londin, and I. Rigoutsos, *IsomiR expression profiles in human lymphoblastoid cell lines exhibit population and gender dependencies.* Oncotarget, 2014. **5**(18): p. 8790.

89. Cho, S., et al., *MiRGator v3. 0: a microRNA portal for deep sequencing, expression profiling and mRNA targeting.* Nucleic acids research, 2012. **41**(D1): p. D252-D257.

90. Sablok, G., et al., *isomiRex: Web-based identification of microRNAs, isomiR variations and differential expression using next-generation sequencing datasets.* FEBS letters, 2013. **587**(16): p. 2629-2634.

91. Zhang, Y., et al., *CPSS: a computational platform for the analysis of small RNA deep sequencing data.* Bioinformatics, 2012. **28**(14): p. 1925-1927.

92. Pantano, L., X. Estivill, and E. Martí, *SeqBuster, a bioinformatic tool for the processing and analysis of small RNAs datasets, reveals ubiquitous miRNA modifications in human embryonic cells.* Nucleic acids research, 2010. **38**(5): p. e34-e34.

93. Trapnell, C., L. Pachter, and S.L. Salzberg, *TopHat: discovering splice junctions with RNA-Seq.* Bioinformatics, 2009. **25**(9): p. 1105-1111.

94. Barturen, G., et al., *sRNAbench: profiling of small RNAs and its sequence variants in single or multi-species high-throughput experiments.* Methods in Next Generation Sequencing, 2014. **1**(1): p. 21-31.

95. Desvignes, T., et al., *miRNA analysis with Prost! reveals evolutionary conservation of organ-enriched expression and post-transcriptional modifications in three-spined stickleback and zebrafish.* Scientific reports, 2019. **9**(1): p. 1-15.

96. Bofill-De Ros, X., et al., *QuagmiR: a cloud-based application for isomiR big data analytics.* Bioinformatics, 2019. **35**(9): p. 1576-1578.

97. Desvignes, T., et al., *Unification of miRNA and isomiR research: the mirGFF3 format and the mirtop API.* Bioinformatics, 2020. **36**(3): p. 698-703.

98. O'Brien, J., H. Hayder, and Y. Zayed, *Overview of microRNA biogenesis, mechanisms of actions, and circulation, Front. Endocrinol., 2018, vol. 9, no. 402*. 2018.

99. Stavast, C.J. and S.J. Erkeland, *The non-canonical aspects of MicroRNAs: many roads to gene regulation.* Cells, 2019. **8**(11): p. 1465.

100. Havens, M.A., et al., *Biogenesis of mammalian microRNAs by a non-canonical processing pathway.* Nucleic acids research, 2012. **40**(10): p. 4626-4640.

101. Annese, T., et al., *microRNAs Biogenesis, Functions and Role in Tumor Angiogenesis.* Frontiers in Oncology, 2020. **10**.

102. Mori, M.A., et al., *Extracellular miRNAs: from biomarkers to mediators of physiology and disease.* Cell metabolism, 2019. **30**(4): p. 656-673.

103. McKelvey, K.J., et al., *Exosomes: mechanisms of uptake.* Journal of circulating biomarkers, 2015. **4**: p. 7.

104. Zhang, J., et al., *Exosome and exosomal microRNA: trafficking, sorting, and function.* Genomics, proteomics & bioinformatics, 2015. **13**(1): p. 17-24.

105. Michell, D.L. and K.C. Vickers, *Lipoprotein carriers of microRNAs.* Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 2016. **1861**(12): p. 2069-2074.

106. Vickers, K.C., et al., *MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins.* Nature cell biology, 2011. **13**(4): p. 423-433.

107. Rüegger, S. and H. Großhans, *MicroRNA turnover: when, how, and why.* Trends in biochemical sciences, 2012. **37**(10): p. 436-446.

108. Kato, M., *Target RNA-directed microRNA degradation; which controls which?* Non-coding RNA investigation, 2018. **2**.

10. Supplements

10.1. Copies of published articles related to the topic of this doctoral thesis

I. Matoušková, P., **Hanousková, B**., & Skálová, L. (2018). MicroRNAs as potential regulators of glutathione peroxidases expression and their role in obesity and related pathologies. *International journal of molecular sciences*, *19*(4), 1199. Review. (IF 2018: **4,355**)

II. **Hanousková, B.**, Vávrová, G., Ambrož, M., Boušová, I., Karlsen, TA., Skálová, L., & Matoušková, P. (2021) MicroRNAs mediated regulation of glutathione peroxidase 7 expression and its changes during adipogenesis. *BBA – gene regulatory mechanisms*. Accepted (IF: **4,49**)

III. **Hanousková, B.**, Neprašová, B., Skálová, L., Maletínská, L., Zemanová, K., Ambrož, M., & Matoušková, P. (2019). High-fructose drinks affect microRNAs expression differently in lean and obese mice. *The Journal of nutritional biochemistry*, *68*, 42-50. (IF 2019: **4,873**)

IV. Skála, M., **Hanousková, B.**, Skálová, L., & Matoušková, P. (2019). MicroRNAs in the diagnosis and prevention of drug-induced cardiotoxicity. *Archives of toxicology*, *93*(1), 1-9. Review (IF: **5,741**)

V. **Hanousková, B.**, Skála, M., Brynychová, V., Zárybnický, T., Skarková, V., Kazimírová, P., ... & Matoušková, P. (2019). Imatinib-induced changes in the expression profile of microRNA in the plasma and heart of mice - A comparison with doxorubicin. *Biomedicine & Pharmacotherapy*, *115*, 108883. (IF: **4,545**)