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Doctoral Thesis

Small noncoding RNAs and their prognostic value in myelodysplastic syndromes

Malé nekódující RNA a jejich prognostický význam u myelodysplastického syndromu

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PROHLÁŠENÍ

Prohlašuji, že jsem tuto práci zpracovala samostatně a že jsem uvedla všechny použité zdroje informací. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze

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1 CONTENTS

2 LIST OF ABBREVIATIONS

AGO2	argonaute 2
AKAP12	A-kinase anchoring protein 12
AML	acute myeloid leukaemia
AML-MRC	acute myeloid leukaemia with myelodysplasia-related changes
BM	bone marrow
CDR	commonly deleted region
CEBPA	CCAAT enhancer binding protein alpha, transcription factor
CLL	chronic lymphocytic leukaemia
CPX-351	liposomal daunorubicin-cytarabine
CR	complete remission
c-FOS	proto-oncogene, the human homolog of the retroviral oncogene v-FOS
c-MYB	avian myeloblastosis virus oncogene cellular homolog
ddPCR	droplet digital PCR
DRAM1	damage regulator autophagy modulator 1
ECM	extracellular matrix
ERK1	extracellular signal-regulated kinase 1, also known as mitogen-activated protein kinase 3 (MAPK3)
ERK2	extracellular signal-regulated kinase 2, also known as mitogen-activated protein kinase 1 (MAPK1)
EVs	extracellular vesicles
FLI1	friend leukaemia virus integration 1 transcription factor
HDL	high density lipoproteins
HI	haematologic improvement
HSCs	haematopoietic stem cells
HSCT	haematopoietic stem cell transplantation
HSPCs	haematopoietic stem/progenitor cells
IHBT	Institute of Hematology and Blood Transfusion
lncRNAs	long noncoding RNAs

MCL1	myeloid cell leukaemia sequence 1
mCR	marrow complete response
MDS	myelodysplastic syndromes
MDS-EB	MDS with excess blasts
MDS-MLD	MDS with multilineage dysplasia
MDS-RS	MDS with ring sideroblasts
MDS-SLD	MDS with single lineage dysplasia
MDS-U	MDS unclassifiable
miRNAs	microRNAs
mtDNA	mitochondrial DNA
MTHFD2	methylenetetrahydrofolate dehydrogenase 2
NF-κB	nuclear factor-κB
NGS	next generation sequencing
NPM1	nucleophosmin 1
ORR	overall response rate
OS	overall survival
PB	peripheral blood
PD	progressed disease
PFS	progression-free survival
piRNAs	piwi-interacting RNAs
PR	partial remission
PTEN	tumour supressor, phosphatase and tensin homologue deleted on chromosome 10
PUS7	pseudouridine synthase 7
qPCR	quantitative PCR
RA	refractory anaemia
RAEB	refractory anaemia with excess blasts
RARS	refractory anaemia with ring sideroblasts
RCMD	refractory cytopenia with multilineage dysplasia
RCUD	refractory cytopenia with unilineage dysplasia
RISC	RNA-induced silencing complex

RN	refractory neutropenia
RPS14	ribosomal protein S14
RT	refractory thrombocytopenia
RT-qPCR	reverse transcription quantitative PCR
SD	stable disease
siRNAs	small interfering RNAs
SMAD7	mothers against decapentaplegic homolog 7
sncRNAs	small noncoding RNAs
snoRNAs	small nucleolar RNAs
snRNAs	small nuclear RNAs
TET2	tet methylcytosine dioxygenase 2
TGF-β	transforming growth factor β
TIRAP	toll-interleukin-1 receptor domain-containing adaptor protein
TNF-α	tumour necrosis factor α
TRAF6	tumour necrosis factor receptor-associated factor-6
tRNAs	transfer RNAs
tsRNAs	tRNA-derived small RNAs
TWIST1	twist basic helix-loop-helix transcription factor 1

3 ABSTRACT

Myelodysplastic syndromes (MDS) are a heterogeneous group of bone marrow (BM) disorders characterized by ineffective haematopoiesis, BM dysplasia, and peripheral blood cytopenia. In recent years, substantial progress has been made towards understanding the molecular pathogenesis of MDS that has brought new possibilities in MDS diagnostics, prognostics, and treatment.

Small noncoding RNAs (sncRNAs), especially microRNAs (miRNAs), are in the field of scientific interest in terms of their expression, function, role in disease development, and potential utilization as disease biomarkers. Special attention has been focused on extracellular sncRNAs present in blood circulation, so called ‘circulating’ sncRNAs, which may become easily accessible biomarkers of disease state or risk of progression.

We have conducted several studies on intracellular and extracellular sncRNA profiles of CD34+ BM cells and blood plasma, respectively, from MDS patients using microarrays or next generation sequencing (NGS). We aimed to identify specific sncRNA profiles associated with MDS and search for sncRNA biomarkers predictive of the patient prognosis and response to treatment with azacitidine (AZA). Another goal was to characterize and compare circulating sncRNA profiles of two different extracellular materials, total plasma and plasma-derived extracellular vesicles (EVs), in order to determine their usefulness as sources of MDS biomarkers.

Initially, using microarrays, we identified significantly lower levels of miR-27a-3p, miR-199a-5p, and miR-223-3p in total plasma of higher-risk MDS patients compared to lower-risk MDS patients. Further analyses indicated that the low levels of miR-223-3p and miR-451 are associated with unfavourable overall survival (OS) and progression-free survival, respectively.

Using NGS, we found other deregulated sncRNAs, including non-miRNA species, between early and advanced stages of MDS. We observed increased levels of many circulating miRNAs related to haematopoiesis (e. g. miR-103a-3p, miR-103b, miR-107, miR-221-3p, miR-221-5p, and miR-130b-5p) and miRNAs located in chromosomal region 14q32 (e.g. miR-127-3p, miR-154-5p, miR-323b-3p, miR-382-3p, miR-409-5p, and miR-485-3p) in early MDS compared to advanced MDS.

We defined a signature of four sncRNAs (miR-1237-3p, U33, hsa_piR_019420, and miR-548av-5p) whose EV levels were the most significantly associated with OS. Further, a combined score of five plasma miRNAs (miR-423-5p, miR-126-3p, miR-151a-3p, miR-125a-5p, and miR-199a-3p) was determined as a predictor of response to AZA treatment. In CD34+ BM cells, the high level of miR-17-3p and low levels of miR-100-5p and miR-133b before treatment were associated with favourable overall response rate to AZA therapy. Moreover, miR-100-5p was found as a predictor of survival, its low level before treatment associated with favourable OS in AZA treated patients.

Regarding the two extracellular materials, total plasma and EVs, hierarchical cluster analysis showed that RNA content of EV samples is more homogeneous than that of total plasma samples. Further, substantially higher number of deregulated sncRNAs between these two materials was found in MDS patients than in control counterparts.

In conclusion, our results demonstrate distinct sncRNA profiles in total plasma, EVs, and CD34+ cells of MDS patients. These profiles are specific for distinct MDS stages and may predict patient outcome. We identified several sncRNAs, mostly miRNAs, that are associated with patient survival and response to AZA therapy and thus, may be considered as potential biomarkers of the disease.

4 ABSTRAKT

Myelodysplastický syndrom (MDS) je heterogenní onemocnění vyznačující se nedostatečnou krvetvorbou, dysplazií kostní dřeně a cytopenií jedné nebo více krevních řad v periferní krvi. Současný pokrok v porozumění patogenezi MDS na molekulární úrovni přináší nové možnosti v oblasti stanovení diagnózy, prognózy a léčby tohoto onemocnění.

Malé nekódující RNA (sncRNA), obzvláště mikroRNA (miRNA), jsou středem zájmu v oblasti výzkumu jejich funkce, exprese, úlohy při vzniku a vývoji onemocnění a také jejich možného využití coby biomarkerů onemocnění. V poslední době je pozornost věnována také extracelulárním sncRNA a to obzvláště těm, které jsou přítomny v krevním oběhu, tzv. „cirkulujícím“ sncRNA. Cirkulující sncRNA jsou považovány za snadno dostupné potenciální biomarkery stavu onemocnění a rizika progrese.

Základem této disertační práce je několik studií zabývajících se profily sncRNA u MDS pacientů. V rámci těchto studií jsme vyšetřovali jak profily intracelulárních sncRNA z CD34+ buněk kostní dřeně, tak profily extracelulárních sncRNA z celkové krevní plasmy a z extracelulárních vesikulů vyizolovaných z plasmy. K analýze jsme využili tzv. „high-throughput“ technologie, konkrétně microarrays a sekvenování nové generace (NGS). Cílem bylo určit profily sncRNA charakterizující MDS a nalézt konkrétní molekuly, které by byly schopné předpovědět další vývoj onemocnění a kvalitu odpovědi na léčbu azacitidinem (AZA). Také jsme porovnali profily cirkulujících sncRNA ze dvou extracelulárních materiálů (tj. z celkové plasmy a extracelulárních vesikulů) s cílem zjistit, jestli jsou vhodnými zdroji sncRNA biomarkerů pro MDS.

Pomocí microarrays jsme nalezli významně snížené hladiny miR-27a-3p, miR-199a-5p a miR-223-3p v celkové plasmě pacientů s vysokorizikovým MDS oproti pacientům s nízkorizikovým MDS. Další analýzy ukázaly, že nízká hladina miR-223-3p předpovídá kratší dobu celkového přežití, zatímco nízká hladina miR-451 naznačuje kratší dobu do progrese onemocnění.

Pomocí NGS jsme nalezli další sncRNA s odlišnými hladinami mezi pacienty s časnými a pokročilými stadii MDS. Kromě miRNA jsme zachytili i jiné druhy sncRNA. U pacientů s pokročilejšími stadii MDS jsme pozorovali zvýšené hladiny mnoha cirkulujících miRNA, které se účastní regulace krvetvorby (např. miR-103a-3p, miR-103b, miR-107, miR-221-3p,miR-221-5p a miR-130b-5p) a také miRNA lokalizovaných v chromosomální oblasti 14q32 (např. miR-127-3p, miR-154-5p, miR-323b-3p, miR-382-3p, miR-409-5p a miR-485-3p).

Identifikovali jsme čtyři sncRNA (miR-1237-3p, U33, hsa_piR_019420 a miR-548av-5p), jejichž kombinované skóre vypočtené z naměřených hladin v extracelulárních vesikulech má spojitost s trváním celkového přežití pacientů. Dále jsme určili pět miRNA (miR-423-5p, miR-126-3p, miR-151a-3p, miR-125a-5p a miR-199a-3p) z celkové plasmy, jejichž kombinované skóre předpovídá kvalitu odpovědi na léčbu AZA. V CD34+ buňkách kostní dřeně jsme pozorovali, že vysoká hladina miR-17-3p a nízké hladiny miR-100-5p a miR-133-b stanovené u pacientů před léčbou AZA ukazují na příznivou odpověď pacientů na tuto léčbu. Nízká hladina miR-100-5p před léčbou AZA navíc naznačuje delší celkové přežití léčených pacientů.

Co se týče porovnání dvou extracelulárních materiálů, tj. celkové plasmy a extracelulárních vesikulů, klastrovací analýza ukázala, že obsah RNA je více homogenní u vzorků extracelulárních vesikulů oproti vzorkům celkové plasmy. Dále jsme zaznamenali podstatně více sncRNA s odlišnými hladinami mezi těmito dvěma materiály u MDS pacientů oproti zdravým jedincům.

Z našich výsledků vyplývá, že celková plasma, extracelulární vesikuly izolované z plasmy a CD34+ buňky kostní dřeně MDS pacientů mají rozdílné profily sncRNA. Tyto profily jsou specifické pro různá stadia MDS a mohou předpovídat další vývoj onemocnění. Určili jsme několik sncRNA, většinou miRNA, jejichž deregulované hladiny souvisí s přežitím pacientů a odpovědí na léčbu AZA. Tyto sncRNA tedy mohou být považovány za potenciální biomarkery předpovídající další vývoj onemocnění u pacientů s MDS.

5 PREFACE

Myelodysplastic syndromes (MDS) are a group of bone marrow (BM) disorders characterized by ineffective haematopoiesis leading to low numbers of mature blood cells. Substantial progress in understanding of MDS pathogenesis has been made in recent years. A vast literature has emerged regarding the spectrum of cytogenetic abnormalities, gene mutations, epigenetic modifications, gene expression patterns, and signalling pathways associated with the disease.

Small noncoding RNAs (sncRNAs) include various species of endogenous RNAs. They are implicated in regulation of essential cellular processes. Cumulative evidence has shown that sncRNAs represent important regulators of haematopoiesis and their deregulation is also implicated in the pathogenesis of MDS. Generally, sncRNAs are considered to be potentially promising molecular biomarkers of disease development.

We performed a comprehensive analysis of sncRNA profiles in all MDS subtypes and searched for sncRNAs with specific expression patterns that may be of clinical relevance in terms of diagnostics, prognostics, and prediction of treatment response. These sncRNAs as auxiliary biomarkers may contribute to better disease and therapy management, prediction of patient outcome, and prevention of the burden associated with ineffective drug application. The improvement in all these aspects would support a personalized approach to the MDS patients.

The analyses were performed on different materials, i.e. CD34+ BM progenitor cells and blood plasma. The CD34+ cells are pivotal in the MDS pathogenesis and provide insight into the complete transcriptome of cellular sncRNAs specific for the disease. Identification of deregulated sncRNAs in the CD34+ cell population may reveal other factors contributing to the disease development or progression. However, the aspiration of BM is an invasive process carrying a risk of complication, such as persistent bleeding and infection. Thus, less invasive approach for continuous monitoring of patient condition would be beneficial. Extracellular sncRNAs circulating in blood (so called 'circulating' sncRNAs) meet this requirement and have become the centre of interest in the field of noninvasive biomarkers. In this context, we analysed circulating sncRNA profiles in different fractions of blood, i.e. total blood plasma and separated extracellular vesicles (EVs) circulating in plasma. It is the very first study comparing sncRNA profiles in paired samples of the two extracellular materials (total plasma and EVs) in MDS.

For investigation, we used high-throughput technologies, specifically microarrays and next generation sequencing (NGS). The initial studies were performed by microarray profiling, enabling detection of thousands of sncRNA transcripts limited only to known sequences provided by databases (predominantly microRNAs). In the most recent study, due to easier access, we have employed the NGS technology, allowing the capture of complete range of RNA transcripts present in a sample and identification of novel sncRNA molecules. Together with the advantage of its wide dynamic range, NGS provides a global view on the complexity of sncRNA patterns and a deeper insight into the molecular background of MDS.

This thesis summarizes the results from three publications (listed below), which analyse sncRNA profiles across MDS subtypes in BM cells, total plasma, and plasma-derived EVs. The thesis provides the novel data on sncRNAs in MDS, and explores the possibility of utilizing sncRNAs as molecular biomarkers of MDS.

Publications that underlie this thesis

This thesis is based on the following publications. They are listed in such order that keeps the continuity of the thesis, not according to the year they were published. In the text they are referred to as P1, P2, and P3 for Publication 1, Publication 2, and Publication 3, respectively.

Publication 1

Dostalova Merkerova M, **Hrustincova A**, Krejcik Z, Votavova H, Ratajova E, Cermak J, Belickova M. Microarray profiling defines circulating microRNAs associated with myelodysplastic syndromes. Neoplasma. 2017;64(4):571–8. PMID: 28485163

Publication 2

Hrustincova A, Krejcik Z, Kundrat D, Szikszai K, Belickova M, Pecherkova P, Klema J, Vesela J, Hruba M, Cermak J, Hrdinova T, Krijt M, Valka J, Jonasova A, Dostalova Merkerova M. Circulating Small Noncoding RNAs Have Specific Expression Patterns in Plasma and Extracellular Vesicles in Myelodysplastic Syndromes and Are Predictive of Patient Outcome. Cells. 2020 Mar 26;9(4):794. PMID: 32224889

Publication 3

Krejcik Z, Belickova M, **Hrustincova A**, Votavova H, Jonasova A, Cermak J, Dyr J E, Dostalova Merkerova M. MicroRNA profiles as predictive markers of response to azacitidine therapy in myelodysplastic syndromes and acute myeloid leukemia. Cancer Biomarkers. 2018;22(1):101–10. PMID: 29630523

6 NOTE

The term 'sncRNAs' used in this thesis stands for multiple sncRNA species (e.g. miRNAs, piRNAs, and tsRNAs), which are described in the Introduction. Actually, most of the information and results in this thesis cover only miRNAs, because miRNAs are the species that have opened a new perspective on noncoding RNA functions and have been exclusively studied up to recently. Due to NGS, data on other sncRNA species have emerged and these species have also become of scientific interest. Some of our results include data on other sncRNA species and they are introduced and discussed as well. Thus, for simplification, the general term 'sncRNAs' is used throughout the thesis when appropriate.

7 INTRODUCTION

7.1 Myelodysplastic syndromes

MDS are a heterogeneous group of clonal disorders of haematopoietic stem cells (HSCs) and their microenvironment, characterized by ineffective haematopoiesis, and manifested by BM dysplasia and peripheral cytopenia in one or more lineages. The incidence ranges from 3 to 4 per 100,000 per year and increases with age. Rates per 100,000 per year in older patients increase from 7 in those aged 60 to 69 years to 35 in those aged over 80 years (1). MDS carry considerable risk of transformation to acute myeloid leukaemia (AML). On average, 30 percent of MDS patients develop AML during the course of the disease (2).

7.1.1 Pathogenesis

MDS are clonal disorders of HSCs caused by various genetic abnormalities. Chromosomal aberrations, gene mutations, copy number alterations, and abnormal gene expression are common in MDS. The initial abnormality leads to the formation of a pathological HSC clone with a certain growth advantage over its normal counterparts, resulting in a clonal haematopoiesis in BM. Increased genome instability of the abnormal HSC clone leads to acquisition of further aberrations. Additional abnormal clones can appear during the disease course and are associated with worsening conditions (3).

Cytogenetic abnormalities are present in 35 to 50 percent of *de novo* MDS cases (4). It is well established that they are very heterogeneous in MDS. The most frequent chromosomal abnormalities in MDS are del(5q), del(7q)/-7, +8, del(11q), del(12p), del(17p), del(20q), and -Y (5). Hundreds of rare cytogenetic abnormalities have been reported in MDS, including for example 3q abnormalities, +13/del(13q), i(17q), +21/–21, and –X (6). Importantly, cytogenetic patterns are not stable in MDS, and a substantial number of patients acquire additional cytogenetic aberrations during the course of the disease, worsening the prognosis and increasing the risk of transformation to AML (7).

Gene mutations participating in the MDS pathogenesis and progression affect RNA splicing machinery, DNA methylation, histone modifications, transcription factors, signal transduction proteins, and components of the cohesion complex. Mutations in RNA splicing and DNA methylation genes occur early and are considered founding mutations, whereas others that occur later are regarded as subclonal mutations (8).

In MDS, the malignant cellular population with impaired ability to differentiate (9) rapidly expands, however hyperproliferation is accompanied by excessive apoptosis (10,11). This leads to insufficient peripheral blood (PB) cell count, i.e. cytopenia, in one or more lineages (anaemia, thrombocytopenia, and neutropenia) that is variable in degree and combination depending on the lineages most affected by increased levels of apoptosis. The discrepancy between hyperproliferation in BM and cytopenias in PB is the characteristic feature of early MDS. During MDS progression, the induction of apoptosis is decreased (12,13), resulting in gradual increase of blast number in BM and often transformation towards AML.

Previous studies reported that AML evolves from an ancestral MDS subclone that acquires further genetic mutations mostly in a linear and hierarchical manner (14,15). Recent results by Chen et al. (16), however, suggest a model of non-linear clonal evolution arising from the stem cell level. Accumulation of mutations in stem cell compartments gives rise to a highly diverse subclonal architecture in MDS stem cells. Certain subclones provide a shared basis for both MDS development as well as AML transformation. Formation of MDS blasts or AML stem cell is then driven by different additional mutations in a non-linear and rather parallel manner (16).

Further, chronic inflammation plays a critical role in the MDS pathogenesis and may act as a trigger of MDS development (17). The inflammatory signalling within the BM microenvironment may drive the functional repression of normal HSCs and subsequently the induction, selection, and expansion of mutant clones that are likely to malignant transformation. This indicates that MDS should be considered a disease of tissue rather than a disease of haematopoietic cells in isolation (18).

7.1.2 Diagnosis and classification

The risk of MDS increases with advancing age. The typical age at diagnosis of MDS is between 60 and 75 years. Diagnoses are rare in adults under 50 years and children. Males are slightly more commonly affected than females. Clinical presentation of MDS is nonspecific and varies considerably depending on subtypes and severity of cytopenias. Symptoms are connected to the most affected cell lineages and may include fatigue, weakness, pallor (secondary to anaemia), infections and fever (secondary to neutropenia), and increased bleeding and bruising (secondary to thrombocytopenia). The diagnosis is generally suspected based on the presence of an abnormal complete blood count and is confirmed by performing BM aspiration and biopsy demonstrating morphological evidence of dysplasia. A number of additional tests, including cytogenetics, flow cytometry and molecular genetics, are needed to complete the laboratory evaluation of patients with MDS (19). Dysplastic changes are the most important diagnostic features of MDS. A BM cell lineage is considered picture of MDS if more than ten percent of cells are affected. Cytogenetic examination of BM aspirate plays a key role in determining clonality of cells (20). In the appropriate clinical context, some cytogenetic abnormalities are sufficient evidence to determine MDS diagnosis. In addition, cytogenetics is also an important tool to assess prognosis and to choose the most effective form of therapy (4).

MDS are very heterogeneous group of disorders with substantial diversity of conditions across the entire spectrum of MDS. Moreover, the boundaries between MDS and related myeloid disorders can be vague and difficult to characterize (21). To improve the specificity of the diagnosis and prognosis, classification systems have been developed.

According to the World Health Organization (WHO), MDS subtype classification is based on determination of the blast cell percentage in BM and PB, PB cytopenias, presence or absence of ringed sideroblasts in BM (sideroblasts are erythroblasts with iron-loaded mitochondria found exclusively in pathological conditions (22)), presence of specific chromosomal abnormalities, and cytogenetic detection of del(5q). The most emphasized criteria for classification are the degree of dysplasia and blast percentages, whereas specific cytopenias have only minor impact on MDS classification. The WHO classification guidelines from 2008 (23) define following MDS subtypes: refractory cytopenia with unilineage dysplasia (RCUD, comprising refractory anaemia (RA), refractory neutropenia (RN), and refractory thrombocytopenia (RT)), refractory anaemia with ring sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), refractory anaemia with excess blasts 1 and 2 (RAEB-1, RAEB-2), MDS with isolated del(5q), and MDS unclassifiable (MDS-U). The subtypes are described in Table 1 (23). The last edition of the WHO classification guidelines from 2016 (24) refines MDS subtypes as follows: MDS with single lineage dysplasia (MDS-SLD), MDS with multilineage dysplasia (MDS-MLD), MDS with ring sideroblasts (MDS-RS), MDS with excess blasts (MDS-EB), MDS with isolated del(5q), and MDS unclassifiable (MDS-U). The subtypes are described in Table 2 (24).

Table 1. MDS classification according to the WHO guidelines from 2008 (23). Peripheral blood (PB) and bone marrow (BM) findings in particular MDS subtypes.

MDS subtype	**PB findings**	**BM findings**
Refractory cytopenia with unilineage dysplasia (RCUD): refractory anaemia (RA), refractory neutropenia (RN), refractory thrombocytopenia (RT)	Unicytopenia or bicytopenia*[1], no or rare blasts (< 1 %)*[2]	Unilineage dysplasia: ≥ 10 % of the cells in one myeloid lineage, < 5 % blasts, < 15 % of erythroid precursors are ring sideroblasts
Refractory anaemia with ring sideroblasts (RARS)	Anaemia, no blasts	≥ 15 % of erythroid precursors are ring sideroblasts, erythroid dysplasia only, < 5 % blasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenia(s), no or rare blasts (< 1 %)*[2], no Auer rods*[3], < 1 × 10^9/l monocytes	Dysplasia in ≥ 10 % of the cells in two or more myeloid lineages (neutrophil and/or erythroid precursors and/or megakaryocytes), < 5 % blasts in BM, no Auer rods, ± 15 % ring sideroblasts
MDS with isolated del(5q)	Anaemia, usually normal or increased platelet count, no or rare blasts (< 1 %)	Normal to increased megakaryocytes with hypolobated nuclei, < 5 % blasts, isolated del(5q) cytogenetic abnormality, no Auer rods
Refractory anaemia with excess blasts-1 (RAEB-1)	Cytopenia(s), < 5 % blasts*[2], no Auer rods, < 1 × 10^9/l monocytes	Unilineage or multilineage dysplasia, 5 %-9 % blasts*[2], no Auer rods
Refractory anaemia with excess blasts-2 (RAEB-2)	Cytopenia(s), 5 %-19 % blasts*[4], Auer rods present or not*[4], < 1 × 10^9/l monocytes	Unilineage or multilineage dysplasia, 10 %-19 % blasts*[4], Auer rods present or not*[4]
MDS unclassifiable (MDS-U)	Cytopenias, < 1 % blasts*[2]	Unequivocal dysplasia in < 10 % of cells in one or more myeloid lineages when accompanied by a cytogenetic abnormality considered as presumptive evidence for a diagnosis of MDS, < 5 % blasts

*[1] Bicytopenia may occasionally be observed. Cases with pancytopenia should be classified as MDS-U.

*[2] If the BM blast percentage is < 5 % but there are 2-4 % blasts in PB, the diagnostic classification is RAEB-1. Cases of RCUD and RCMD with 1 % blasts in PB should be classified as MDS-U.

*[3] Rod-shaped inclusions formed by crystallisation of cytoplasmic azurophilic granules (25).

*[4] Cases with Auer rods and < 5 % blasts in PB and < 10 % in BM should be classified as RAEB-2. Although the finding of 5-19 % blasts in PB is diagnostic of RAEB-2, cases of RAEB-2 may have < 5 % blasts in PB if they have Auer rods or 10-19 % blasts in BM or both. Similarly, cases of RAEB-2 may have < 10 % blasts in BM but may be diagnosed by the other two findings, Auer rods and/or 5-19 % blasts in PB.

Table 2. MDS classification according to the WHO guidelines from 2016 (24). Peripheral blood (PB) and bone marrow (BM) findings and cytogenetics in particular MDS subtypes.

MDS subtype	Dysplastic lineages	Cytopenias*1	Ring sideroblasts as % of BM erythroid elements	BM and PB blasts	Cytogenetics by conventional karyotype analysis
MDS with single lineage dysplasia (MDS-SLD)	1	1 or 2	< 15 %/< 5 %*2	BM < 5 %, PB < 1 %, no Auer rods*3	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with multilineage dysplasia (MDS-MLD)	2 or 3	1-3	< 15 %/< 5 %*2	BM < 5 %, PB < 1 %, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with ring sideroblasts (MDS-RS)					
MDS-RS with single lineage dysplasia (MDS-RS-SLD)	1	1 or 2	≥ 15 %/ ≥ 5 %*2	BM < 5 %, PB < 1 %, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS-RS with multilineage dysplasia (MDS-RS-MLD)	2 or 3	1-3	≥ 15 %/ ≥ 5 %*2	BM <5 %, PB <1 %, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with isolated del(5q)	1-3	1-2	None or any	BM < 5 %, PB < 1 %, no Auer rods	del(5q) alone or with 1 additional abnormality except −7 or del(7q)

Table 2.

MDS subtype	Dysplastic lineages	Cytopenias*[1]	Ring sideroblasts as % of BM erythroid elements	BM and PB blasts	Cytogenetics by conventional karyotype analysis
MDS with excess blasts (MDS-EB)					
MDS-EB-1	0-3	1-3	None or any	BM 5-9 % or PB 2-4 %, no Auer rods	Any
MDS-EB-2	0-3	1-3	None or any	BM 10-19 % or PB 5-19 % or Auer rods	Any
MDS unclassifiable (MDS-U)					
with 1 % blood blasts	1-3	1-3	None or any	BM < 5 %, PB = 1 %*[4], no Auer rods	Any
with single lineage dysplasia and pancytopenia	1	3	None or any	BM < 5 %, PB < 1 %, no Auer rods	Any
based on defining cytogenetic abnormality	0	1-3	< 15 %*[5]	BM < 5 %, PB < 1 %, no Auer rods	MDS-defining abnormality

*[1] Cytopenias defined as: haemoglobin, < 10 g/dl; platelet count, < 100 × 10^9/l; and absolute neutrophil count, < 1.8 × 10^9/l. Rarely, MDS may present with mild anaemia or thrombocytopenia above these levels. PB monocytes must be < 1 × 10^9/l.
*[2] If SF3B1 mutation is present.
*[3] Rod-shaped inclusions formed by crystallisation of cytoplasmic azurophilic granules (25).
*[4] One percent PB blasts must be recorded on at least two separate occasions.
*[5] Cases with ≥ 15 % ring sideroblasts by definition have significant erythroid dysplasia, and are classified as MDS-RS-SLD.

MDS can also be classified according to their prognostic variables. To assess prognosis of primary untreated adult patients with MDS, the International Prognostic Scoring System (IPSS) was generated in 1997 (26). Based on cytogenetics, percentage of BM blasts and number of cytopenias (Table 3), the IPSS predicted disease outcome for survival and evolution to AML (Table 4). In 2012, the IPSS was refined and the Revised International Prognostic Scoring System (IPSS-R) was developed (27,28). The IPSS-R takes into account the risk of specific cytogenetic abnormalities present (Table 5), the number and degree of cytopenias, and proportion of blasts in BM. Score values for each prognostic variable are evaluated (Table 6) and the total score determines the risk category (Table 7). The IPSS-R stratifies patients into five risk categories: very low, low, intermediate, high, and very high. The two lowest categories are often referred to as 'lower-risk', whereas the two highest categories are referred to as 'higher-risk' MDS. The intermediate category is heterogeneous with some patient characteristics similar to lower-risk MDS and others to higher-risk MDS. Kaplan-Meier curves for clinical outcomes of patients within the five IPSS-R categories are shown in Figure 1 (27).

To see how well prognostication or treatment work, overall survival (OS), progression-free survival (PFS), and overall response rate (ORR) are measured.

Table 3. IPSS prognostic score values (26).

Prognostic variable	**Score value**				
	0	**0.5**	**1**	**1.5**	**2**
BM blasts (%)	< 5	5-10	—	11-20	21-30
Cytopenia*[1]	0-1	2-3			
Cytogenetics*[2]	Good	Intermediate	Poor		

— indicates not applicable
*[1] Number of lineages affected by cytopenia. Cytopenias defined as: haemoglobin, < 10 g/dl; platelet count, < 100 × 10^9/l; and absolute neutrophil count, < 1.8 × 10^9/l.
*[2] Good: normal, -Y, -20q, -5q, Intermediate: all other, Poor: chromosome 7 aberration and/or ≥ 3 chromosomal aberrations.

Table 4. IPSS risk categories, scores, and clinical outcomes (26).

IPSS category	IPSS score	Overall survival*[1]		AML 25% evolution*[2]	
		age at diagnosis (years)			
		≤ 70	> 70	≤ 70	> 70
Low	0	9	3.9	> 9.4 (NR)	> 5.8 (NR)
Intermediate-1	0.5-1.0	4.4	2.4	5.5	2.2
Intermediate-2	1.5-2	1.3	1.2	1.0	1.4
High	> 2.5	0.4	0.4	0.2	0.4

*[1] medians, years
*[2] median time (years) to 25% AML evolution
NR indicates not reached

Table 5. MDS cytogenetic scoring system for IPSS-R (27).

Cytogenetic prognostic subgroups	Cytogenetic abnormalities
Very good	-Y, del(11q)
Good	Normal, del(5q), del(12p), del(20q), double including del(5q)
Intermediate	del(7q), +8, +19, i(17q), any other single or double independent clones
Poor	-7, inv(3)/t(3q)/del(3q), double including -7/del(7q), complex: 3 abnormalities
Very poor	> 3 abnormalities

Table 6. IPSS-R prognostic score values (27).

Prognostic variable	Score value						
	0	**0.5**	**1**	**1.5**	**2**	**3**	**4**
Cytogenetics	Very good	—	Good	—	Intermediate	Poor	Very poor
BM blasts (%)	≤ 2	—	>2 - <5	—	5-10	> 10	—
Hemoglobin (g/dl)	≥ 10	—	8 - < 10	< 8	—	—	—
Platelets (x10^9/l)	≥ 100	50 - < 100	< 50	—	—	—	—
Neutrophils (x10^9/l)	≥ 0.8	< 0.8	—	—	—	—	—

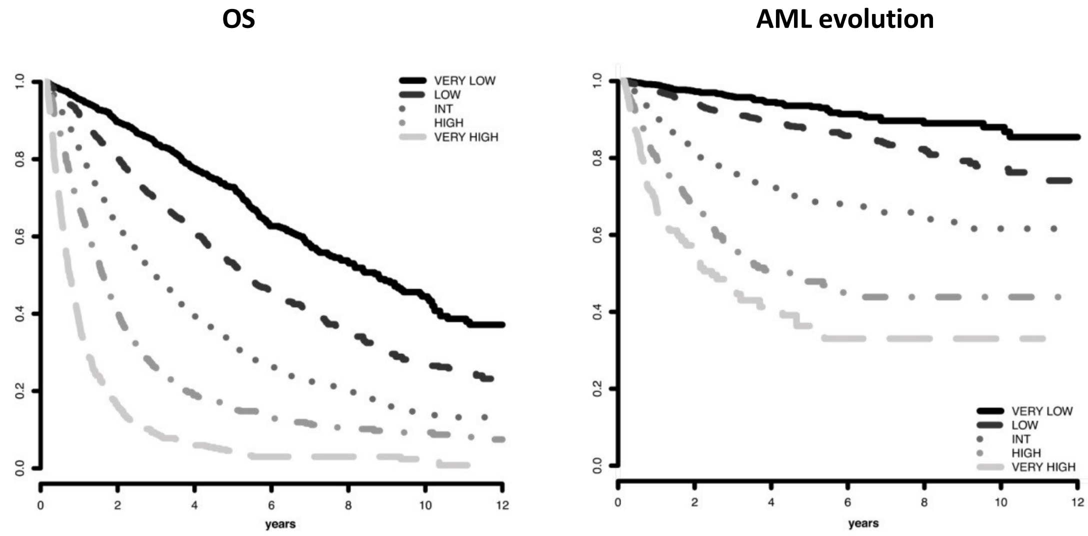
— indicates not applicable

Table 7. IPSS-R risk categories, scores, and clinical outcomes (27).

IPSS-R category	IPSS-R score	Overall survival*[1]	AML 25%*[2]
Very low	≤ 1.5	8.8	NR
Low	> 1.5-3	5.3	10.8
Intermediate	> 3-4.5	3	3.2
High	> 4.5-6	1.6	1.4
Very high	> 6	0.8	0.7

*[1] medians, years
*[2] median time (years) to 25% AML evolution
NR indicates not reached

Figure 1. OS duration (left) and time to AML evolution (right) for patients within the five IPSS-R categories (27).



7.1.3 Somatic mutations

Application of high throughput technologies such as next generation sequencing (NGS) has identified recurrent somatic mutations in MDS cells. Somatic mutations appear to have a great impact on MDS progression and prognosis, indicating their potential to contribute to better diagnosis and prognosis prediction. In 80 to 90 percent of MDS patients, recurring somatic mutations have been found in a number of genes involved in transcriptional regulation (TP53, RUNX1, GATA2, ETV6), signal transduction (JAK2, KRAS, CBL), DNA methylation (DNMT3A, TET2, IDH1/2), chromatin modification (EZH2, ASXL1), and RNA splicing (SF3B1, U2AF1, SRSF2) (29,30). Many specific mutations are strongly associated with the disease outcome in MDS, and the addition of mutation data improves the prognostic value of existing risk-stratification schemes in MDS (31). In particular, point mutations in TP53, EZH2, ETV6, RUNX1 and ASXL1 genes have been shown to be associated with specific clinical features and poor OS, independent of established risk factors (32). For example, TP53 mutations are associated with an aggressive course of MDS in general and appear to predict poorer response to lenalidomide treatment in patients with del(5q) (33). Evaluation for TP53 mutation status is recommended in patients with MDS with isolated del(5q) to identify an adverse prognostic subgroup (34,35) in this prognostically favourable MDS subtype.

Analysis of SF3B1 mutation is the first example of a genetic mutation included into the diagnostic criteria for MDS according to the 2016 WHO classification (24), as it is considered the important diagnostic method for diagnosis of MDS with ring sideroblasts (MDS-RS). Recurrent mutations in spliceosome SF3B1 gene are frequent and are associated with the presence of ring sideroblasts, which indicates more favourable prognosis of MDS-RS (36).

In conclusion, patterns of somatic mutations are diverse in MDS. Even though majority of MDS patients carry at least one oncogenic mutation (29), there is a long list of mutations in more than 50 genes with often unclear aetiology, complicating the use of somatic mutations as simple and universal markers of MDS prognosis.

7.1.4 Treatment

The heterogeneous nature of MDS demands a complex and personalized variety of therapeutic approaches. Among them, the only potentially curative option is haematopoietic stem cell transplantation (HSCT). Because MDS affect mostly the elderly, who are often frail with multiple comorbidities and cannot tolerate intensive therapeutic approaches, HSCT is accessible to only a limited number of fit patients. For majority of the patients with MDS, treatment strategies are nonintensive and risk-adapted, involving the definition of different aims of therapy according to the risk status of the patient (by the IPSS-R). These approaches are noncurative, but aimed at improving cytopenias and quality of life, and delaying disease progression. In all risk groups, supportive care with transfusions and antimicrobial drugs remains important.

In the case of lower-risk MDS, therapy is mainly aimed at improving cytopenias, preventing complications, such as bleeding and severe infections, decreasing transfusion burden, and improving quality of life. In a substantial number of patients with mild and asymptomatic level of cytopenia, there is no need to go beyond supportive care, including blood transfusions and using antibiotics. For lower-risk patients with symptomatic anaemia, treatment with erythropoiesis-stimulating agents (ESAs) or immunomodulatory agent lenalidomide is appropriate. Studies suggest that treatment with ESAs leads to significant erythroid responses in 20 to 70 percent of unselected MDS patients and in approximately 40 percent of patients with LR-MDS. A median duration of treatment response is two years without an increasing risk of leukaemic progression. Lenalidomide was proven to be effective in the treatment of MDS patients with isolated del(5q) and lower-risk MDS patients, particularly in cases with 5q deletions. Some lower-risk patients may be candidates for immunosuppressive therapy, thrombopoiesis-stimulating agents, or DNA hypomethylating agents

(HMAs) azacitidine (AZA) and decitabine. All treatment decisions have to take into account a potential drug-induced deterioration of the patient's clinical status (37–39).

Among higher-risk patients, transplant candidates should undergo HSCT as soon as possible, with HMAs useful as a bridge to transplantation. For those patients who are not eligible for HSCT, HMAs therapy represents the only approved therapeutics and current standard of care (40). Further, HMAs and particularly AZA have been proven to be a valuable treatment also for patients who relapsed after HSCT (41). HMAs do not eradicate transformed cells, but can decrease clonal burden and may therefore improve haematopoiesis. They have been shown to improve OS, clinical outcomes, and quality of life of patients with advanced MDS (37,38). It was evaluated, however, that the ORR to AZA treatment ranges only between 40 to 50 percent (42,43) and the outcome of patients after treatment failure is very poor.

Two HMAs approved for treatment of MDS, AZA and decitabine, are cytidine analogues which inhibit a group of enzymes called DNA methyltransferases (DNMTs) leading to demethylation of the cytosine residues in the promoter-associated CpG islands (40). The hypomethylation and subsequent turnaround in transcription of tumour suppressors and DNA repair genes is considered to be the main mechanism of HMAs action. In addition to epigenetic modulation, HMAs exhibit also immunomodulating effects and are able to incorporate into DNA and RNA molecules, but the contribution of these features to their clinical activity in MDS has not been established yet (40,44,45).

7.1.5 Acute myeloid leukaemia

AML is the most common acute leukaemia in adults. In majority of cases, it appears as a *de novo* malignancy in healthy individuals. However, it can also develop from an underlying haematologic disorder, or arise as a consequence of prior therapy. Regardless of its aetiology, the pathogenesis of AML involves the abnormal proliferation and differentiation of a clonal population of myeloid stem cells. The diagnosis of AML is established by the presence of 20 percent or more blasts of myeloid origin in BM or PB (46).

AML is a highly heterogeneous disease and is classified into multiple categories by WHO according to cytogenetic and molecular abnormalities, degree of myeloid lineage differentiation, and dysplastic changes (24). Although AML patients can be stratified into favourable, intermediate, and adverse prognostic risk groups based on their cytogenetic profile, prognosis within these categories varies widely. AML associated with a prior haematologic disorder and therapy-related AML carry a significantly poor prognosis. If AML is left untreated, patients usually die from infection or bleeding within months from diagnosis (46).

About 30 percent of MDS patients develop AML (23), which is thus classified as AML with myelodysplasia-related changes (AML-MRC) (24). AML-MRC represents 25 to 34 percent of all AML diagnoses (47) and up to 48 percent of AML diagnoses in adults (48). It associates with adverse prognosis with lower remission rates and shorter OS compared to other AML categories. AML-MRC patients are treated with liposomal daunorubicin-cytarabine (CPX-351). Patients who are unable to tolerate intensive treatment with CPX-351 are commonly treated with AZA (47,48).

7.2 Small noncoding RNAs

At the end of the last millennium, the importance of noncoding RNAs was completely unknown. Up to that point, the scientific community had believed that noncoding RNAs are just transcriptional trash and had been focused exclusively on protein coding genes. Since the discovery of the first microRNA (miRNA), lin-4 from Caenorhabditis elegans, in 1993 (49,50), researchers have identified plenty of previously unknown noncoding RNA species and started to reveal their multiple functions affecting various biological processes and features of cells.

7.2.1 Function and biogenesis of small noncoding RNAs

Small noncoding RNAs (sncRNAs) refer to noncoding RNA species that are less than 200 nucleotides in length and share some molecular features and mechanisms of action in regulating of biological processes. SncRNAs are involved in the regulation of gene expression, RNA splicing, epigenetic processes, and chromatin structure remodelling. Thus, sncRNAs affect a variety of essential biological processes, such as cell proliferation, differentiation, apoptosis etc., and are critical for normal development. They are often expressed in a tissue-specific manner (51,52). A large amount of evidence indicates that sncRNA deregulation is associated with development of cancer and various diseases (53–56), including haematologic malignancies (57).

So far, the most explored sncRNA species in humans are miRNAs. However, the current interest moves also towards non-miRNA sncRNA species such as piwi-interacting RNAs (piRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), small interfering RNAs (siRNAs), transfer

RNAs (tRNAs), and tRNA-derived small RNAs (tsRNAs) (Table 8). All these versatile sncRNA species are known to be key components of molecular interactions and gene regulation in eukaryotes (58).

Although sncRNA families are different in their origin, they share specific steps in their biosynthetic pathways and regulatory mechanisms. They are produced by pathways containing specialized enzymes with nuclease activity able to excise small RNAs from specific RNA transcripts. The mechanism of sncRNA action is ensured by a group of effector proteins, that are commonly engaged within high molecular weight protein-RNA complexes, responsible for the stabilization, transport, and regulatory activity of sncRNAs.

The following paragraphs provide a brief characterization of sncRNA species that are commonly detected within sncRNA profiling studies (i.e. miRNAs, piRNAs, snoRNAs, tRNAs, and tsRNAs) and are nowadays of special interest as potential disease biomarkers.

Table 8. Basic characteristics of sncRNA species.

sncRNA species		**Function**	**Length**
miRNAs	microRNAs	RNAs that usually suppress the translation of target mRNA by binding to 3' UTR through RNA interference pathway.	21-25 nt
piRNAs	piwi-interacting RNAs	RNAs involved in retrotransposon silencing through interactions with piwi proteins.	26-31 nt
siRNAs	small interfering RNAs	RNAs that guide sequence-specific degradation of target mRNAs through RNA interference pathway.	10-25 bp
snRNAs	small nuclear RNAs	RNAs located in the nucleus, involved in spliceosome formation (e.g., U1, U2, U5, U4, and U6) and RNA processing. Also commonly referred to as U-RNAs.	~ 150 nt
snoRNAs	small nucleolar RNAs	RNAs located in the nucleolus, mostly involved in modification of other RNAs, such as ribosomal RNAs or spliceosomal RNAs.	~ 60-250 nt
tRNAs	transfer RNAs	RNAs that transfer amino acids to the ribosome for protein construction.	76-90 nt
tsRNAs	tRNA-derived small RNAs	Recently identified RNA species with versatile roles in regulation of translation and ribosome biogenesis.	14-50 nt

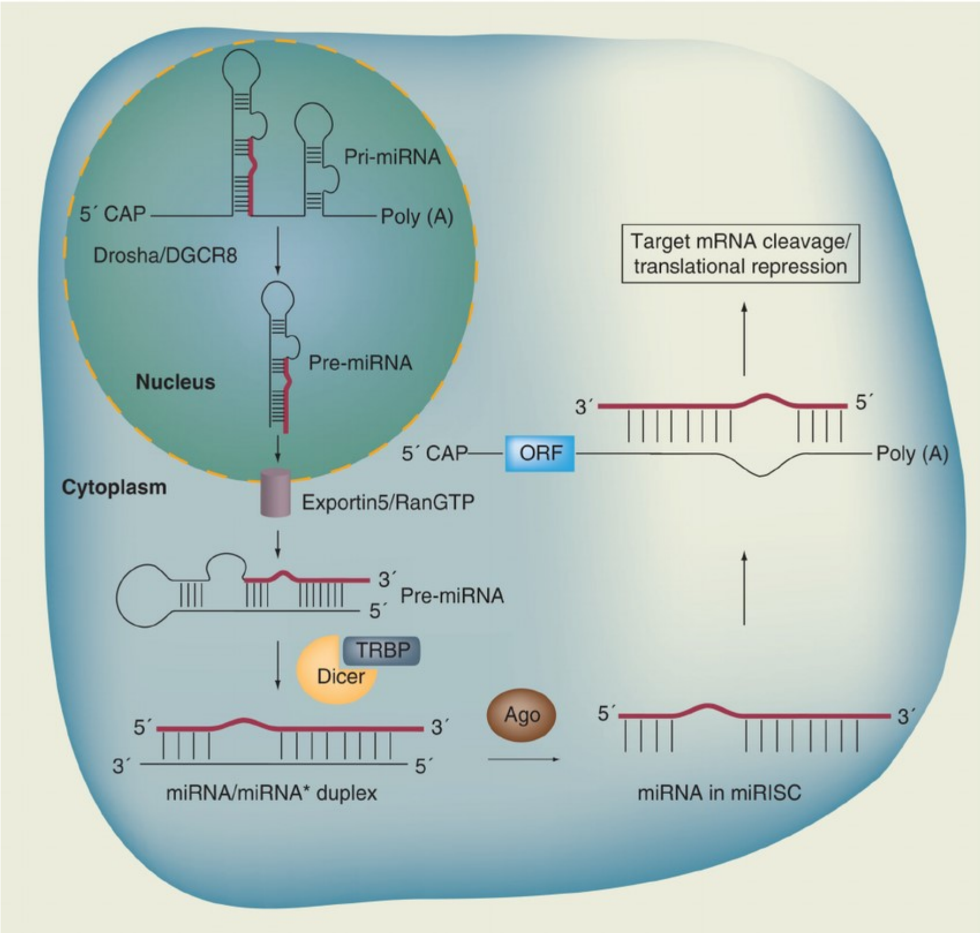
7.2.2 microRNAs

miRNAs are short (~ 22 nucleotides) endogenous single stranded noncoding RNA molecules and their sequences are highly conserved throughout various organisms. miRNAs play an essential role in the regulation of gene expression at the posttranscriptional levels. An individual miRNA is able to regulate the expression of more target mRNAs and each mRNA can be controled by several miRNAs. It is estimated that in humans, miRNAs regulate over 30 percent of protein coding genes (59). The ability of miRNAs to regulate thousands of mRNAs has raised an intensive interest in their role in physiological and pathological processes. It has been repeatedly proven that miRNAs play crucial roles in a wide variety of biological processes such as development, differentiation, proliferation, and apoptosis. Since they influence the expression of genes involved in fundamental signalling pathways, their deregulation often triggers various pathological processes and subsequent development of different diseases and cancers (60). In oncogenesis, some of deregulated miRNAs act as oncogenes or tumour suppressors (61). The majority of miRNAs are expressed in a tissue-specific manner. For example, miR-122 is preferentially expressed in liver (62), miR-124 in neurological tissues (63), miR-133 in muscles (64), and miR-208a in heart (65). Moreover, changes in the spectrum of tissue miRNAs correlate with various pathophysiological conditions (66).

About half of all currently identified miRNAs are intragenic, mostly present in the intronic regions of protein coding genes, while the remaining miRNAs are intergenic, regulated by their own promoters. miRNAs can be localized in clusters and transcribed as one long transcript. In the dominant miRNA biogenesis pathway, the 'canonical' pathway (Figure 2), primary miRNA transcripts (pri-miRNAs) are cleaved into precursor miRNAs (pre-miRNA, ~60 nt) by the ribonuclease III enzyme, Drosha. Pre-miRNAs are exported to the cytoplasm by Exportin 5, where they are cleaved into two mature (~22 nt) miRNA molecules by the RNase III endonuclease Dicer generating a mature miRNA duplex. The directionality of the miRNA strand determines the name of the mature miRNA form. The 5p strand arises from the 5′ end of the pre-miRNA hairpin while the 3p strand originates from the 3′ end. Either of the strands derived from a mature miRNA duplex can be loaded into the Argonaute (AGO) family of proteins in an ATP-dependent manner to form the RNA-induced silencing complex (RISC). Although one of the strands is usually preferentially incorporated, the proportion of AGO-loaded 5p or 3p strand varies depending on the cell type or cellular environment, ranging from near equal proportions to predominantly one or the other. The sequence of the incorporated strand determines the targets that are recognized by RISC (67). Further, multiple non-canonical miRNA biogenesis pathways have been elucidated. These pathways use the proteins involved in the canonical pathway, mainly Drosha, Dicer, Exportin 5, and AGO2, but they use them in different combinations. In general, the non-canonical miRNA biogenesis can be grouped into Drosha-independent (68) and Dicer-independent (69) pathways.

Posttranscriptional regulation of gene expression is performed by binding of miRNAs to a specific sequence at the 3′ UTR of target mRNAs to trigger their degradation or repression depending on the miRNA/mRNA complementarity. Pairing with complete complementary target causes cleavage and subsequent degradation of the target mRNA, whereas imperfect complementarity leads to the RNA interference and translational repression. miRNAs bind also to 5′ UTR and coding regions, that have silencing effects on gene expression, and to promoter regions causing induction of transcription (67).

Figure 2. The canonical pathway of miRNA biogenesis. Original figure from Wang et al. 2012 (70). The mature miRNA is highlighted in red. pri-miRNAs are processed by Drosha/DGCR8 into pre-miRNAs. The pre-miRNA is transported from the nucleus to the cytoplasm by Exportin-5 and Ran-GTP, where it is cut by a Dicer complex (Dicer/TRBP) into a miRNA/miRNA* duplex. One strand of the duplex is preferably incorporated into miRISC. DGCR8 – DiGeorge syndrome critical region 8, TRBP – TAR RNA-binding protein, miRISC – miRNA-induced silencing complex, ORF – open reading frame.



7.2.3 piwi-interacting RNAs

piRNAs are short (24-31 nucleotides) single stranded RNA molecules longer than miRNAs. They bind to the piwi subfamily of Argonaut proteins forming piRNA silencing complex (piRISC), which is guided to the target nucleic acids in a sequence dependent manner. They arise from piRNA clusters located in genomic regions enriched in transposable and other repetitive elements, and are transcribed as long primary RNAs that are further processed to primary piRNAs through primary processing pathway. To enforce piRNA expression, primary piRNAs can subsequently enter an amplification system called the 'ping-pong' cycle, where they act as guides for the generation of secondary piRNAs (71). Unlike miRNAs, piRNAs have been described to function primarily through epigenetic modifications (71–73) rather than transcript targeting. While miRNAs are widely expressed in most mammalian cells and tissues, piRNAs are expressed mainly in the germline where they suppress transposable and repetitive elements to maintain genomic integrity. Nevertheless, it has been shown that piRNAs are expressed also in somatic tissues and tumour tissues in a tissue or cancer-specific manner (74,75).

7.2.4 small nucleolar RNAs

snoRNAs are noncoding RNAs that contribute to ribosome biogenesis and RNA splicing by modifying ribosomal RNA (rRNA) and spliceosomal RNAs, respectively. snoRNAs are longer than abovementioned sncRNA species and range from 60 to 300 nucleotides. They are components of small nucleolar ribonucleoproteins (snoRNPs), which are complexes controlling posttranscriptional modifications of ribosomal rRNA, snRNAs and probably other RNA species. These modifications include 2'-O-methylation and pseudouridylation facilitating RNA folding and stability. snoRNAs are responsible for targeting the assembled snoRNPs to a specific target and directing the site-specific modifications of RNAs. Interestingly, instead of being transcribed from independent genes, most snoRNAs are processed from introns of precursor messenger RNAs (pre-mRNAs) (76,77).

7.2.5 transfer RNAs and tRNA-derived small RNAs

tRNAs help decode a messenger RNA (mRNA) sequence into a protein. Specific tRNAs transfer the appropriate amino acids to the ribosome for protein synthesis (78). Fragmentation of tRNAs generates a family of small RNAs collectively known as tRNA-derived small RNAs (tsRNAs). Although these tsRNAs were initially discarded as an artifact of RNA sequencing technology, there is an increasing amount of evidence pointing to tsRNAs as biological entities with roles in translational regulation (79), gene silencing (80,81), and modulation of gene expression (82). They affect responses to various stress conditions (83–85), regulate mammalian development, haematopoietic stem cell fate and immune response (86), and play a role in pathological processes leading to cancer and other diseases development (87,88). Interestingly, some tsRNAs associate with Argonaute proteins and perform their functions as miRNAs (89). The biogenesis of tsRNAs is conserved and regulated process. They are generated from mature or precursor tRNAs by specific cleavage at different sites by specific RNases, including Dicer, RNase Z, and angiogenin (90,91). By different RNases, various types of tsRNAs are produced *via* different biogenesis pathways, which have not been fully uncovered yet.

The nomenclature for tsRNAs is still inconsistent due to their recent identification. Based on cleavage sites of tRNAs and the length of generated tsRNAs, they can be divided into two main types: tRNA-derived fragments (tRFs), also called tRNA-derived RNA fragments (tdRs), and tRNA halves, also called stress-induced tRNA fragments (tiRNAs) as they are often generated in response to stress conditions. The first, tRFs, are about 14-30 nucleotides in length and are derived from the mature or pre-tRNAs. Up until now, four subtypes of tRFs have been identified and characterized by their location in the tRNA structure: 5-tRFs, 3-tRFs, 1-tRFs, and 2-tRFs. The second, tRNA halves, are 30-50 nucleotides in length and produced by specific cleavage in the anticodon loop of mature tRNAs. Two subtypes of tRNA halves have been discovered, 5'-tRNA halves and 3'-tRNA halves. In summary, different types of tsRNA appear to accumulate during different biological processes *via* different biogenesis pathways (88).

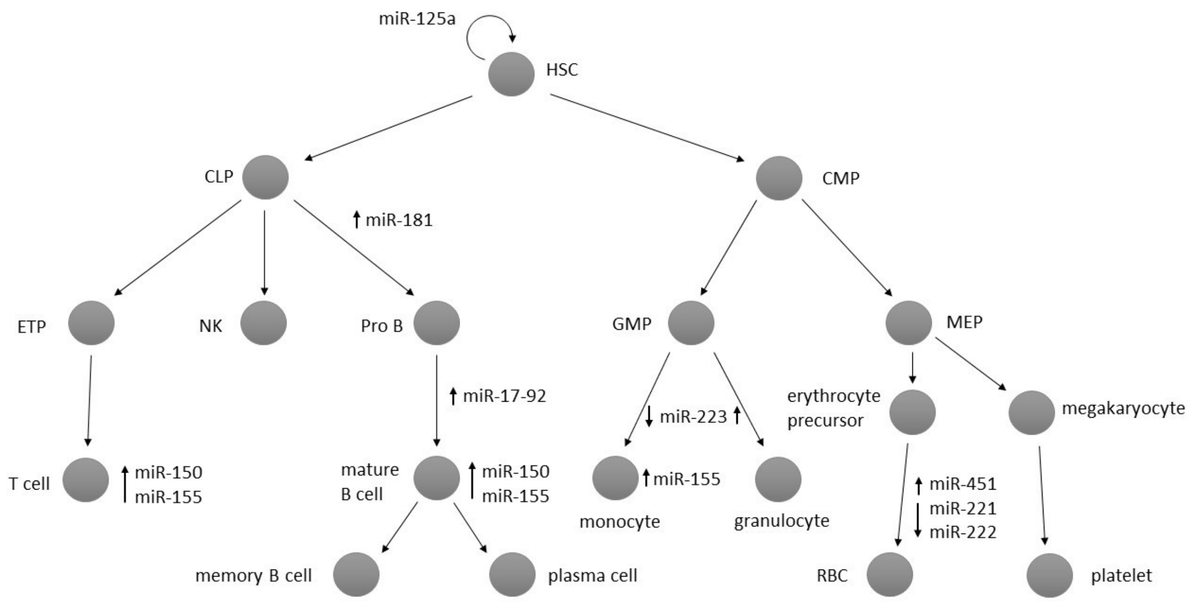
7.2.6 sncRNAs in normal haematopoiesis

Stable haematopoiesis requires complex and careful regulation to maintain proper cellular proliferation and cell death modulation, cellular differentiation, and lineage commitment. During the last decade, the function of miRNAs in haematopoiesis was extensively studied, and many miRNAs playing a critical role in the development of both normal and malignant haematopoiesis were discovered. The role of other sncRNA species in haematopoiesis remain to be elucidated.

In 2004, the first study exploring a role of miRNAs in the haematopoietic lineage differentiation showed that forced expression of miR-181 in haematopoietic stem/progenitor cells (HSPCs) markedly increases B cell differentiation, inhibits production of CD8+ T cells, and miR-142 decreases production of both CD4+ and CD8+ T cells, but does not affect B cells (92). Since then, many miRNAs specific for the maintenance of the ‘stemness’ of HSCs and for the development of individual blood cell lineages have been determined (Figure 3). For example, it was shown that miR-125a and miR-29a are highly expressed in HSCs and modulates their self-renewal and numbers (93,94). Further, miR-29a is a key regulator of normal myeloid differentiation with tumour-suppressive function (95). miR-223 regulates granulocytic differentiation and function. This miRNA shows a highly lineage-specific pattern of expression with its low levels in HSCs and common myeloid progenitors. The expression of miR-223 miRNA is steadily upregulated during differentiation to granulocytes and is repressed during differentiation to the alternative monocytic fate (96). MiR-451 is expressed predominantly in erythroid cells, its expression increasing during their maturation. In contrast, miR-221 and miR-222 are downregulated during erythroid differentiation. This downregulation enables the expression of their target gene encoding Kit receptor, whose activation triggers erythroblast expansion (97). Another miRNA, miR-150, is highly expressed in mature lymphocytes, whereas it is not active in HSCs (98). The target gene of miR-150 is transcription factor MYB that regulates lymphocyte development (99,100). miR-155, with its high levels in activated B-cells, T-cells, and monocytes, also participates in lymphoid differentiation (101). Further, the development of B-cells is positively regulated by miRNAs encoded in cluster miR-17-92. This cluster of miRNAs inhibits the expression of the apoptotic protein Bim and thus, plays a key role in pro-B cells to pre-B cells transition (102). Regarding specificity of miRNA profiles in haematopoietic cell lineages, expression analysis of 13 haematopoiesis related miRNAs in individual cell types from the PB of healthy individuals enables discrimination of individual blood cell lineages and determination of the cellular origin of *in vitro* cultured lines (103).

Regarding recently acknowledged other sncRNA species, also snoRNAs were shown to be expressed in a lineage and development specific manner during haematopoiesis. Most significantly, snoRNAs located in DLK-DIO3 and SNURF/SNRPN imprinted loci are highly expressed in haematopoietic progenitors and downregulated during myeloid differentiation (104).

Figure 3. Schema of lineage differentiation in haematopoiesis and miRNAs involved in the process. Original figure from Hrustincova et al. 2019 (105). CLP – common lymphoid progenitor, CMP – common myeloid progenitor, ETP – early thymic progenitor, GMP – granulocyte macrophage progenitor, HSC – haematopoietic stem cell, MEP – megakaryocyte erythroid progenitor, NK – natural killer cell, RBC – red blood cell, ↑ indicates increased level of miRNA, ↓ indicates decreased level of miRNA



7.2.7 sncRNAs in malignant haematopoiesis

Many miRNAs, including the abovementioned, have been found to play a critical role in the pathogenesis and progression of haematopoietic disorders. For example, miR-125a knockout mice were shown to develop myeloproliferative disorders (106). *In vitro*, miR-125a-5p induces granulocytic differentiation in different human AML cell lines as well as in normal human primary HSPCs (107). Upregulation of miR-125a and miR-99b in macrophages lead to their polarization and secretion of inflammatory cytokines to kill tumour cells (108). Levels of miR-10a regulating myeloid differentiation are increased in AML (109) and atypical myeloproliferative neoplasms (110). Reduced levels of miR-29a and miR-142-3p were observed to be involved in AML development (95). Another study suggested that miR-29a initiates AML by converting myeloid progenitors into self-renewing leukaemia stem cells (94). Further, miR-221 and miR-222 were found to be consistently overexpressed in AML (111). In AML cell lines, miR-223 was demonstrated to inhibit cell proliferation and enhance cell apoptosis, whereas it was observed to be suppressed in AML patients (112).

miR-126 inhibits cell apoptosis and increases viability of AML cells *in vitro* (113) and its higher level correlate with poorer prognosis in AML patients (114). Highly expressed miR-181a and miR-181b were identified in association with CEBPA gene mutations in cytogenetically normal AML patients (115). Finally, increased miR-150 expression was found to contribute to myelodysplastic haematopoiesis in MDS-del(5q) *via* its negative regulation of the transcription factor MYB (116). More details on sncRNAs in MDS are introduced in the chapter 7.3.

The data on the role of other sncRNA species in haematopoietic diseases are still limited. A study focusing on snoRNA profiling in AML found a subset of snoRNAs that show consistent differential expression between AML and normal CD34+ cells, with the great majority of them being decreased in the AML samples (104). Another study found two tsRNAs downregulated in chronic lymphocytic leukaemia (CLL) patients. The authors performed further experiments to determine whether other tsRNAs are involved in CLL and revealed specific tsRNA signatures indicating that tsRNAs, like miRNAs, may have an oncogenic and/or tumour suppressor function in haematopoietic malignancies (117). In conclusion, it is evident that miRNAs and other sncRNA species are involved in haematopoiesis maintenance and their deregulation is associated with development of haematopoietic disorders.

7.2.8 Circulating sncRNAs

First extracellular sncRNAs were observed in blood in 2004 (118). In 2008, miRNAs were found in serum of patients suffering from diffuse large B-cell lymphoma (119). Since then, a great effort has been devoted to the research of this phenomenon. Nowadays, it is evident that miRNAs and other sncRNA species, originally found in tissues, are present not only in intracellular environment but also extracellularly in various body fluids such as blood plasma (120), serum (121), saliva (122), and urine (123). Moreover, their sncRNA profiles are significantly different. Examination of 12 human body fluids shows a distinct composition of the miRNA spectrum in various fluid types (124). The emergence of novel high-throughput technologies has allowed to analyse complete sncRNA profiles in different tissues as well as in biological fluids from healthy and diseased individuals.

Extracellular sncRNAs released into blood circulation are referred to as ‘circulating’ sncRNAs. They are of special interest, because they originate from every tissue in the body, and their spectrum reflects overall condition of an organism. Thus, they are believed that they may serve as good blood-based biomarkers of various diseases (125,126). Regarding this possibility, it was demonstrated that the quantity of some sncRNA species in blood would be sufficient to use them as biomarkers. Except for most abundant miRNAs, also piRNAs and tRNAs are highly represented sncRNA species in body fluids (127,128). Most of the studies on circulating sncRNAs have been performed on total blood plasma or serum so far. With the extending knowledge that circulating RNAs are included in extracellular vesicles (EVs), which appear to have function in cell-to-cell communication, the studies move to exploring sncRNA contents also in plasma-derived EVs. Lots of studies were conducted in order to explore circulating sncRNA stability, functions, profiles, and their potential to serve as disease biomarkers. The knowledge is introduced in the following subsections.

7.2.9 sncRNA stability, carriers, and sorting

Several studies focused on sncRNA stability in human blood plasma and serum and found that they are remarkably stable under distinct conditions such as storage in room temperature, multiple freeze-thaw cycles (129), and long-term storage (130). It has been shown that circulating sncRNAs are protected from endogenous RNase activity (129), that degrades exogenously added RNA within seconds (131). This indicates that extracellular sncRNAs are unlikely to exist in an unprotected state. In the last decade, different sncRNA carriers and ways of sncRNA release from cells were revealed. Specifically, sncRNAs have been found to be included in EVs (132,133), incorporated in high density lipoproteins (HDL) (134), and associated with proteins such as AGO2 (135), the effector component of the RISC complex that directly binds miRNAs, or nucleophosmin 1 (NPM1) (136), which is involved in the biogenesis of ribosomes (Figure 4).

Several types of EVs have been identified differing in their sizes and biogenesis. Exosomes (30-150 nm) (137) are formed as intraluminal vesicles within multivesicular bodies and are released into the extracellular space upon fusion of multivesicular bodies with the plasma membrane, whereas microvesicles (100-1000 nm) (138,139) are shed from the plasma membrane by outward blebbing (140). Another type of vesicles containing sncRNAs are apoptotic bodies (500-2000 nm) (141,142), which are released from the cell that undergoes apoptosis.

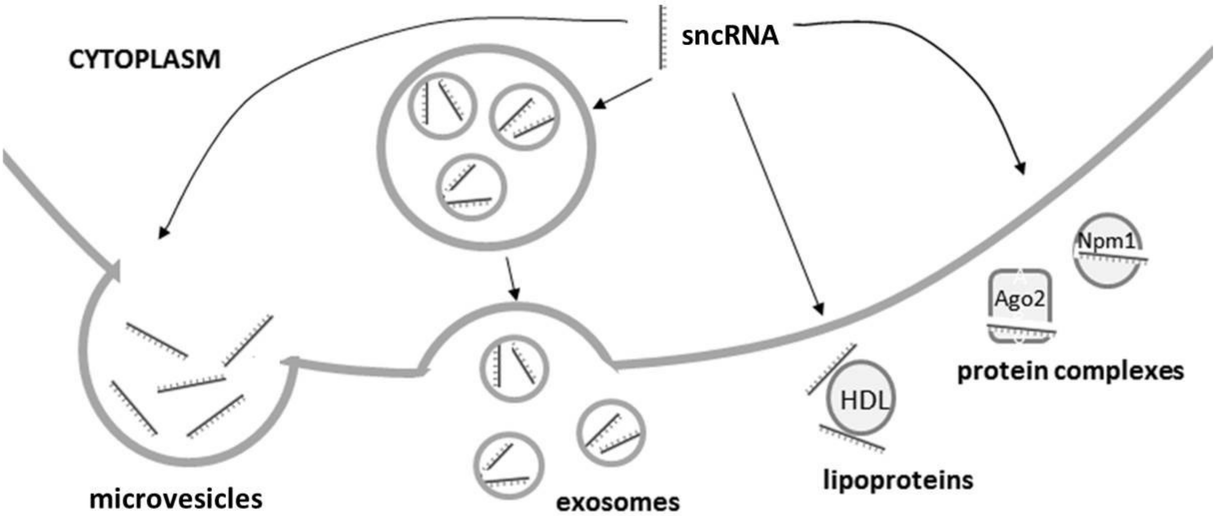
Savelyeva et al. (143) found that a variety of blood plasma RNAs are comparable to the variety of cellular RNA species. Not only extensive set of miRNAs but also fragments of other cellular RNAs, such as rRNAs, tRNAs, mRNAs, lncRNAs, sncRNAs, and RNAs encoded by mitochondrial DNA (mtDNA) can be detected within EVs as well as in EV-depleted plasma (143). For example, a study profiling circulating RNAs of healthy individuals and cancer patients showed that in EVs, piRNAs are almost equally abundant as miRNAs (127).

Interestingly, EV-associated miRNAs appear to be more stable than those not associated with EVs (144). Another study suggested that exosomes are more protective of RNA degradation and provide more consistent source of miRNAs compared to intracellular and exosome-free plasma/serum miRNA contents (145).

The sorting of sncRNAs into EVs and their secretion appear to be selective and controlled process. For example, HDL-associated miRNA profiles significantly differ from the exosome profiles both in miRNA spectrum and abundance (134). Another study revealed a significant difference in miRNA spectrum between microvesicles and their maternal cells, suggesting selective sorting of miRNAs into microvesicles (132). It was found that the sorting of miRNAs into exosomes is controlled by recognition of specific sequence motifs present in these miRNAs (146). Gámbaro et al. (147) investigated how sncRNA stability influences the sorting. They transfected cells with highly stable glycine 5' tRNA halves, which belong to the group of stress-induced tRNA fragments frequently detected in extracellular space and biofluids, and found that in contrast to unstable RNAs, these tRNA halves are present in EVs and in recipient cells in amounts proportional to the concentration of RNA used for transfection. Thus, the results suggested that even in the presence of selective sorting, the concentration of specific RNAs, which is given also by their stability, affects sorting of specific RNAs into EVs and their delivery in recipient cells (147).

All these observations indicate that extracellular sncRNAs are not only mere leftovers of cellular metabolism as was believed not long ago, but they appear to play a critical role in intercellular communication that is mediated by EVs, and modulation of recipient cell features and functions.

Figure 4. Different ways of sncRNA release from cells to the extracellular environment. Edited figure from Hrustincova et al., 2015 (148). sncRNAs are included in EVs such as microvesicles and exosomes, incorporated in high density lipoproteins (HDL), and associated with proteins such as AGO2 and NPM1.



7.2.10 EVs mediate cell-to-cell communication

Nowadays, it is accepted that EVs released into extracellular environment enable a transfer of their molecular cargo to the recipient cells in the immediate vicinity or at distant locations *via* blood circulation and thus, EVs mediate cell-to-cell communication (149–151). In 2007, Valadi et al. (152) found that RNA cargo from exosomes can be delivered to another cell and in this recipient cell, it can be functional (152). Zhang et al. (153) reported that in human blood cells and cultured cells, miR-150 is selectively packaged into microvesicles, actively secreted, and delivered into recipient cells, where it can participate in regulatory processes (153). It was observed that in AML, both primary cells and cell lines release exosomes that are received by neighboring cells. The exosomes are enriched for several RNAs, including miRNAs, relevant to AML pathogenesis. Moreover, these miRNAs regulate the biological functions of recipient cells (154). Another study indicated a direct effect of miR-150 and miR-155 from AML exosomes on HSPCs. These miRNAs are enriched in AML exosomes and suppress translation of c-MYB, a transcription factor involved in HSPC differentiation and proliferation (155).

Further findings of Huan et al. (156) suggested that AML exosomes regulate not only functions of HSPC directly, but also modulate BM compartmental signalling. Both *in vivo* and *in vitro* results showed that AML cells increase exosome production under physiological oxygen conditions, which inhibits expression of HSC maintenance factors by stromal cells. Moreover, AML exosomes from extramedullary myeloid tumours appeare to traffic to BM niche and dysregulate niche signalling (156). Kumar et al. (157) showed that AML blast-derived exosomes transform the BM niche into a leukaemia growth-permissive microenvironment. These exosomes increase the number of mesenchymal stromal progenitors, block osteolineage development and bone formation *in vivo*, and accelerate AML growth. Conversely, disruption of exosome secretion in AML cells significantly delays AML development. Furthermore, AML-derived exosomes suppress production of HSC maintenance factors by stromal cells and thus, reduce their ability to support normal haematopoiesis (157). Regarding treatment, Chen et al. (158) indicated that AML-derived exosomes protect AML cells against chemotherapy *via* induction of IL-8 production in BM stromal cells. In addition, the study showed that IL-8 inhibition increases the sensitivity of AML cells to cell death (158). Another study showed that pre-therapy plasma of refractory or relapsed AML patients contains elevated levels of exosomes carrying immunosuppressive cargo that interfere with anti-leukaemia functions of activated immune cells used for adoptive cell therapy and reduce their therapeutic potential (159). In summary, exosomes mediate disruption of normal haematopoiesis in AML. Strategies to block their formation, secretion, and/or their modulatory effect on recipient cells might be utilized as a novel therapeutic approach in AML (160).

The crosstalk between HPCs and their microenvironment was observed also in MDS. EVs derived from mesenchymal stromal cells of MDS patients were shown to carry different cargo compared to that of controls, including upregulated miR-10a and miR-15a. Further, they are able to incorporate into CD34+ cells, where their cargo modifies gene expression of MDM2 and p53, and induces cell viability and clonogenic capacity (161). Interestingly, miRNAs and piRNAs from BM mesenchymal stem cell EVs induce cell survival and inhibit cell differentiation of HSCs in umbilical cord blood (162). Because umbilical cord blood is an alternative source of HSCs for patients with haematologic diseases, who can be cured by allogeneic HSCT (163), this observation provides a new insight into the biology of umbilical cord blood transplantation.

In conclusion, many studies have reported the significance of cell-to-cell communication through delivery of signal and regulatory molecules mediated by EVs. It has been shown that EVs carry a vast number of various molecules such as different RNA species (mRNAs, sncRNAs, and long noncoding RNAs), oncoproteins and oncopeptides, DNA fragments, and lipids, which induce phenotypic changes in recipient cells (164). There is comprehensive information on how the EVs derived from stem/progenitor cells inhibit or enhance immune response by affecting natural killer cells, dendritic cells, monocytes/macrophages, microglia, T cells, and B cells (165). Thus, EVs have crucial roles in processes associated with cancer development (166–168), including leukaemia (169), where they modulate BM microenvironment, haematopoiesis, and the immune system to facilitate the malignancy.

7.2.11 Circulating sncRNAs as biomarkers in haematologic malignancies

The existing observations indicate that circulating sncRNAs play a critical role in (patho)physiological processes occurring in an organism. As they can be secreted from every cell and tissue in the body, they may very good reflect the actual condition of an organism and thus, may serve as valuable blood-based biomarkers of various diseases and cancers (125,126). Moreover, blood plasma and EVs are of special interest as a source of biomarkers because they can be obtained noninvasively, offering a novel feasible alternative to routine invasive BM biopsies, which can be demanding, especially for elderly patients with many comorbidities.

The first evidence of the potential applicability of circulating miRNAs as noninvasive diagnostic markers was reported by Lawrie et al. in 2008 (119). They demonstrated that the levels of three tumour-associated miRNAs (miR-155, miR-210 and miR-21) were higher in the serum from patients with diffuse large B-cell lymphoma (DLBCL) than in controls. Moreover, they showed high level of miR-21 to be associated with relapse-free survival (119). Another study reported increased levels of miR-21 in DLBCL cell lines and serum of DLBCL patients compared to controls (170), suggesting its potential utilization as biomarker for DLBCL diagnosis. On the contrary, Fang et al. (171) did not observe deregulated serum levels of miR-21 in DLBLC patients, but demonstrated increased levels of miR-15a, miR-16-1, miR-29c, and miR-155, and decreased level of miR-34a in DLBCL patients compared to controls (171).

In AML, Fayyad-Kazan et al. (172) reported that miR-150 and miR-342 are significantly downregulated in plasma, and indicated that they may be potentially utilized as predictors of AML relapse (172). Plasma levels of miR-92a, miR-143 and miR-342 are decreased in AML patients and according to their specificity and sensitivity values, these miRNAs appear to be promising circulating biomarkers in clinical detection of AML (173). Further, Gado et al. (174) demonstrated decreased levels of miR-29a-3p and miR-92a-3p in both plasma and BM from AML patients along with increased expression of their target gene myeloid cell leukaemia sequence 1 (MCL1) in blood cells, which indicates diagnostic and therapeutic potential of these miRNAs (174).

Low levels of circulating miR-92a were observed not only in AML (173–176), but also in multiple myeloma (177) and non-Hodgkin's lymphoma (178), suggesting that measurement of miR-92a level in plasma may be useful for monitoring disease status or initiation of therapy in haematologic malignancies in general. miR-92a is a component of mir-17-92 cluster coding for a polycistronic transcript with pleiotropic functions. mir-17-92 is considered a polycistronic miRNA oncogene, whose overexpression is frequently observed in a variety of tumour types (179). Interestingly, Gu et al. (180) showed that miR-92a is downregulated in AML cell lines and its overexpression suppresses proliferation and induces apoptosis of AML cells through directly targeting methylenetetrahydrofolate dehydrogenase 2 (MTHFD2). Further, miR-92a overexpression in mice dramatically decreases tumour growth and MTHFD2 expression *in vivo*. The authors suggested that miR-92a may act as a tumour suppressor in AML and may be a promising therapeutic target for AML patients (180).

Regarding circulating EVs and their sncRNA content, Hornick et al. (181) suggested that AML-derived exosomes and their miRNA content may be promising early biomarkers of AML as they can be detected in blood in advance of circulating blasts (181). Another study investigated the role of increased miR-125b level in circulating exosomes of intermediate risk AML patients. The results showed that high level of miR-125b is associated with higher-risk of relapse and overall death (182).

Relevance of circulating miRNAs to be blood-based disease biomarkers was further tested on other haematologic malignancies such as multiple myeloma (177,183–185), Hodgkin lymphoma (186), T-cell leukaemia (187), or chronic lymphocytic leukaemia (188–191). All of these studies suggested individual circulating miRNAs, such as miR-16-5p, miR-34a-5p, hsa-miR-29a-3p, hsa-miR-150-5p, hsa-miR-155-5p, miR-181a, miR-221, and miR-223-3p, to be useful diagnostic, prognostic, predictive, and survival biomarkers of specific diseases and also potential targets for therapy.

7.2.12 Methodological aspects of sncRNA detection

sncRNAs, especially those circulating in blood, appear to represent promising disease biomarkers. However, methods for sncRNA isolation, measurement, and quantification have specific limitations and critical technical hotspots, such as sample processing, inefficient isolation, haemolysis in blood samples, variable efficiency of reverse transcription and PCR, inconsistency in reference genes, and variability in genome-wide platforms used for sncRNA detection (148). Glinge et al. (192) highlighted the importance of proper and systematic sample collection, handling, and storage when measuring circulating sncRNAs. The study demonstrated that the results are affected by factors such as a type of collection tube (192). Variance in all procedures and methodological aspects lead to a discrepancy of the reported results. The inconsistent results obtained by various methods make it difficult to define consensus disease biomarkers that may be implicated in clinical use. Thus, the methods are still being under development in order to improve their sensitivity, specificity, and reproducibility to avoid confounding variables influencing the results. More information on methodological aspects of sncRNA detection and quantification are discussed in our review (148).

7.3 Small noncoding RNAs in myelodysplastic syndromes

Nowadays, miRNAs and other sncRNA species are accepted as important regulators of haematopoiesis. Their abnormal function has serious implications for haematopoietic cell features and phenotypes. sncRNA deregulation has been found in haematopoietic disorders and naturally, it also contributes to the development and progression of MDS. Properties of sncRNAs such as stability and tissue specificity make these molecules highly promising diagnostic and prognostic biomarkers as well as interesting therapeutic targets. Particularly circulating sncRNAs are of special interest as they can be obtained noninvasively, unlike BM biopsies, which can be demanding for MDS patients. So far, miRNAs are the most explored sncRNA species in MDS. Although many studies have been conducted to investigate miRNA profile in MDS, there are not much overlapping results regarding deregulated individual miRNAs. This inconsistency may reflect large heterogeneity of MDS, but it may also be explained by nonuniformity in sample handling and processing procedures, and variability in platforms used for miRNA detection. Nevertheless, there is evidence that sncRNAs are aberrantly expressed in MDS, but their functions in MDS pathophysiology are mostly unknown and remain to be elucidated. Identifying the putative targets of specific sncRNAs is critical for better understanding of MDS. This section summarizes our knowledge on the contribution of sncRNAs to the pathogenesis of MDS and discusses their potential applicability in the assessment of disease diagnosis and prognosis.

7.3.1 miRNAs deregulated in MDS and in particular MDS subtypes

The initial studies in the field of miRNAs in MDS focused on characterization of miRNA profiles that are common in MDS or specific for individual MDS subtypes. In 2009, Pons et al. (193) measured expression levels of 25 haematopoiesis-related miRNAs in BM and PB of MDS patients. The study reported differentially expressed miRNAs between MDS and controls, such as an overexpression of miR-17-92 cluster in both BM and PB of MDS (193). Hussein et al. (194) demonstrated that the miRNA expression profile in BM cells can distinguish MDS patients with chromosomal alterations from those with normal karyotypes (194). Another study examined global miRNA expression in BM mononuclear cells and observed high levels of miR-222 and miR-10a and low levels of miR-146a, miR-150, and let-7e in MDS (195). Dostalova Merkerova et al. (196) analysed miRNA expression at the genome-wide level in CD34+ BM cells and found significant differences in miRNA expression between early and advanced MDS subtypes. They further identified strong upregulation of miR-34a in early subtypes of MDS (196), corresponding with the results of another study, which reported substantial overexpression of miR-34a in MDS patients with del(5q) (197).

MDS with isolated del(5q), formerly referred to as 5q-syndrome, is one of the best characterized MDS subtype. MDS with del(5q) is determined by haploinsufficiency of specific genes within commonly deleted region (CDR) localized in 5q31.3-5q33 locus, which is essential for its specific phenotype. In CDR, besides protein coding genes, 13 genes encoding miRNAs are located. In MDS with del(5q), decreased levels of miR-143-5p, miR-146a, and miR-378, mapped within CDR, were detected (196,197). Importantly, Starczynowski et al. (198) showed that the deletion of chromosome 5q correlates with loss of miR-145 and miR-146a, which are abundant in HSPCs. TIRAP and TRAF6 were identified as respective targets of these miRNAs. It is known that TIRAP lies upstream of TRAF6 in an innate immune signalling pathway. Knockdown of miR-145 and miR-146a together or enforced expression of TRAF6 in mouse HSPCs result in thrombocytosis, mild neutropenia and megakaryocytic dysplasia. A subset of mice transplanted with TRAF6-expressing BM were observed to progress either to BM failure or to AML (198). Kumar et al. (199) showed that miR-145 loss in MDS with del(5q) affects megakaryocyte and erythroid differentiation. They reported that miR-145 functions through the repression of FLI1, a megakaryocyte and erythroid regulatory transcription factor. Inhibition of miR-145 increases the production of megakaryocytic cells relative to that of erythroid cells (199). Another study showed substantial overexpression of miR-34a in MDS patients with del(5q) (197). The expression of miR-34a is induced by p53 and it promotes apoptosis through inhibition of BCL2 gene (200). Thus, high expression of miR-34a in MDS patients with del(5q) is likely to be related to an increased apoptosis of BM progenitors (201).

7.3.2 miRNAs as MDS biomarkers

Many studies have also focused on miRNA relevance and significance as potential MDS biomarkers. The earliest study in this area showed close association of miRNA profiles with IPSS score of MDS. A unique signature consisiting of ten miRNAs permitted discrimination between lower-risk and higher-risk disease. In addition, miR-181 family members were selectively overexpressed in higher-risk MDS and associated with shorter patient survival (195). Another study suggested that increased expression of miR181a/b/d-5p may predict MDS to AML transformation (202). Pons et al. (193) explored the levels of haematopoiesis related miRNA in MDS and AML in order to seek a link between these miRNAs and MDS progression to AML. The results showed that the expression levels of miR-222 and miR-181a are higher in AML than in MDS in both BM and PB. Further, differential expression of miR-15a and miR-16 between lower-risk and higher-risk MDS groups was observed, suggesting that all these miRNAs may be implicated in the progression of MDS to AML (193). Lovat et al. (203) reported that double knockout of the two miR-15/16 loci in mouse leads to the development of AML and indicated that decreased levels of miR-15a/-15b/-16 in MDS patients predict the progression to AML (203). Finally, miR-22 upregulation in MDS appears to correlate with poor patient survival (204).

7.3.3 miRNA functions in MDS

Several studies focused on regulatory pathways and specific targets of individual miRNAs in MDS. For example, it was shown that overexpression of miR-125a in MDS CD34+ cells modulates NF-κB activation and enhances erythroid differentiation arrest (205). Upregulation of miR-34a in MDS reduces c-FOS expression leading to TNF-α overproduction, which is considered to cause ineffective haematopoiesis (206). miR-21 mediates ineffective haematopoiesis in MDS by activating TGF-β signalling *via* reducing expression of SMAD7 (207). Deregulation of miR-10a/b is controlled by TWIST1 and appeares to promote apoptosis *via* NF-κB and p53 (208). Another study reported induction of apoptosis in MDS *via* p53 activation by upregulated miR-661 (209). Similarly, miR-143 induces apoptosis in MDS through the Fas/FasL pathway (210). Downregulation of microRNA-144 inhibits proliferation and promotes apoptosis of MDS cells through the activation of the AKAP12-dependent ERK1/2 signalling pathway (211). Overexpression of miR-205-5p induces cell proliferation by suppressing PTEN (212). Finally, miR-22 targets TET2 tumour suppressor (204) leading to increased HSC self-renewal accompanied by defective differentiation (204).

7.3.4 non-miRNA sncRNA species in MDS

With the development of high-throughput sequencing technology, the first studies observing other sncRNA species have emerged, investigating their expression and potential as disease biomarkers. Beck et al. (213) determined sncRNA and coding gene expression in primary BM cells of MDS patients and performed integrative analysis of the data. The analysis showed that in early MDS, the sncRNA profile is enriched in piRNAs, potentially protecting DNA from the accumulation of mutations.

In advanced MDS, tRNAs are enriched, possibly contributing to the reduction of apoptosis, which is characteristic for advanced stage of the disease (213). Guo et al. (202,214) demonstrated the utility of tsRNAs as predictive biomarkers for both, response to therapy with HMAs (214) and MDS progression to AML (202). In the first study (214), the authors reported that tsRNAs are one of the most common sncRNA species and some of them are associated with response to the treatment (214). In the second study (202), they identified miRNAs (miR-181a/b/d-5p, miR-199b-5p, and miR-486-5p) and tsRNAs that predict progression of MDS to AML independently of increased blast percentage, emphasizing their value as true predictive markers of future transformation instead of just documentation of the disease progression. In addition, the results indicated that the production of tsRNAs is specific (202). Interestingly, it was shown that pseudouridylation of tsRNAs mediated by pseudouridine synthase 7 (PUS7) directs them to inhibit translation and this phenomenon critically governs HSC commitment, HSPCs differentiation and haematopoietic lineage specification. Moreover, deregulation of this regulatory pathway appears to be common for MDS with the deletion of chromosome 7, where PUS7 gene is located (215).

7.3.5 Circulating sncRNAs in MDS

To date, only a few studies have investigated circulating sncRNAs in MDS. First studies focused on plasma/serum levels of specific miRNAs. Guo et al. (216) found that the levels of let-7a and miR-16 are decreased in MDS plasma compared to controls, and associated with patient PFS and OS (216). Kim et al. (217) investigated the level of miR-21 in serum of MDS patients treated with HMAs. The results showed that serum level of miR-21 differentiate responders from nonresponders and is associated with ORR and PFS of MDS patients treated with HMAs (217). Thus, initial studies showed that in MDS, specific circulating miRNAs may serve as predictive biomarkers of survival and response to HMAs therapy.

With developing high-throughput technologies, analysis of up to thousands of predefined miRNAs became available. Zuo et al. (218) were the first who investigated the global profile of circulating miRNAs in MDS plasma. They analysed 800 miRNAs and identified a 7-miRNA signature (let-7a, miR-16, miR-25, miR-144, miR-451, miR-651, and miR-655) as an independent predictor of survival in MDS patients with normal karyotypes (218). With unravelling the role of EVs as mediators of intercellular communication, research has focused on investigation of the EV cargo. Giudice et al. (219) explored the possible diagnostic and prognostic potential of plasma exosomal miRNAs and found 21 miRNAs that appear to be strongly associated with MDS.

Further, with the development of next generation sequencing, other sncRNA species became detectable. Enjeti et al. (220) sequenced sncRNA content in microvesicles of MDS patients and revealed that the sncRNA cargo of MDS microvesicles is approximately twice as high as that of microvesicles of controls. Furthermore, miRNA content and characteristics of microvesicle population of MDS patients were significantly different than those of controls (220).

To conclude, circulating sncRNAs have a great potential to become a noninvasive biomarkers of the disease, which would highly contribute to improving MDS diagnostics, prognosis assessment, treatment, and patient comfort. Thus, there is a great effort to uncover the real value and features of these molecules, and find a way to utilize them in clinical practice.

8 AIMS

We aimed to analyse sncRNA expression using high-throughput technologies to identify sncRNA profiles associated with MDS development, progression, and treatment, and to search for novel prognostic and predictive biomarkers of the disease and response to AZA therapy, respectively.

8.1.1 Specific aims

1. Characterization of specific sncRNA profiles associated with MDS subtypes and risk groups and determination of sncRNAs with different levels between

i. MDS patients and healthy individuals

ii. patients with different MDS subtypes

iii. patients with lower-risk and higher-risk MDS

iv. MDS and AML-MRC patients

v. MDS patients responsive and nonresponsive to AZA therapy.

2. Characterization of sncRNA profiles in different materials and their comparison between

i. BM cells and total plasma (i.e. intracellular *versus* extracellular sncRNAs)

ii. total plasma and EVs (different extracellular materials)

in order to define source-related specificities in sncRNA levels, their association with the patient outcome and to find which of the two extracellular materials would be suitable as possible source of circulating sncRNA biomarkers.

3. Determination of sncRNAs that may serve as auxiliary MDS biomarkers for prediction of patient outcome and AZA therapy responsiveness. Achieving this goal included

i. correlation of specific sncRNA signatures with clinical data, particularly with regard to patient follow-up (i.e. patient survival, progression of the disease, and response to AZA therapy)

ii. assessing of predictive value of selected sncRNAs at the level of both individual molecules and multiple molecule signature

iii. validation of the high-throughput data of particular sncRNAs on an independent or extended sample cohorts using quantitative methods to prove the accuracy of the results and to study association of these sncRNAs with clinical characteristics more precisely.

4. Identification of biological pathways potentially affected by the deregulated sncRNAs that may underlie the pathophysiology of MDS.

9 METHODS

Most of the methods described below were applied in all the three publications that underlie this thesis. When applied under specific conditions in the particular publication, it is indicated as P1, P2, and P3. For references see the Preface.

9.1.1 Patient samples

The PB and BM aspirates were collected from patients with MDS and AML-MRC with no known history of previous malignancy, chemotherapy, or radiation therapy. The samples were obtained from the Clinical Departments of the Institute of Hematology and Blood Transfusion and the General University Hospital in Prague. None of the patients had received drug therapy for their disease or HSCT prior to blood collection, except for patients involved in the study of AZA treatment response. The patient's diagnoses were assessed based on the standard 2008 or 2016 WHO classification criteria (23,24), and all the patients were classified according to the IPSS (26) or IPSS-R categories (27).

PB samples and BM aspirates from age-matched healthy donors with no adverse medical history were used as controls. Written informed consents were obtained from all tested subjects in accordance with the ethical standards of the Declaration of Helsinki and its later amendments. The study was approved by the Institutional Scientific Board and the Local Ethics Committee.

Regarding AZA therapy, AZA was administered at 75 mg/m2/day for seven consecutive days every 28 days. The haematologic evaluation of the response to the treatment was performed after the fourth cycle according to the International Working Group (IWG) criteria for MDS (221) and AML (222). Patients who achieved complete remission (CR), partial remission (PR), marrow complete remission (mCR), or haematologic improvement (HI) were considered responders, whereas patients with stable disease (SD) or progressive disease (PD) were considered nonresponders.

The detailed characteristics of individual sample cohorts are included in the appropriate Results sections of P1, P2, and P3.

9.1.2 Cell separation

Mononuclear cells (MNCs) were separated from the BM aspirates by Ficoll-Paque density centrifugation (GE Healthcare, Munich, Germany). CD34+ cells were isolated from MNCs using the Direct CD34 Progenitor Cell Isolation MACS Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Both procedures were performed according to the manufacturer's instructions.

9.1.3 Separation of blood plasma

PB was collected in EDTA tubes, and blood plasma was separated from the PB by centrifugation at 460 g for 10 min. The absence of haemolysis in plasma samples was confirmed spectrophotometrically by measuring oxyhaemoglobin absorbance at 414 nm and by qPCR evaluating the ratio of miR-451 to miR-23a (delta Cq (miR-23a - miR-451a)) (223,224) using StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Only samples with the oxyhaemoglobin absorbance less than 0.2 and delta Cq (miR-23a - miR-451a) less than 5 were included. Plasma samples were further centrifuged at 12,000 g at 4°C for 15 min to remove cell debris and after gradual freezing stored at -80°C.

9.1.4 Separation of extracellular vesicles

EVs were extracted from plasma using the ExoQuick Plasma Prep and Exosome precipitation kit (System Biosciences, Palo Alto, CA, USA). Briefly, plasma samples were pretreated with thrombin (5 U/mL) and centrifuged to dispose of fibrin and cell debris. Then, EVs were precipitated from 250 µL of pretreated plasma using ExoQuick according to the manufacturer's protocol.

9.1.5 Transmission electron microscopy

Transmission electron microscopy (TEM) was used to confirm the presence and size of EVs. It was done in cooperation with Romana Hadravová from the Institute of Organic Chemistry and Biochemistry of Academy of Sciences of the Czech Republic. EVs were visualized by negative staining. Briefly, Parlodion-carbon-coated grids were floated on the top of a 5 µl drop of the sample for 5 min. Then, the grids were transferred on the top of a drop of 2% phosphotungstic acid (pH 7.4), stained for 2×1 min and dried. Photomicrographs were taken with JEOL JEM-1011 electron microscope (JEOL, Peabody, MA, USA) operated at 80 kV.

9.1.6 Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was used to define size and quantity of EVs. It was done in cooperation with Jaroslav Hanuš from the University of Chemistry and Technology in Prague. NTA was performed using Malvern NanoSight NS300 instrument (Malvern Panalytical, Malvern, UK). Briefly, purified EVs were diluted 5 x 10^3 in PBS and tracked using NTA analysis software. Each sample was analysed 3 times, and the counts were merged.

9.1.7 Western blotting

Western blotting was performed to confirm the presence of exosomes in isolated EV fractions. It was done in cooperation with Tereza Hrdinová and Matyáš Krijt from the Department of Proteomics, Institute of Hematology and Blood Transfusion (IHBT) in Prague. Briefly, EVs and K562 total cell lysate (used as a positive or a negative control) were lysed in 200 µl of NaCl-HEPES + 0,15% Triton (Sigma-Aldrich, St. Louis, MO, USA) and incubated on ice for 20 min. Total protein concentration was quantified by the Bradford protein assay (Bio-Rad, Hercules, CA, USA) and 30 µg of proteins were separated on 4 - 15% Mini-PROTEAN TGXTM gels (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with SuperBlock blocking buffer (Thermo Fisher Scientific) and immunostained. The following primary antibodies were used: mouse anti-CD81 antibody (1:1,000) (B11, sc-166029, Santa Cruz Biotechnology, Dallas, Texas, USA), rabbit anti-CD9 antibody (1:1,000) (EXOAB Kit 1, System Biosciences, Palo Alto, CA, USA) and rabbit anti-calnexin (C5C9) antibody (1:1,000) (2679, Cell Signaling Technology, Danvers, MA, USA). Secondary horseradish peroxidase-conjugated anti-rabbit (7074P2, Cell Signaling Technology) or anti-mouse (7076P2, Cell Signaling Technology) antibodies were used. For imaging, Westar Supernova ECL substrate was used (Cyanagen, Bologna, Italy).

9.1.8 RNA extraction

Different methods of RNA extraction were used to meet the requirements of downstream applications on RNA purity and input.

In P1 and P3, RNA from total plasma and CD34+ cells, respectively, were isolated using a phenol-chloroform extraction (225). In P1, Trizol LS reagent (Invitrogen, Basel, Switzerland) was used and the procedure was modified according to Filková et al. (226).

In P2, RNA from total plasma and EVs was extracted using the miRNeasy Serum/Plasma Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

RNA concentration was quantified with NanoDrop spectrophotometer (Thermo Fisher Scientific) and Qubit 2.0 fluorometer using Qubit microRNA Assay Kit, Qubit RNA HS Assay Kit, and Qubit RNA BR Assay Kit (Thermo Fisher Scientific). Integrity of RNA from CD34+ cells was evaluated with Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

9.1.9 miRNA profiling with microarrays and data analysis

miRNA profiles were determined using Agilent Human miRNA Microarrays (Sure Print G3 Unrestricted miRNA 8x60K, Release 19.0, Agilent Technologies). The input amounts of total RNA from plasma (P1) and from cells (P3) were 350 ng and 200 ng per sample, respectively. The samples were processed according to the manufacturer's protocol. Scanning was performed on an Agilent Microarray Scanner.

Data extraction and quality control were performed by Agilent Feature Extraction Software (v10.7.3.1). The data were normalized and processed in R statistical software (www.r-project.org) with the AgiMicroRNA (227) and its web interface MagiCMicroRna (228) packages in cooperation with Viktor Stránecký from the Institute of Inherited Metabolic Disorders in Prague.

Differential gene expression analyses were performed in R using the gtools package (|FC| and its modifications). Subsequent data analyses were performed using MeV v4.8.1 software (229). Analysis of variance (ANOVA) was performed to identify differentially expressed miRNAs between different groups of samples. Welch's approximate t-test was applied to determine differentially expressed miRNAs between two sample groups. Multiple testing correction was performed using the Holm-Bonferroni method. Hierarchical clustering of the miRNA expression data was done by using average linkage and Euclidean distance.

9.1.10 Small RNA-seq and data analysis

All sequencing libraries were constructed from 5 µl of RNA (RNA was isolated from plasma (200 µl) and EVs (precipitated from 250 µl of plasma) and was eluted to 30 µl Rnase-free water). The libraries were prepared, amplified, and purified using a QIAseq miRNA Library Kit (QIAGEN) following the manufacturer's protocol. The concentration and size of the purified libraries were measured with Qubit 2.0 fluorometer using Qubit dsDNA HS Assay Kit and Agilent 4200 TapeStation using D1000 Screen Tape Assay (Agilent Technologies), respectively. Libraries were adjusted to 4 nM, pooled together, and sequenced on HiSeq 2500 sequencer (Illumina, San Diego, CA, USA) as single reads for 83 cycles.

After quality control of raw data using the FastQC tool (230), the sequences were processed using the QIAseq miRNA Primary Quantification pipeline (QIAGEN) available *via* The GeneGlobe Data Analysis Center. Briefly, 3' adapter and low-quality bases were trimmed using Cutadapt (231), and the insert sequences and unique molecular indices (barcodes) were identified. The reads shorter than 16 nucleotides were discarded from the analysis. Sequences were then aligned using a sequential alignment strategy to map to different databases (perfect match to miRBase mature, miRbase hairpin, noncoding RNA, mRNA and other RNA, and finally the second mapping to miRBase mature, in which up to two mismatches were tolerated) using Bowtie (232). At each step, only unmapped sequences were passed to the next step. miRBase v21 was used for annotation of miRNAs, and piRNABank was used for piRNAs. All remaining unaligned sequences were mapped to the GRCh38 genome.

De novo miRNAs were predicted using the miRdeep2 tool. The origin of nonhuman RNAs was identified by metagenome analyzer MEGAN (233). Due to an excessive amount of data, the nonhuman RNAs were analysed only on some of the samples (11 patients and 3 controls) using 50,000 randomly selected reads (the number was assessed as sufficient for the analysis based on a taxonomy rarefaction plot).

Annotated read counts were subsequently processed in R statistical environment. Data normalization and subsequent statistical analyses were performed using the edgeR package (234). Binary logarithms of fold changes (logFC) and q values (False Discovery Rate (FDR) adjusted p value) were generated as an output of edgeR package for differential expression analysis of the data. Analyses were considered statistically significant if $q < 0.05$. Hierarchical cluster analysis was performed using the pvclust package (235) with average correlation. RNA-seq analysis was done in cooperation with David Kundrát, Department of Genomics, IHBT, Prague.

9.1.11 Reverse transcription quantitative PCR

Reverse transcription quantitative PCR (RT-qPCR) was performed for relative quantification of individual sncRNA levels. Reverse transcription reactions were prepared with TaqMan MicroRNA cDNA synthesis kit (Thermo Fisher Scientific) following the manufacturer´s instructions.

A no template control and a negative control for each RT reaction were included in every assay. qPCR reactions were prepared using TaqMan MicroRNA assays and TaqMan Universal Master Mix II, no UNG (both Thermo Fisher Scientific) and performed on StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The assays were designed and verified by the producing company (Thermo Fisher Scientific) and are commercially available. The individual assays are specified in the Results. The data were normalized to RNU48 and processed by the $2^{-\Delta\Delta CT}$ method (236).

9.1.12 Droplet digital PCR

Droplet digital PCR (ddPCR) was performed to quantify absolute levels of individual sncRNAs. RT reactions were prepared with TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer´s instructions. A no template control and a negative control for each RT reaction were included in every assay. PCR reactions were prepared using ddPCR Supermix for Probes (Bio-Rad) and TaqMan MicroRNA assays (Thermo Fisher Scientific). The assays were designed and verified by the producing company (Thermo Fisher Scientific) and are commercially availabe. The individual assays are specified in the Results. Droplets were prepared using QX200 Automated Droplet Generator. After PCR, signals were detected on QX200 Droplet Reader with QuantaSoft software (all from Bio-Rad).

9.1.13 Mutational screening and data analysis

Mutational screening was performed as a part of routine clinical assessment using the TruSight Myeloid Sequencing Panel Kit (Illumina) containing 568 amplicons of 54 genes associated with myeloid malignancies. It was done in cooperation with Jitka Veselá, Monika Hrubá, and Katarina Szikszai, Department of Genomics, IHBT, Prague. The amplicon library was constructed according to the manufacturer's recommendations. The libraries were 2x150 bp paired-end sequenced on a MiSeq instrument (Illumina), and the data were analysed using NextGENe software (SoftGenetics, State College, PA, USA). The clinical significance of each variant was verified in several genomic databases (UCSC, COSMIC, ExAC, and PubMed). The arbitrary cut off was set at three percent of variant allele frequency (VAF).

9.1.14 Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Unpaired or paired tests (when appropriate) were used to compare continuous variables between different groups of samples. Based on data normality distribution (according to Shapiro-Wilk test), appropriate parametric or nonparametric variants of testing were utilized. The chi-squared test was applied for the comparison of categorical clinical variables. Pearson correlation analysis was performed to identify possible dependence between continuous variables. The sensitivity and specificity of the optimum cut-off points were defined as the values that maximized the area under the receiver operating characteristic (ROC) curve (AUC). The progression-free survival (PFS) and overall survival (OS) curves were generated by the Kaplan-Meier method, and the differences between groups were assessed by the log-rank test. Multivariate analysis was performed using the Cox proportional regression model. Results were considered statistically significant if $p < 0.05$.

All statistical analyses were done in cooperation with Pavla Pecherková, Department of Biostatistics and Bioinformatics, IHBT, Prague. Analyses with more details are specified in the Results.

9.1.15 miRNA pathway analysis

Pathway analysis was done to identify signalling pathways that may be affected by specific miRNAs. The analysis was based on the data on significant differences in miRNA levels using DIANA-miRPath v3.0 (237). Within the analysis, target prediction of miRNAs was computed using the DIANAmicro T-CDS, and the most significantly affected KEGG pathways were identified.

10 RESULTS

The Results section comprises three subsections presenting the results from the three publications (Publication 1, Publication 2, and Publication 3 as indicated in the Preface).

10.1 Publication 1

Dostalova Merkerova et al., Microarray profiling defines circulating microRNAs associated with myelodysplastic syndromes, Neoplasma, 2017

In this study, we investigated levels of 2,006 predefined miRNAs in total blood plasma from MDS patients using microarrays. We found that specific miRNAs are significantly deregulated between lower-risk and higher-risk MDS patients and associate with progression and survival, suggesting that specific circulating miRNAs may serve as biomarkers of MDS patient outcome.

10.1.1 Patient cohort

The study was conducted on a cohort of 60 individuals, who included 40 MDS patients and 20 healthy individuals as controls. Based on the WHO classification criteria from 2008 (23), the diagnoses of MDS patients were as follows: 2 RCUD, 3 RARS, 17 RCMD, 4 RAEB-1, and 14 RAEB-2. The detailed clinical characteristics of all patients are summarized in Table 9.

Table 9. Characteristics of the patients. The data are presented as the mean and range for all continuous variables.

Number of patients		40
Sex (male/female)		30/10
Age (years)		66 (40-85)
Diagnosis (RCUD/RARS/RCMD/RAEB-1/RAEB-2)		2/3/17/4/14
IPSS category (low/intermediate-1/intermediate-2/high)		4/20/10/6
IPSS karyotype (good/intermediate/poor)		25/9/6
Cytogenetics		
normal karyotype		23
isolated del(20q)		2
isolated +8		2
complex		6
other		7
BM blasts (%)		6.1 (0.0-19.6)
Haemoglobin (g/l)		100 (51-138)
Neutrophils ($x10^9$/l)		1.9 (0.2-8.6)
Platelets ($x10^9$/l)		123 (13-528)
Follow-up	number of patients	38
	mean follow-up (months)	22.7 (2.3-78.5)
i. stable disease	number of patients	16
	mean follow-up (months)	27.7 (3.2-78.5)
ii. progression	number of patients	22
	mean time from diagnosis (months)	14.3 (2.3-58.0)
iii. HSCT	number of patients	7
	mean time from diagnosis (months)	6.7 (2.3-13.4)
iv. death	number of patients	11
	mean time from diagnosis (months)	18.5 (3.6-46.7)

10.1.2 miRNA profiling in MDS plasma

miRNA profiling was performed using microarrays. For this high-throughput analysis, only 21 samples (14 patients and 7 controls) were selected, due to limited budget. The complete raw and normalized data have been deposited in the NCBI Gene Expression Omnibus (GEO) database and are accessible through GEO Series accession number GSE76775.

Among 2,006 miRNAs present on the array, expression of 207 and 201 miRNAs was detectable in MDS and control samples, respectively. Only the miRNAs that were detected in more than 4 samples were included for further analysis. The Welch t-test was applied to determine miRNAs differentially expressed between two groups of samples: (i) patients and controls and (ii) lower-risk and higher-risk patients. Comparative analysis identified 48 miRNAs showing significantly ($p < 0.01$)

altered levels in plasma of MDS patients (irrespective of their clinical parameters) compared to those in plasma of controls (Figure 5). Among these miRNAs, 19 were downregulated (e.g. miR-451a, miR-92a-3p, miR-320a/b/d/e, and miR-142-3p/5p), and 29 were upregulated (e.g. miR-150-5p, miR188-5p, and miR-371b-5p) in patients. Between lower-risk (N = 8) and higher-risk (N = 6) MDS patients, 17 miRNAs were identified as deregulated. For example, miR-16-5p, miR-17-5p, miR-27a-3p/b-3p, miR-223-3p were downregulated and miR-188-5p, miR-623 were upregulated in higher-risk patients (Figure 6).

10.1.3 Quantification of individual miRNA levels by ddPCR

Levels of specific miRNAs were quantified by ddPCR in the cohort of all samples. Based on the microarray results, we selected six haematopoiesis and/or oncology related miRNAs (miR-16-5p, miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a) that showed deregulated levels in MDS plasma. In concordance with the microarray data, plasma miR-150-5p was increased and miR-16-5p, miR-27a-3p, miR-199a-5p, and miR-451a were decreased in MDS patients compared to controls. Comparison of miRNA levels between higher-risk and lower-risk patients revealed significantly ($p < 0.05$) lower levels of miR-27a-3p, miR-199a-5p, and miR-223-3p in higher-risk patients (Figure 7).

Figure 5. miRNAs deregulated in MDS plasma compared to control plasma. The heatmap shows miRNAs with deregulated levels ($p < 0.01$) in all MDS patients compared to controls. Color gradient intensity scale indicates binary logarithm of fold change (logFC) of signal intensities compared with the mean signal intensity of controls. Red indicates increased level of miRNA, green indicates decreased level of miRNA. Each column represents a patient, and each row represents a miRNA. LR – lower-risk MDS patient, HR – higher-risk MDS patient

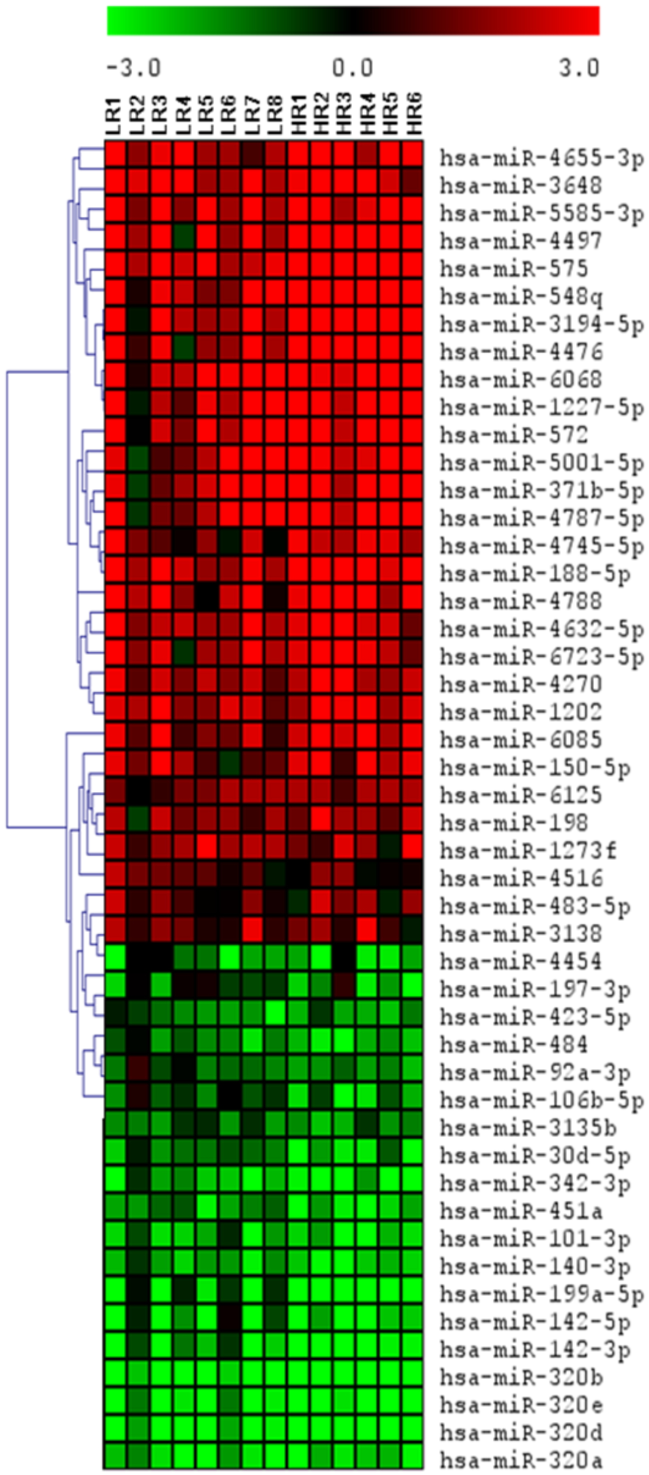


Figure 6. miRNAs altered between lower-risk and higher-risk MDS in plasma. The heatmap shows miRNAs with deregulated levels (p < 0.01) between lower-risk and higher-risk MDS patients. Color gradient intensity scale indicates logFC of signal intensities compared with the mean signal intensity of controls. Red indicates increased level of miRNA, green indicates decreased level of miRNA. Each column represents a patient, and each row represents a miRNA. LR – lower-risk MDS patient, HR – higher-risk MDS patient

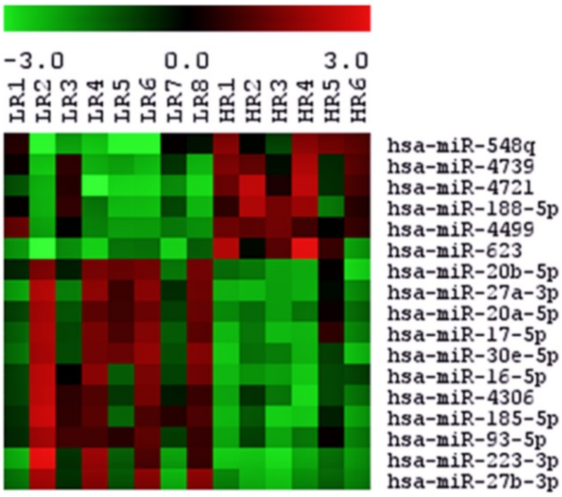
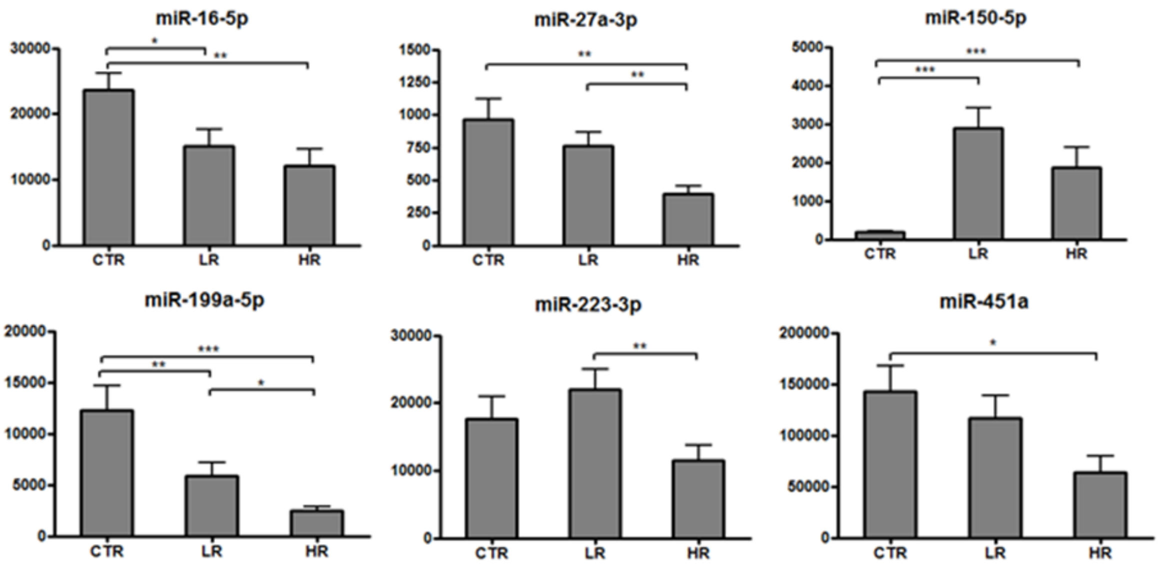


Figure 7. Levels of specific miRNAs in plasma. Absolute amounts of miR-16-5p, miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a were quantified with ddPCR. CTR – control, LR – lower-risk MDS, HR – higher-risk MDS, * p < 0.05, ** p < 0.01, *** p < 0.001



10.1.4 Correlation of circulating miRNA levels with clinical variables

A series of univariate analyses was performed for various clinical variables and for each of the six selected miRNAs to evaluate whether their plasma levels are potential predictors of patient outcomes. The mean patient follow-up was 22.7 months, and within the monitoring period, 22 patients progressed, and 11 patients died. The univariate analysis indicated significantly different PFS ($p < 0.05$) associated with the following parameters: IPSS karyotype, IPSS score, amount of cytopenias, WHO-based diagnosis, and the levels of five miRNAs (miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a). The univariate analysis for OS showed distinct stratification of MDS patients based on miR-27a-3p and miR-223-3p plasma levels. The cut-off values for each miRNA (assessed based on ROC curve analysis), mean PFS and OS values, 95% confidence intervals (CIs) and p values are listed for each of the tested variables in Table 10.

Multivariate Cox analyses confirmed that miR-451a plasma level (cut off $100x10^4$ copies/µl of plasma) and amount of cytopenias are associated with PFS and that plasma level of miR-223-3p (cut off $17x10^4$ copies/µl of plasma) are associated with OS (Table 11). Kaplan-Meier curves (Figure 8) showed that patients with low miR-451a expression have significantly decreased PFS (25.7 months, 95% CI 10.4 to 41.1) compared to those with high expression (41.5 months, 95% CI 29.1 to 54.0) and that patients with low miR-223-3p expression have significantly decreased OS (27.7 months, 95% CI 13.4 to 41.9 months) compared to those with high expression (70.6 months, 95% CI 57.1 to 84.1 months). Thus, the results suggest that miR-451a and miR-223-3p may potentially serve as predictive biomarkers of patient outcome.

Further, we performed Pearson correlation analysis for the six selected miRNAs with clinical variables (numbers of erythrocytes, platelets, neutrophils, monocytes, leukocytes, and lymphocytes, haemoglobin concentration, and percentage of blasts in BM). However, we did not find any significant correlation between tested miRNA levels and clinical variables. The absence of correlation between miRNA levels and blood cell counts suggests that miRNAs are not simply released from one type of blood cells, but their release from cells may be more complex and selective process.

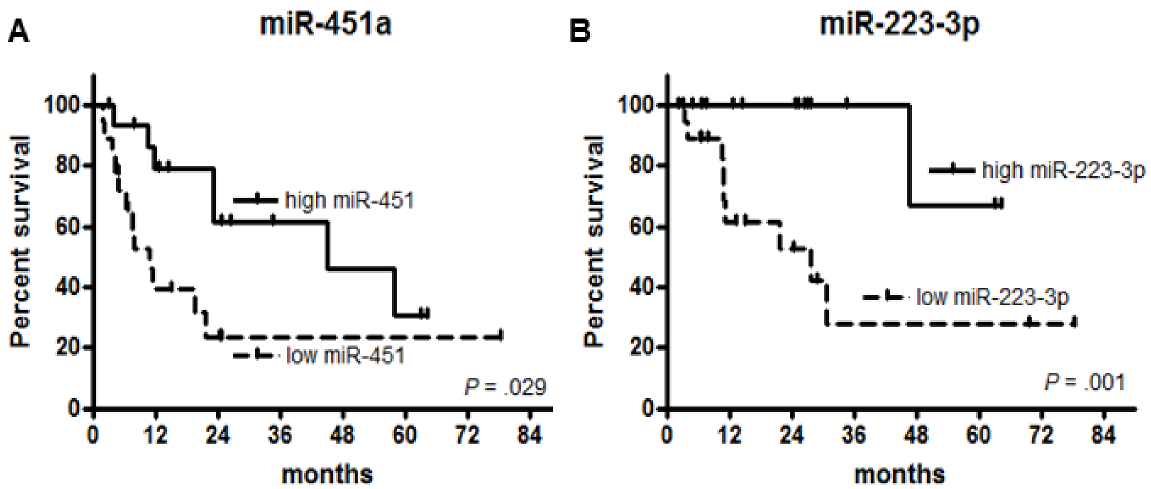
Table 10. Univariate analysis for PFS and OS.

Variable		PFS			OS		
		Mean est. (mo)	95% CI	p value	Mean est. (mo)	95% CI	p value
Age	< 65 years	26.0	9.5-42.4	.155	52.1	29.9-74.3	.739
	≥ 65 years	35.9	23.3-46.6		42.6	27.6-57.6	
Gender	male	36.8	23.3-50.4	.375	46.3	33.0-65.6	.507
	female	23.8	8.6-39.1		49.3	29.4-63.1	
Diagnosis	RCUD/ RARS	41.7	19.5-63.9	**.038**	41.7	19.5-63.9	.958
	RCMD	41.2	24.6-57.8		50.6	32.4-68.9	
	RAEB-1/ RAEB-2	13.2	7.5-18.9		26.8	20.1-33.5	
IPSS category	lower-risk	42.2	27.9-56.4	**.013**	50.8	35.2-66.5	.709
	higher-risk	12.7	6.9-18.6		26.3	19.2-33.5	
IPSS karyotype	good	42.5	27.9-57.2	**.005**	53.6	36.8-70.5	.082
	intermediate	24.7	10.6-38.9		42.0	30.7-53.3	
	poor	6.4	3.3-9.6		10.2	6.4-13.9	
Blasts	< 5%	38.5	24.0-53.0	.191	45.3	28.9-61.7	.314
	≥ 5%	22.0	8.8-35.1		47.6	28.4-66.8	
Haemoglobin	< 100 g/l	26.2	12.9-39.5	.082	48.5	29.5-67.5	.699
	≥ 100 g/l	37.0	22.7-51.2		42.3	27.7-57.0	
Neutrophils	< $1.5x10^9$/l	29.4	15.1-43.7	.467	40.7	24.1-57.3	.183
	≥ $1.5x10^9$/l	34.5	20.7-48.3		59.7	46.9-72.4	
Platelets	< $150x10^9$/l	27.3	15.0-39.5	.126	43.8	26.7-60.7	.179
	≥ $150x10^9$/l	38.8	23.2-54.4		49.6	32.4-66.8	
Cytopenia	0-1 lineage	46.8	31.0-62.5	**.024**	59.0	49.2-68.7	.084
	2-3 lineages	24.9	13.7-36.1		40.0	23.8-56.2	
miR-16-5p	< $12x10^4$ copies/µl of plasma	25.6	11.4-39.8	.338	49.8	29.1-70.4	.769
	≥ $12x10^4$ copies/µl of plasma	33.6	20.3-47.0		41.8	25.7-58.0	
miR-27a-3p	< $2.7x10^3$ copies/µl of plasma	11.2	6.2-16.2	**.005**	18.0	11.8-24.1	**.001**
	≥ $2.7x10^3$copies/µl of plasma	41.9	28.0-55.8		60.7	45.7-75.6	
miR-150-5p	< $25x10^3$ copies/µl of plasma	26.9	14.0-40.0	**.038**	44.9	27.6-62.2	.089
	≥ $25x10^3$ copies/µl of plasma	50.1	36.0-64.1		60.0	43.4-76.4	
miR-199a-5p	< $2.6x10^3$ copies/µl of plasma	21.9	8.9-34.9	**.048**	37.9	19.4-56.5	.100
	≥ $2.6x10^3$ copies/µl of plasma	37.4	24.3-50.5		47.6	30.9-64.4	
miR-223-3p	< $17x10^4$ copies/µl of plasma	18.3	9.0-27.7	**.008**	27.7	13.4-41.9	**.001**
	≥ $17x10^4$ copies/µl of plasma	49.6	32.1-67.2		70.6	57.1-84.1	
miR-451a	< $100x10^4$ copies/µl of plasma	25.7	10.4-41.1	**.029**	56.3	38.6-74.1	.709
	≥ $100x10^4$ copies/µl of plasma	41.5	29.1-54.0		47.6	33.3-61.9	

Table 11. Multivariate Cox analysis for PFS and OS. HR – hazard ratio, CI – confidence interval, n.a. – not analysed

	PFS			OS		
Variable	HR	95% CI	p value	HR	95% CI	p value
Diagnosis	0.512	0.040-6.488	.300	0.923	0.148-5.744	.932
IPSS category	6.398	0.835-49.032	.074	1.089	0.089-13.369	.947
IPSS karyotype	1.207	0.358-4.064	.762	0.151	0.014-1.693	.125
Cytopenia	12.647	1.578-101.358	**.017**	2.128	0.172-26.287	.556
miR-27a-3p	0.177	0.026-1.201	.076	0.682	0.103-4.511	.691
miR-150-5p	2.983	0.376-23.685	.301	n.a.	n.a.	n.a.
miR-199a-5p	4.133	0.527-32.404	.177	n.a.	n.a.	n.a.
miR-223-3p	0.739	0.155-3.513	.704	0.039	0.002-0.856	**.032**
miR-451a	0.072	0.011-0.467	**.006**	n.a.	n.a.	n.a.

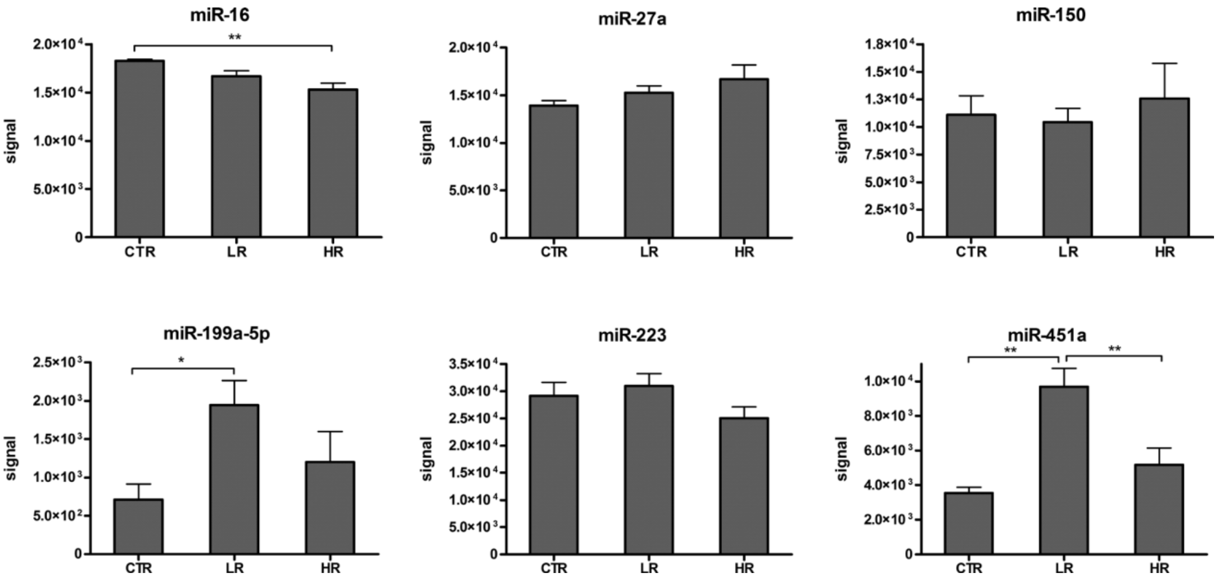
Figure 8. Patient survival according to miRNA levels. Kaplan-Meier curves for PFS based on miR-451a plasma level ($p < 0.05$) (A) and OS according to miR-223-3p plasma level ($p = 0.01$) (B).



10.1.5 Comparison of circulating miRNA levels with their levels in CD34+ cells

Previously, we have assessed miRNA profiles in CD34+ MDS cells using microarrays (196). We used these data to evaluate the cellular levels of the six miRNAs deregulated in MDS plasma (miR-16-5p, miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a). We observed some expression changes between controls, higher-risk, and lower-risk patient groups (Figure 9). However, we did not find any apparent relation of circulating miRNA levels to their levels in CD34+ cells, again suggesting that miRNA release from cells may be specific process.

Figure 9. miRNA levels in CD34+ MDS cells. Relative expression of miR-16-5p, miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a in CD34+ MDS cells were calculated based on microarray data (196). CTR – controls, LR – lower-risk, HR – higher-risk MDS patients, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$



10.2 Publication 2

Hrustincova et al., Circulating small noncoding RNAs have specific patterns in plasma and extracellular vesicles in myelodysplastic syndromes and are predictive of patient outcome, Cells, 2020

In this study, we continued to analyse sncRNA profiles in blood plasma. Notably, we moved from microarray profiling to the next generation sequencing (NGS), which provides identification of all sncRNAs present in a sample, including non-miRNA sncRNA species. Thus, except for the most abundant miRNAs, we detected also other sncRNA species such as piRNAs, tsRNAs, and snoRNAs. Importantly, we investigated not only samples of total blood plasma, but also samples of plasma-derived EVs. It is the very first study in the field of MDS that analyses sncRNA profiles in paired samples from two extracellular sources (total plasma and EVs) and draws a comparison between them. We identified sncRNAs that may be useful as prognostic and predictive biomarkers of MDS outcome and AZA treatment response, respectively.

In order to keep clarity of this thesis, the large amount of results presented in the publication were reduced to only a major link. All the data can be found in the publication and its supplemental information.

10.2.1 Patient cohorts

The study was conducted on 114 blood plasma samples that were randomly divided in two cohorts – discovery and validation cohorts. The discovery cohort analysed by NGS comprised 59 individuals, including 31 MDS, 11 AML-MRC patients, and 17 healthy controls. Based on the WHO classification criteria from 2016 (24), the diagnoses of MDS patients were as follows: 5 MDS-MLD, 5 MDS-RS, 3 MDS with isolated del(5q), 5 MDS-EB1, and 13 MDS-EB2. The diagnoses MDS-MLD, MDS-RS, and MDS with isolated del(5q) were considered early MDS and the diagnoses MDS-EB1 and MDS-EB2 were considered advanced MDS. The time from diagnosis ranged from zero months (i.e. diagnostic samples) to five years from the initial assessment of the diagnosis, with the majority of samples obtained within one year from the diagnosis (81 %). The detailed clinical characteristics of all patients are summarized in Table 12. An independent validation cohort analysed by ddPCR comprised 55 individuals, including 36 MDS, 7 AML-MRC patients, and 12 healthy controls (Table 13).

Mutational screening for 54 genes associated with myeloid malignancies was done for 18 of 31 MDS patients in the discovery cohort (58 %). Of them, 83 % bore at least one somatic mutation with 1.8 mutational events per patient on average (range 1-5). The most frequently mutated genes in the cohort were SF3B1 (5 patients, 28 %, variant allele frequency (VAF) ranging from 26 to 50 %) and DNMT3A (5 patients, 28 %, VAF ranging from 26 to 47 %). These two most commonly mutated genes were selected for further investigation.

In the follow-up period, 24 patients received AZA therapy. The mean time from sample collection to AZA treatment initiation was 1 month (0-7 months). The mean number of administered AZA cycles was 9 (2-56 cycles), the mean time to the best response in the responder cohort was 4.5 months (3-6 months), and the mean duration of the response was 15 months (6-56 months). In this AZA cohort, 9 patients were considered responders, i.e. they achieved complete remission (CR), partial remission (PR), marrow complete remission (mCR), or haematologic improvement (HI), 6 patients had stable disease, and 9 patients progressed after AZA treatment initiation. The overall response rate (ORR) including rates for all responders within the cohort was 37.5 %.

Furthermore, we analysed the survival of MDS patients stratified according to clinical variables. We found that diagnosis, IPSS-R category, IPSS-R-based karyotype, BM blasts, haemoglobin level, and platelet count were significantly associated with OS (univariate analysis, $p < 0.05$).

Table 12. Characteristics of the patients in the discovery cohort. The data are presented as the mean and range for all continuous variables.

MDS	**31**
Age (years)	67 (29-87)
Sex (male/female)	20/11
Diagnosis MLD/RS/5q-/EB1/EB2	5/5/3/5/13
IPSS-R category very low/low/intermediate/high/very high/n.a.	3/10/4/6/6/2
BM blasts (%)	7.4 (0.4-19.4)
White blood count ($x10^9$/l)	4.3 (1.2-11.9)
Haemoglobin (g/l)	100 (72-138)
Neutrophils ($x10^9$/l)	2.4 (0.1-8.6)
Platelets ($x10^9$/l)	136 (13-390)
Cytogenetic features normal karyotype isolated del(5q) complex other n.a.	 13 3 4 8 3
IPSS-R karyotype very good/good/intermediate/poor/very poor/n.a.	0/18/4/2/4/3
Somatic mutations No. of analysed patients No. of mutations per patient: 0/1/2/3/4/5 The most frequent mutations SF3B1/DNMT3A/RUNX1/ASXL1/EZH2/SETBP1	 18 (58 %) 3/5/6/2/1/1 5/5/4/3/3/3
Follow-up mean follow-up (months)	22 (1-61)
Deceased, number of patients mean time to death (months)	19 (61 %) 19 (1-43)
Follow-up treatment with AZA, number of patients No. of responders No. of patients with stable disease No. of patients with progressed disease	24 9 6 9
AML-MRC	**11**
Age	69 (58-77)
Sex (male/female)	6/5
BM blasts (%)	26.0 (20.1-41.0)
White blood count ($x10^9$/l)	3.0 (0.8-8.1)
Haemoglobin (g/l)	95 (77-127)
Neutrophils ($x10^9$/l)	1.0 (0.1-3.4)
Platelets ($x10^9$/l)	116 (24-258)
Cytogenetic features normal karyotype isolated del(5q) complex other n.a.	 5 1 1 2 2

n.a. – not analysed

Table 13. Characteristics of the patients in the validation cohort. The data are presented as the mean and range for all continuous variables.

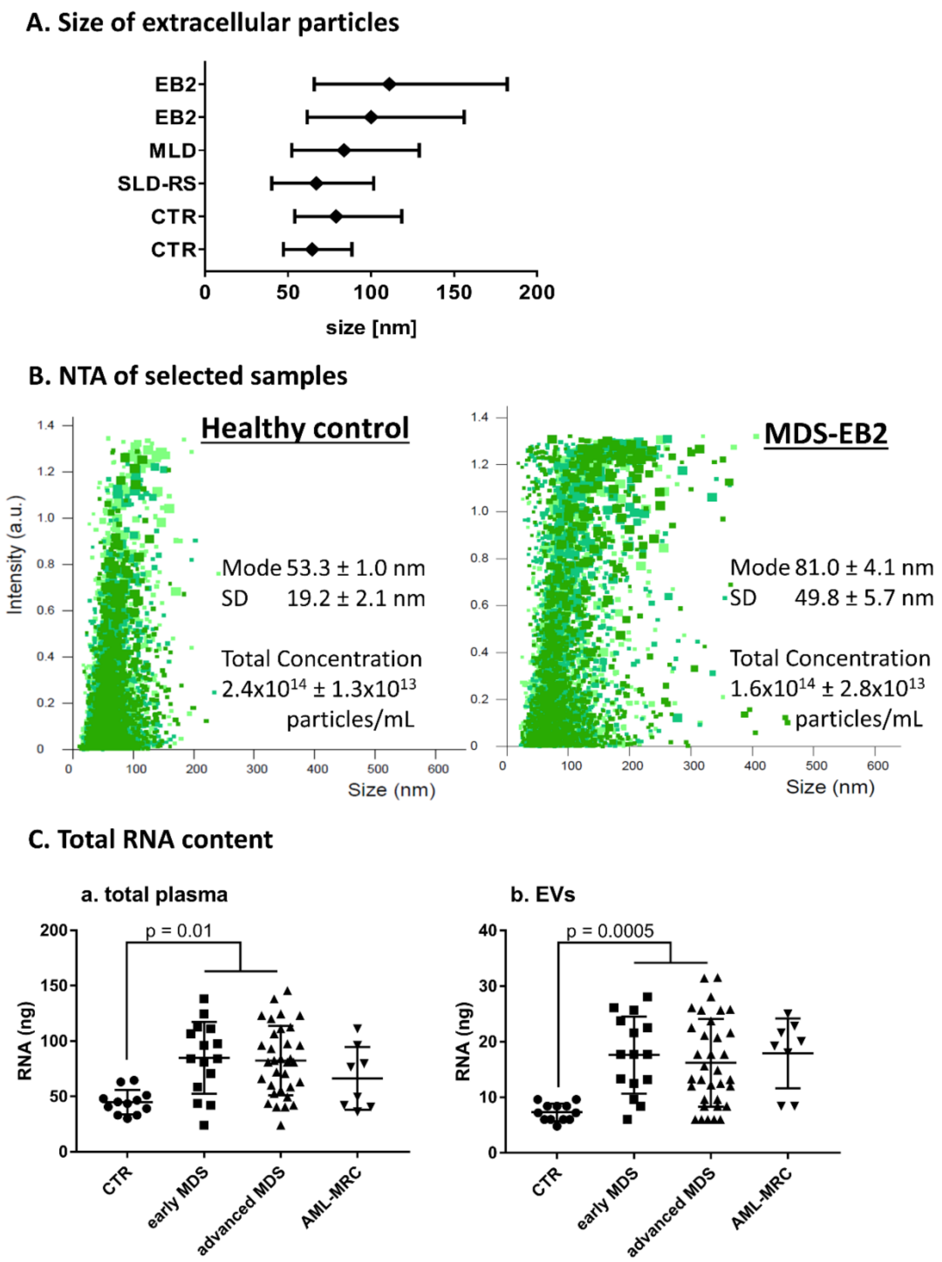
MDS	**36**
Age	60 (29-81)
Sex (male/female)	26/10
Diagnosis SLD/MLD/RS/5q-/EB1/EB2	2/10/2/1/5/16
IPSS-R category very low/low/intermediate/high/very high/n.a.	6/10/5/4/11
BM blasts (%)	7.1 (0.2-19.8)
White blood count (x10^9/l)	3.8 (0.9-11.4)
Haemoglobin (g/l)	101 (59-138)
Neutrophils (x10^9/l)	1.8 (0.1-7.6)
Platelets (x10^9/l)	126 (26-273)
Cytogenetic features normal karyotype isolated del(5q) complex other	 16 2 9 9
IPSS-R karyotype very good/good/intermediate/poor/very poor	1/20/3/3/9
Follow-up mean follow-up (months)	11 (0-77)
Deceased, number of patients mean time to death (months)	17 (47 %) 7 (1-23)
AML-MRC	**7**
Age	68 (62-76)
Sex (male/female)	5/2
BM blasts (%)	36.3 (23.0-62.0)
White blood count (x10^9/l)	2.2 (1.0-4.8)
Haemoglobin (g/l)	100 (85-127)
Neutrophils (x10^9/l)	0.8 (0.1-2.9)
Platelets (x10^9/l)	91 (10-202)
Cytogenetic features normal karyotype complex other	 2 3 2

n.a. - not analysed

10.2.2 Characterization of extracellular vesicles in MDS plasma

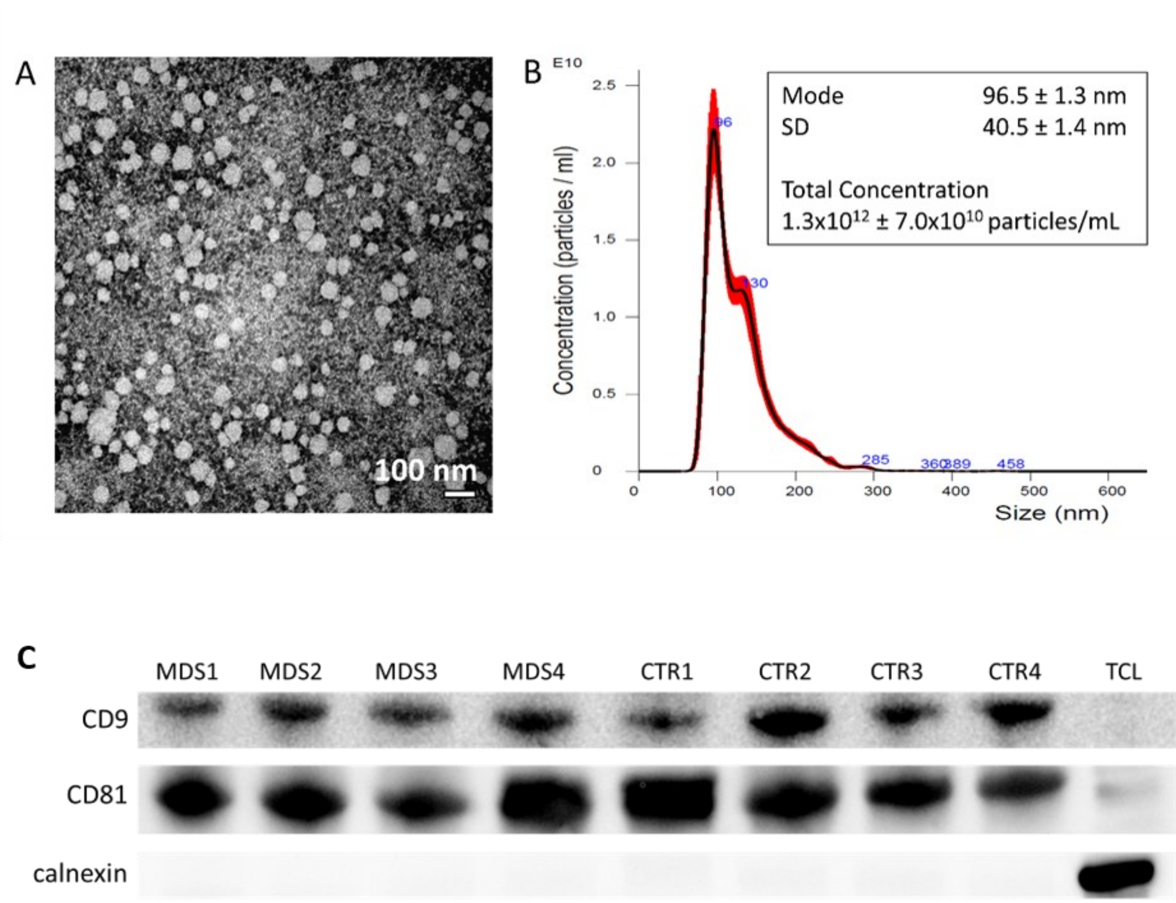
To characterize the EVs in MDS plasma, we analysed several total plasma samples by nanoparticle tracking analysis (NTA). We observed that neither particle counts, nor their cumulative volumes were associated with the disease. However, we observed a fraction of particles with larger sizes specifically in the plasma of higher-risk MDS patients (Figure 10, A and B). More importantly, we obtained significantly higher amounts of RNA material from patient samples compared to controls when isolated from total plasma samples (44.7 ± 3.2 ng/ml of plasma in controls *vs.* 92.9 ± 8.8 ng/ml in patient samples, p = 0.01) as well as from EV fractions (7.3 ± 0.5 ng/ml of plasma in controls *vs.* 17.5 ± 1.3 ng/ml in patient samples, p = 0.0005) (Figure 10C).

Figure 10. Characterization of extracellular particles in plasma. (A) Particle size (mean size with 10th and 90th percentiles) measured by nanoparticle tracking analysis (NTA). (B) NTA report of representative samples of total plasma from one control and one MDS-EB2 patient. (C) RNA yield obtained from (a) 1 ml of total plasma and (b) EV samples that were isolated from 1 ml of the plasma is plotted.



After separation, we reanalysed the isolated EVs by transmission electron microscopy (TEM), NTA, and western blotting. TEM imaging showed that the majority of isolated EVs ranged in size from 50 nm to 100 nm, suggesting sufficient exosome enrichment in the samples (Figure 11A). Based on the NTA measurements, we determined that the obtained EVs had a mode size of 96 nm (the size that has the highest number of recurrences in the sample), with particle sizes ranging from 87 to 180 nm (10^{th} to 90^{th} percentile) (Figure 11B). Using western blotting, we confirmed the presence of exosomes. Common exosome markers CD9 and CD81 were detected in MDS and control EV samples, whereas cell organelle (endoplasmic reticulum) marker calnexin tested as a negative control was not detected (Figure 11C). Based on these data, we conclude that although enriched in exosomes, our precipitated EV fractions contain a heterogeneous mixture of exosomes and, to a lesser extent, microvesicles. This is consistent with outputs from other protocols including those based on high-speed ultracentrifugation, e.g. (238).

Figure 11. Transmission electron microscopy (TEM) (A) and nanoparticle tracking analysis (NTA) (B) of one representative EV sample. Characterization of exosomes in EV samples by western blotting (C). Common exosome markers CD9, CD81, and cell organelle marker calnexin (negative control). K562 Total Cell Lysate (TCL) was loaded as a control.



10.2.3 General overview of circulating sncRNAs in MDS patients

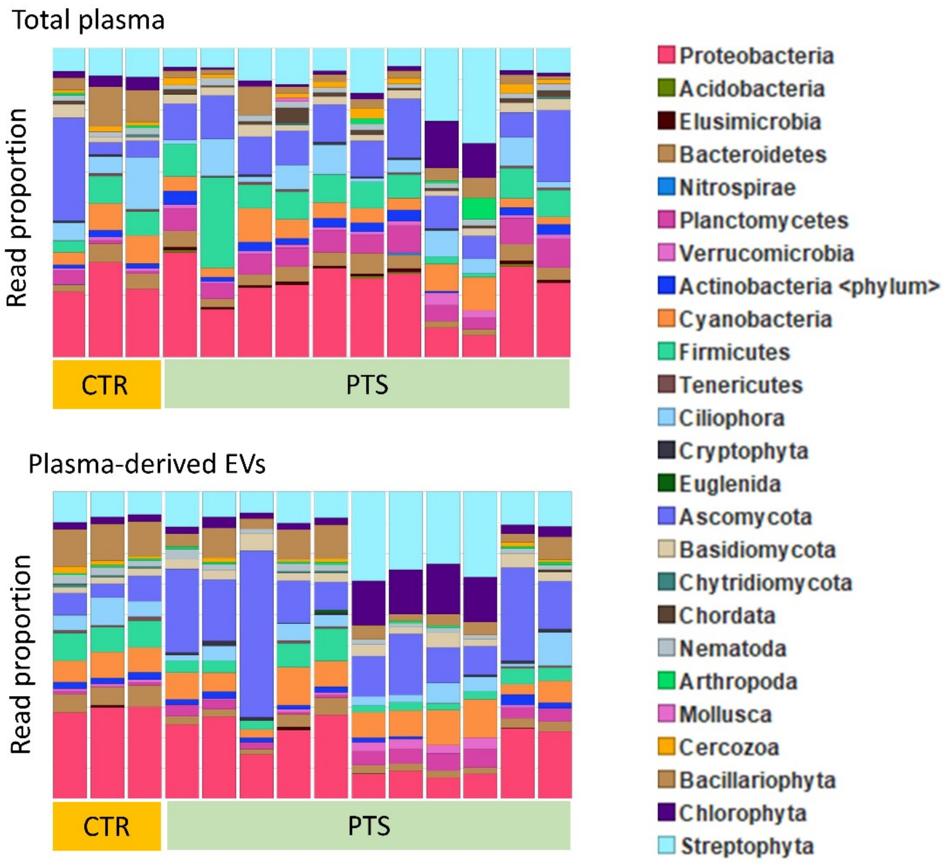
To systematically characterize the sncRNAs circulating in MDS plasma, we performed small RNA-seq analysis in paired samples (118 samples from 59 individuals) of total plasma and plasma-derived EVs. On average, we captured 8.3 millions (M) of total reads per sample (4.0-18.0 M reads) and after filtering, 5.0 M reads on average per sample (2.6-12.6 M reads) were retained in the analysis. In the filtering process, we removed all reads that had low quality scores, defective or missing adapters and/or barcodes, and were shorter than 16 nucleotides. The data have been deposited in the SRA (Sequence Read Archive) database under accession no. PRJNA574254.

The retained sequences were annotated and assigned to several categories of transcripts. The most abundant categories of transcripts were miRNAs (51.0 % of reads on average), followed by rRNAs (4.1 %), piRNAs (0.7 %), tRNAs (0.4 %), and mRNAs (0.4 %). Of the retained reads, 42.2 % were uncharacterized (of these, 8.5 % were mappable and 33.7 % were not mappable to the human genome). In total, we identified 2,543 miRNAs, 141 piRNAs and 364 tRNAs with at least one read in one sample.

Because a large proportion of the mapped reads were uncharacterized, we predicted potential *de novo* miRNAs among these sequences. Using the miRdeep2 tool, we identified 7,667 *de novo* miRNAs in total. On average, we found 168 and 146 *de novo* miRNAs per sample of total plasma and EVs, respectively. These *de novo* identified miRNAs were included in subsequent differential analyses.

Furthermore, we addressed the question of the features and origin of the uncharacterized unmapped reads. Interestingly, their proportion increased with read length. While almost all of the reads with lengths of 20-22 bp mapped to the human genome, the unmapped reads were longer
(> 31 bp). A high percentage of unmapped sequences was annotated as 16S/18S rRNA molecules of nonhuman origin (approx. 20 %), suggesting that numerous RNAs originating from other species are present in human plasma. The majority of these reads were mappable to Proteobacteria, Ascomycota, Streptophyta, and Chlorophyta. No apparent profile specific to MDS patients either in total plasma or in EVs was found (Figure 12). However, a deeper analysis of these nonhuman sequences with respect to differential representation of individual taxa has not been done as is beyond the scope of this study.

Figure 12. Taxonomy profile of reads mappable to nonhuman genomes. The proportion of the reads assigned to individual phyla is shown.

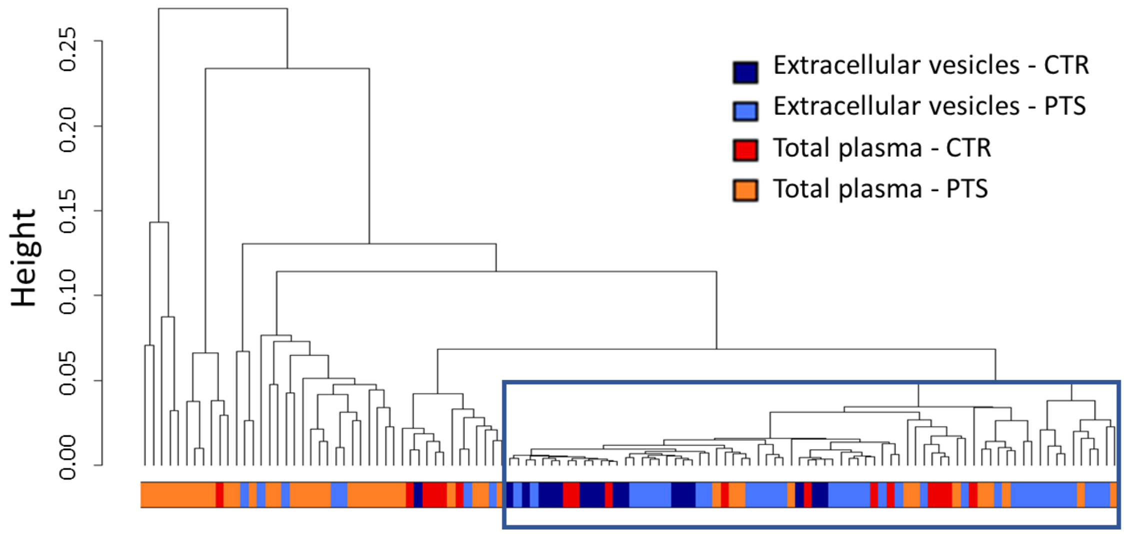


10.2.4 Comparison of total plasma samples with EV samples

We focused on human sncRNAs and observed that total plasma samples had a substantially higher proportion of miRNA reads compared to EV samples (60 % in total plasma and 46 % in EVs on average). In contrast, EV samples had higher proportion of uncharacterized reads than total plasma samples (38 % in EVs and 27 % in total plasma on average). Hierarchical cluster analysis of all samples revealed that EV content was more homogeneous than the sncRNA content of total plasma, preferentially clustering the majority of EV samples into one cluster (Figure 13).

Further, we performed differential expression analysis between paired samples of total plasma and EVs. We identified a striking difference between MDS and control samples. In MDS samples, 419 sncRNAs were differentially represented between total plasma and EVs (|logFC| > 1 and q < 0.05). In controls, only 44 sncRNAs reached this statistical significance cut off and the majority of them were also found in MDS samples. However, 385 differentially represented sncRNAs between total plasma and EVs were uniquely identified only in MDS. Interestingly, levels of miRNAs were proportionally deregulated between the two materials (114 miRNAs were increased in total plasma and 105 miRNAs were increased in EVs), but the levels of piRNAs, tRNAs, and other RNA categories were almost exclusively increased in total plasma (25 piRNAs and 105 tRNAs were increased in total plasma and only four piRNAs and three tRNAs were increased in EVs). These data suggest substantial deregulation of circulating sncRNAs associated with MDS. Moreover, the process of RNA release from cells may be specific for distinct sncRNA species, for example particular sncRNA species may be selectively loaded into EVs.

Figure 13. Hierarchical cluster analysis of total plasma and EV samples based on all RNA-seq data. The blue frame highlights the clustered EV samples. CTR – controls, PTS – MDS/AML-MRC patients



10.2.5 sncRNAs differentially expressed in MDS

To characterize sncRNA profiles specific for MDS, we compared sncRNA levels between MDS patients, AML-MRC patients, and controls in each material separately. The results show relatively higher numbers of differentially represented sncRNAs (|logFC| > 1 and q < 0.05) between MDS patients and controls in total plasma (391 sncRNAs with 316 increased and 75 decreased sncRNAs in MDS) than in EVs (219 sncRNAs with 179 increased and 40 decreased sncRNAs in MDS). Table 14 shows the distribution of deregulated sncRNAs among various sncRNA species. Many of these sncRNAs were uniquely deregulated either in total plasma or EVs. Importantly, we found that the levels of many haematopoiesis-related miRNAs were significantly increased (q < 0.05) in MDS patients compared to controls, mostly in both plasma and EVs (e.g. miR-10a-5p, miR-29a-3p, miR-34a-5p, miR-99b-5p, miR-125a-5p, miR-146b-5p, and miR-150-3p/5p were increased in both total plasma and EVs, and let-7a-3p, miR-21-3p, miR-221-3p, miR-221-3p/5p, and miR-223-3p were increased only in total plasma). Regarding other types of sncRNAs, hsa_piR_019914/gb/DQ597347, hsa_piR_020450/gb/DQ598104, chr2.trna27-GlyCCC, chr18.trna4-LysCTT, SNORD119, and U33 were upregulated in MDS samples (q < 0.05).

On the other hand, there were almost no significant differences between sncRNA profiles of MDS and AML-MRC patients. There were only 9 and 14 differentially represented RNAs in plasma and EVs, respectively (Table 15).

Table 14. Numbers of significantly deregulated (|logFC| > 1 and q < 0.05) circulating sncRNAs in MDS patients compared to controls.

Type of transcripts	**total plasma**		**EVs**	
	increased	decreased	increased	decreased
all annotated RNAs	316	75	179	40
annotated miRNAs	112	36	65	18
***de novo* identified miRNAs**	19	39	26	20
piRNAs	24	0	15	1
tRNAs	120	0	43	1
other annotated RNAs	41	0	30	0

Table 15. Numbers of significantly deregulated (|logFC| > 1 and q < 0.05) circulating sncRNAs in MDS patients compared to AML-MRC patients.

Type of transcripts	**total plasma**		**EVs**	
	increased	decreased	increased	decreased
all annotated RNAs	1	8	0	14
annotated miRNAs	0	3	0	1
***de novo* identified miRNAs**	1	5	0	10
piRNAs	0	0	0	1
tRNAs	0	0	0	2
other annotated RNAs	0	0	0	0

10.2.6 sncRNAs differentially expressed between early and advanced stages of MDS

To define sncRNAs with changed levels during MDS progression, we investigated the differences in sncRNA profiles between early (MDS-MLD, MDS-RS, and MDS with isolated del(5q)) and advanced stages of MDS (MDS-EB1 and MDS-EB2). These results showed 100 and 43 differentially represented sncRNAs (|logFC| > 1 and q < 0.05) in plasma (81 increased and 19 decreased in early MDS) and EVs

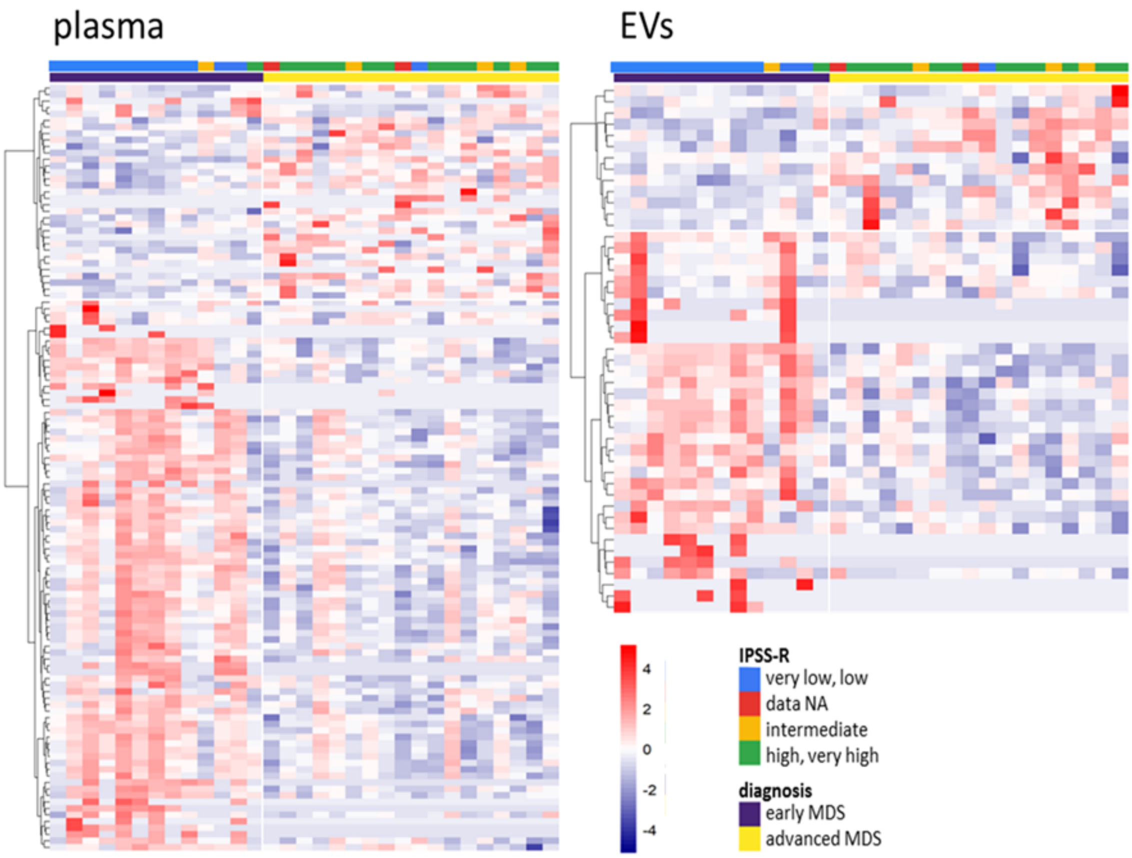
(34 increased and 9 decreased in early MDS), respectively. Their distribution among various sncRNA species is shown in Table 16. Heatmaps in Figure 14 show apparently distinct levels of these sncRNAs associated with the stage of the disease when the patients are divided according to either WHO classification or IPSS-R score.

The detailed examination of significantly deregulated sncRNAs (|logFC| > 1 and q < 0.05) between early and advanced stages of MDS revealed that only 14 sncRNAs were deregulated in both types of materials, total plasma and EVs. Regarding haematopoiesis related miRNAs, levels of some of them (e.g. miR-103a-3p, miR-103b, miR-107, miR-221-3p, miR-221-5p, and miR-130b-5p) were significantly decreased (q < 0.05) in total plasma of advanced MDS compared to early MDS. Interestingly, multiple miRNAs (e.g. miR-127-3p, miR-154-5p, miR-323b-3p, miR-382-3p, miR-409-5p, and miR-485-3p) clustered in chromosomal region 14q32 were found among the significantly upregulated (q < 0.05) miRNAs in total plasma and/or EVs of early MDS. Regarding other sncRNA species, hsa_piR_000805/gb/DQ571003, hsa_piR_019420/gb/DQ596670, chr6.trna152-ValCAC, and chr7.trna5-CysGCA were significantly deregulated (q < 0.05) between early and advanced MDS.

Table 16. Numbers of significantly deregulated (|logFC| > 1 and q < 0.05) circulating sncRNAs in early MDS patients compared to advanced MDS patients.

Type of transcripts	**total plasma**		**EVs**	
	increased	decreased	increased	decreased
all annotated RNAs	81	19	34	9
annotated miRNAs	55	12	24	7
***de novo* identified miRNAs**	12	4	8	0
piRNAs	7	1	1	2
tRNAs	5	1	1	0
other annotated RNAs	2	1	0	0

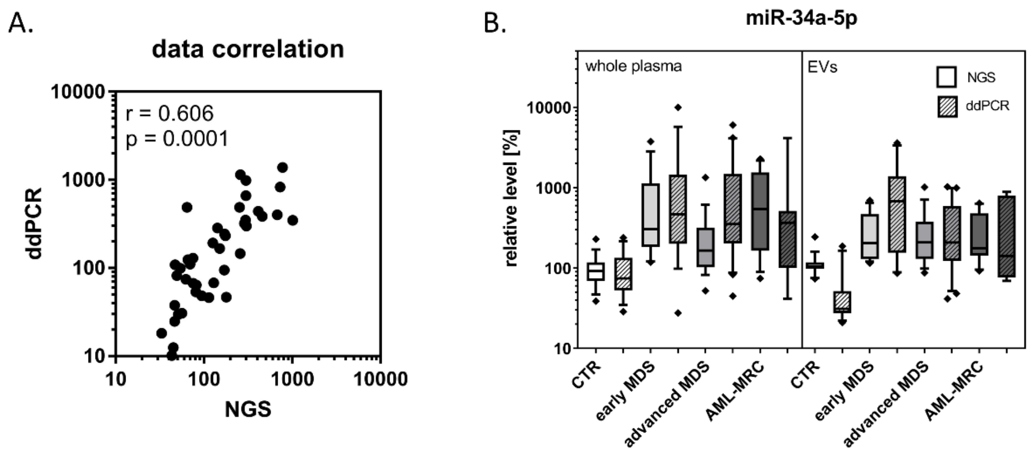
Figure 14. Heatmaps of differentially represented sncRNAs between early and advanced MDS in total plasma (left) and EVs (right) ($q < 0.05$). Color gradient intensity scale shows the row z-score of CPM (binary logarithm) of individual RNAs. Red indicates increased level of RNA, blue indicates decreased level of RNA. Each column represents a patient, and each row represents an RNA.



10.2.7 Quantification of individual sncRNA levels by ddPCR

To validate the results of NGS analysis, we evaluated the levels of miR-16-5p, miR-34a-5p, miR-125a-5p, miR-125b-5p, miR-127-3p, miR-221-3p, and hsa_piR_001170/DQ571526 by ddPCR in an independent validation cohort (36 MDS patients, 7 AML-MRC patients, and 12 controls, Table 13). These sncRNAs were selected based on their previously described relevance for MDS and/or significantly different levels in the small RNA-seq experiments. After absolute quantification of sncRNA levels, we compared these results with those of the small RNA-seq analysis by performing Pearson correlation of the mean expression values measured in the individual sample groups (controls, early MDS, advanced MDS, and AML-MRC). The correlation analysis proved concordance between both methods ($r = 0.606$, $p = 0.0001$) as shown in Figure 15.

Figure 15. Correlation of sncRNA levels measured by different methods in two independent sample cohorts. In the testing cohort, the data were obtained using small RNA-seq (NGS) and in the validation cohort, by ddPCR method. (A) The mean values of sncRNA levels (miR-16-5p, miR-34a-5p, miR-125a-5p, miR-125b-5p, miR-127-3p, miR-221-3p, and hsa_piR_001170/DQ571526) in individual sample groups (controls, early MDS, advanced MDS, and AML-MRC) were plotted in the graph and Pearson correlation was calculated. (B) Detailed comparison of NGS and ddPCR results for miR-34a levels are shown for illustration.



10.2.8 Pathway analysis based on miRNA profiles specific for MDS

To identify biological functions potentially influenced by deregulated miRNAs circulating in MDS plasma, we performed miRNA target prediction coupled with pathway enrichment analysis. The analyses were performed for miRNAs differentially represented (|logFC| > 1, q < 0.05) between MDS and control samples, and between early and advanced MDS samples either in plasma or EVs. As shown in Table 17 and Table 18 in more detail, we found that the deregulated miRNAs were associated with multiple pathways related to cancer (namely, the Ras, TGF-beta, ErbB, and Rap1 pathways), pluripotency of stem cells, extracellular matrix (ECM), and focal adhesion.

Table 17. The most significantly enriched pathways in the sets of deregulated miRNAs between MDS and controls. The top ten pathways with the most significant p values are listed for each dataset.

KEGG pathway	p value
plasma: MDS *vs.* CTR	
Mucin type O-Glycan biosynthesis	9.77e-15
Proteoglycans in cancer	6.05e-09
ErbB signalling pathway	2.80e-08
Ras signalling pathway	2.02e-07
Axon guidance	2.02e-05
Pathways in cancer	2.02e-05
Rap1 signalling pathway	3.30e-05
Lysine degradation	3.33e-05
Glioma	6.23e-05
Signalling pathways regulating pluripotency of stem cells	9.51e-05
EVs: MDS *vs.* CTR	
ECM-receptor interaction	1.16e-26
Fatty acid biosynthesis	1.41e-08
ErbB signalling pathway	1.41e-08
Proteoglycans in cancer	1.57e-08
Axon guidance	3.54e-08
Glioma	5.42e-08
Mucin type O-Glycan biosynthesis	5.18e-06
Estrogen signalling pathway	3.52e-05
Focal adhesion	5.71e-05
Signalling pathways regulating pluripotency of stem cells	5.71e-05

Table 18. The most significantly enriched pathways in the sets of deregulated miRNAs between early and advanced MDS. The top ten pathways with the most significant p values are listed for each dataset.

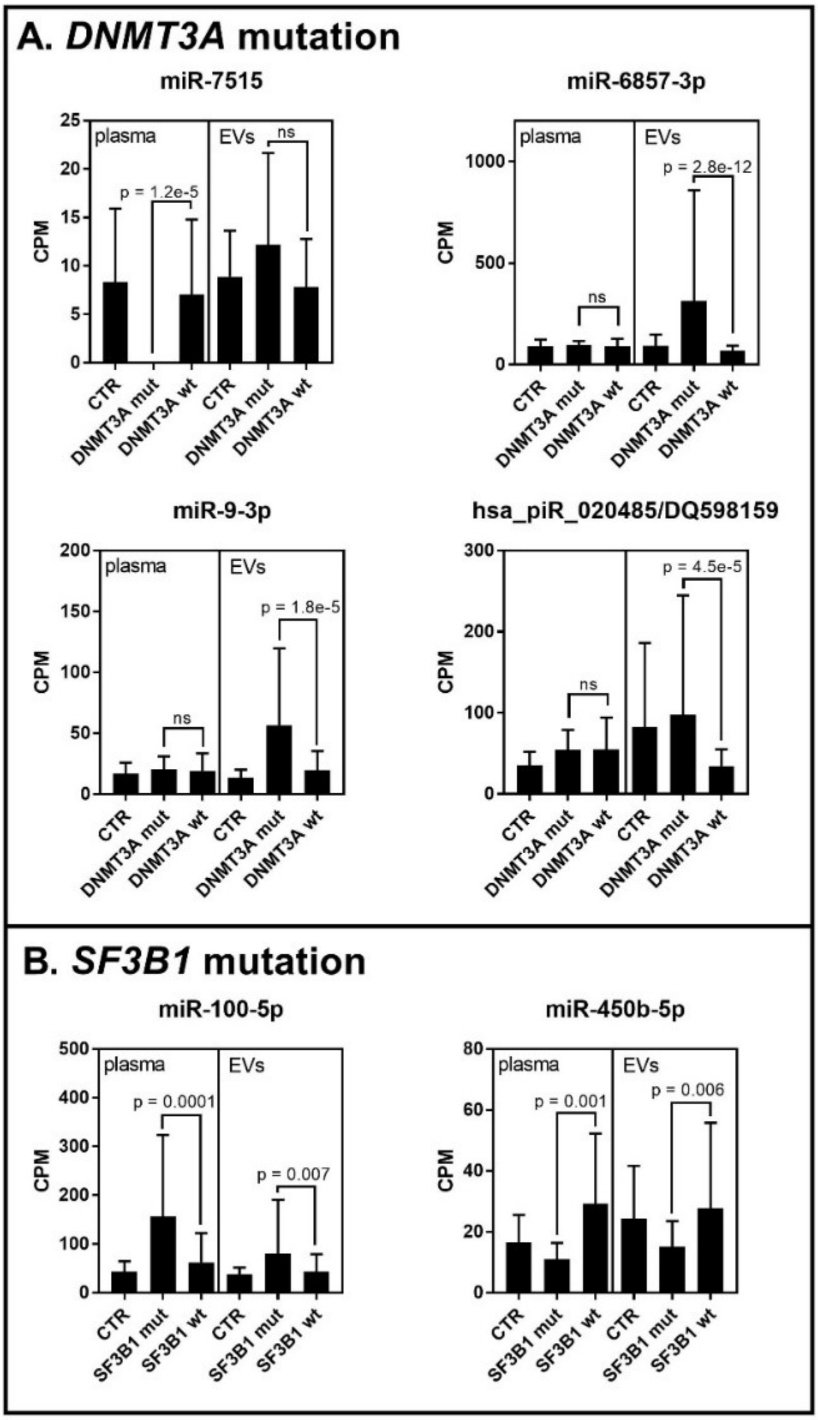
KEGG pathway	p value
plasma: early *vs.* advanced MDS	
Amphetamine addiction	1.56e-07
Signalling pathways regulating pluripotency of stem cells	5.60e-06
Transcriptional misregulation in cancer	1.35e-05
Gap junction	2.31e-05
Glioma	3.38e-05
FoxO signalling pathway	5.34e-05
Hippo signalling pathway	0.000116
ErbB signalling pathway	0.000172
Proteoglycans in cancer	0.000175
TGF-beta signalling pathway	0.000254
EVs: early *vs.* advanced MDS	
Biotin metabolism	0.00716
Central carbon metabolism in cancer	0.00716
Signalling pathways regulating pluripotency of stem cells	0.00716
Lysine degradation	0.00983
TGF-beta signalling pathway	0.00983
Steroid biosynthesis	0.0129
Glioma	0.0139
RNA transport	0.0141
ErbB signalling pathway	0.0145
Morphine addiction	0.0145

10.2.9 Relation between somatic mutations and levels of circulating sncRNAs in MDS

We explored the possible relation between the presence of somatic mutations and the levels of circulating sncRNAs in MDS. Using differential expression analyses, we searched for sncRNAs with differential expression between MDS patients with *vs.* without a mutation in SF3B1 or DNMT3A genes. The analysis identified only a few significantly deregulated sncRNAs (SF3B1: no sncRNAs, DNMT3A: miR-7515 in total plasma, miR-6857-3p, miR-9-3p, and hsa_piR_020485/gb/DQ598159 in EVs, $p < 0.05$, Figure 16A).

To gain better insight into the potential effects of SF3B1 and DNMT3A mutations on levels of circulating sncRNAs, we refined the selection criteria and included all sncRNAs at raw $p < 0.01$ (non-adjusted for multiple testing) with the awareness of potential high numbers of false positive results. Regarding the SF3B1 mutations, we identified 22 and 15 sncRNAs deregulated in total plasma and EVs, respectively. The DNMT3A mutations were associated with the deregulation of 34 sncRNAs in total plasma and 32 sncRNAs in EVs ($p < 0.01$). Finally, we intersected deregulated sncRNAs in the two materials and found that total plasma and EVs displayed different sncRNA profiles. Only miR-100-5p and miR-450b-5p of SF3B1-mutated samples were deregulated in both materials, plasma and EVs (Figure 16B).

Figure 16. Differential sncRNA levels associated with presence of somatic mutations in (A) DNMT3A gene and (B) SF3B1 gene. CTR – controls, mut – mutated, wt – wild type, ns – not significant



10.2.10 Circulating sncRNAs as prognostic biomarkers of MDS survival

The OS-associated sncRNAs were identified by performing univariate Cox regression along with a permutation test using BRB-ArrayTools (239). sncRNAs with permutation p values < 0.001, which were computed based on 10,000 random permutations, were considered significantly associated with survival. Two sets of sncRNAs (separately for the two materials, total plasma and EVs) whose levels significantly correlated with OS were identified. Of the 3,130 sncRNAs uploaded into the analysis tool, 173 and 122 sncRNAs were significantly (univariate $p < 0.05$) associated with OS in total plasma and EVs, respectively. Of these, only the sncRNAs with the highest level of association (permutation $p < 0.001$) were chosen for further analyses, i.e. three sncRNAs in total plasma (miR-1260b, miR-3191-3p, and miR-328-3p) and four sncRNAs in EVs (miR-1237-3p, U33, hsa_piR_019420/gb/DQ596670, and miR-548av-5p). The results are summarized in Table 19 and Kaplan-Meier plots are shown in Figure 17.

To test the combination strategy of multiple survival-associated sncRNAs for better patient stratification, we defined a risk prediction score that combined the effects of the selected sncRNAs above. A formula of survival risk score was constructed by including each of the selected sncRNAs, weighted by their estimated regression coefficients in the univariate Cox regression model. The leave-one-out cross-validation (LOOCV) method was employed to evaluate the accuracy of the score system. The prognostic index of sample was computed by the formula $\sum_i w_i x_i + C$, where w_i is the estimated regression coefficient, x_i is the logged level for the i-th gene, and C is a coefficient for recalculation of the final index to zero.

The coefficients of survival risk formula for these individual sncRNAs contributing to final risk assessment and p values of cross-validation tests are included in Table 19. The final survival risk score of a total plasma sample was calculated based on the following formula:

Plasma risk score = - 0.631 × log2 (level of miR-1260b) - 0.24 × log2 (level of miR-328-3p) + 6.861.

Similarly, the survival risk score of an EV sample was calculated as follows:

EV risk score = 0.615 × log2 (level of miR-1237-3p) + 0.917 × log2 (level of U33) - 0.106 × log2 (level of hsa_piR_019420) - 1.01 × log2 (level of miR-548av-5p) - 4.948.

A higher score (> 0) indicated an increased risk of mortality, whereas a lower score (≤ 0) denoted a better outcome. Thus, the patient cohort was partitioned into two risk groups according to the survival risk score (> 0 for the higher-risk group and ≤ 0 for the lower-risk group).

To evaluate the performance of combined prognostic signatures, Kaplan-Meier curves and receiver operation characteristic (ROC) curves were plotted for the samples divided into higher-risk and lower-risk groups according to the computed score formulas. The results showed that combining sncRNA level scores increased the predictive power of the survival risk model more significantly in EV material (univariate $p < 0.001$, ROC: AUC = 0.860, $p = 0.0009$) than in total plasma (univariate $p = 0.008$, ROC: AUC = 0.636, $p = 0.206$) as shown in Figure 18.

Furthermore, we tested the relation between individual survival-associated sncRNAs and their combination scores with clinical parameters. A series of Pearson correlation tests showed a significant association between the levels of the majority of these sncRNAs and BM blast count and platelet count (Table 20). However, Cox multivariate analysis revealed that the EV combined score (HR = 5.866, 95% CI 2.262 to 15.210, $p < 0.001$) was the variable most significantly associated with OS, even when compared with the IPSS-R score (HR = 1.410, 95% CI 0.840 to 2.366, $p = 0.193$) (Table 21).

Table 19. sncRNAs associated with OS of MDS patients. Prediction model coefficients are applicable to the formula of survival risk score. The survival risk score of a total plasma sample = - 0.631 × log2 (level of miR-1260b) - 0.24 × log2 (level of miR-328-3p) + 6.861. Similarly, the survival risk score of an EV sample = 0.615 × log2 (level of miR-1237-3p) + 0.917 × log2 (level of U33) - 0.106 × log2 (level of hsa_piR_019420) - 1.01 × log2 (level of miR-548av-5p) - 4.948. A sample is predicted as high (low) risk if its prognostic index is > 0 (≤ 0). n.a. – not applicable, n.s. – nonsignificant

sncRNA	Univariate Cox regression analysis			Prediction model	
	Univariate Cox regression, p value	Permutation, p value	Hazard ratio	Coefficient	Cross-validation, p value
Total plasma					
miR-1260b	0.0007	0.0006	0.441	-0.631	0.0002
miR-3191-3p	0.0009	0.0009	0.338	n.a.	n.s.
miR-328-3p	0.0009	0.0008	0.474	-0.24	0.0008
EV fraction					
miR-1237-3p	0.00002	< 1e-07	20.135	0.615	5e-07
U33	0.0006	0.0006	2.499	0.917	0.0002
hsa_piR_019420	0.001	< 1e-07	20.135	-0.106	0.0008
miR-548av-5p	0.001	0.001	0.217	-1.01	0.0009

Figure 17. Kaplan-Meier curves for individual sncRNAs significantly associated with OS of MDS patients in samples of (A) total plasma and (B) EVs.

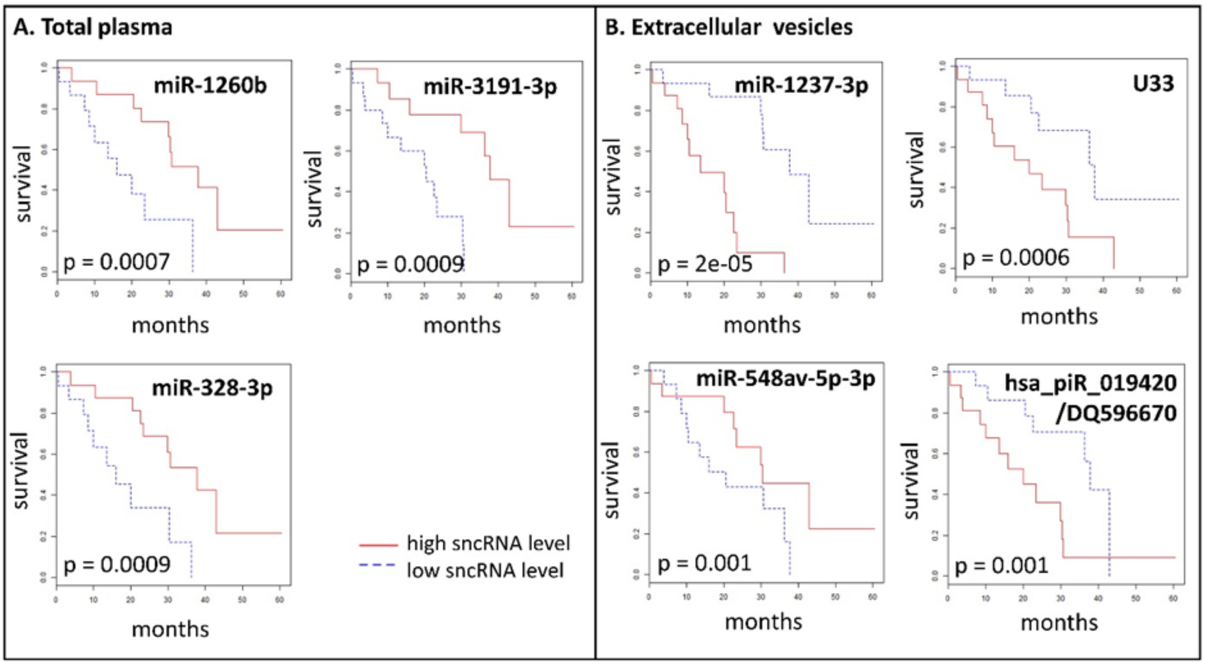


Figure 18. Performance of the combined prognostic model for OS of MDS patients. Kaplan-Meier curves and ROC curves are shown for (A) a two-sncRNA signature (miR-1260b and miR-328-3p) in total plasma and (B) a four-sncRNA signature (miR-1237-3p, U33, hsa_piR_019420, and miR-548av-5p) in EVs.

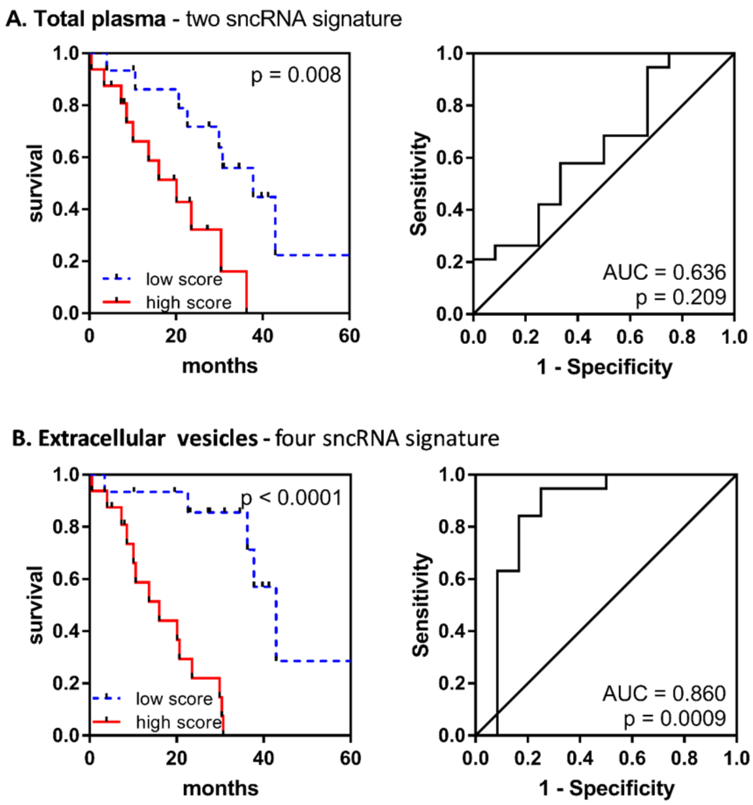


Table 20. Correlation of clinical variables with individual sncRNA levels and with combined scores for OS. The Pearson correlation coefficient is listed. *** p < 0.001, ** p < 0.01, * p < 0.05.

		age	blasts	haemoglobin	neutrophils	platelets	karyotype
Total plasma	miR-1260b	-0.334	-0.510**	0.046	0.015	0.838***	-0.225
	miR-3191-3p	-0.253	-0.199	-0.125	0.061	0.337	-0.300
	miR-328-3p	-0.453*	-0.487**	0.239	0.029	0.826***	-0.203
	combined score (total plasma)	0.284	0.531**	-0.088	-0.053	-0.818***	0.218
EVs	U33	0.090	0.365*	-0.229	0.146	-0.372*	-0.093
	hsa_piR_019420/DQ596670	0.090	0.412*	-0.119	0.140	-0.473**	-0.053
	miR-548av-5p	0.045	-0.207	0.357*	-0.114	0.270	-0.204
	combined score (EVs)	0.168	0.413*	-0.374*	0.066	-0.490**	0.264

Table 21. Cox multivariate analysis for OS of MDS patients.

variable	HR	95.0% CI for HR		p value
		Lower	**Upper**	
age	1.044	0.945	1.153	0.397
blasts	0.882	0.767	1.013	0.076
haemoglobin	1.001	0.961	1.043	0.948
neutrophils	0.778	0.577	1.048	0.099
platelets	1.003	0.993	1.013	0.602
IPSS-R score	1.410	0.840	2.366	0.193
combined score (total plasma)	1.764	0.666	4.677	0.254
combined score (EVs)	5.866	2.262	15.210	**< 0.001**

10.2.11 Circulating sncRNAs predictive of response to AZA therapy

To search for sncRNAs applicable as predictive biomarkers of the AZA treatment response, we analysed RNA-seq data from AZA-treated MDS/AML-MRC patients. Using differential expression analysis, we found only a few sncRNAs significantly (|logFC| > 1, q < 0.05) associated with patient response to AZA. In total plasma, the levels of miR-4774-3p and miR-762 were increased, and the levels of miR-125b-5p, miR-4324, miR-3156-5p, and miR-3692-3p were decreased in relation to later response to AZA treatment. In EVs, different sncRNAs were associated with AZA response. Levels of miR-6857-3p, miR-1299, miR-183-5p and miR-513b-3p were increased, and miR-6832-3p levels were decreased.

Because the results of differential expression analysis were limited to only several miRNAs of low predictive value, we performed additional machine learning analysis (with support of Jiří Kléma, Czech Technical University, Prague) to define a combined sncRNA signature that would predict the AZA response with higher accuracy compared to individual sncRNAs. The Recursive Feature Elimination (RFE) method implemented in Support Vector Machine (SVM) regression model was used to define sncRNA classifiers that discriminated AZA responders and nonresponders based on sncRNA pretreatment levels and interactions between them. Accuracy (Acc) and AUC were calculated to define the optimal number of features. Since the data sample was limited, the LOOCV method was employed to provide an unbiased evaluation of a model fit. The group of best classifiers was further tested and reduced by backward stepwise logistic regression algorithms using the maximum likelihood estimation (MLE) method. The resulting predictive formula was calculated by logistic regression using the KNIME platform and sag solver.

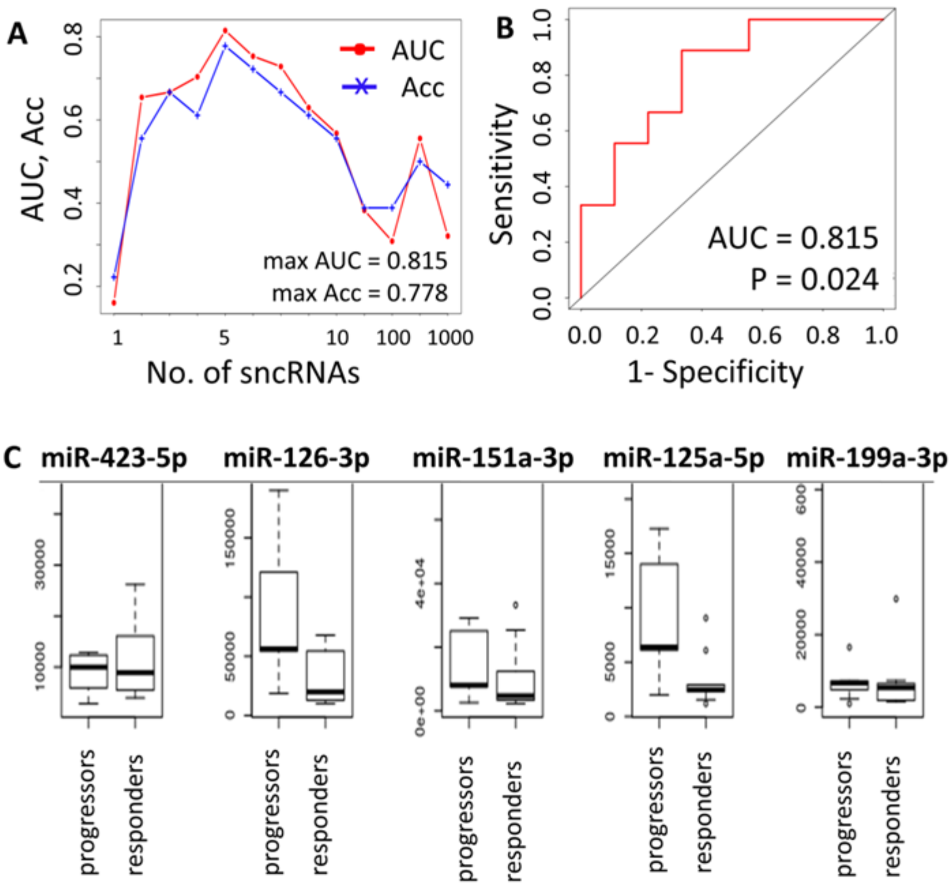
The results from the SVM-RFE regression model showed that the best classification of responders *vs.* progressors could be achieved using cumulative expression data of five sncRNAs measured in total plasma (AUC = 0.815, Acc = 0.778), with 6 to 10 sncRNAs combined being of less predictive value (Figure 19, A and B). The five most common sncRNAs with the best cumulative predictive value determined by SVM were miR-423-5p, miR-126-3p, miR-151a-3p, miR-125a-5p, and miR-199a-3p (Figure 19C). These results, however, were achieved only for the total plasma and for the clearly defined groups of patients (responders *vs.* progressors). When testing the data from EV samples or trials involving patients with stable disease after AZA treatment, no significant differences were detected.

The sncRNAs preselected by differential expression analysis (miR-125b-5p, miR-4324, and miR-4774-3p) and the sncRNAs with the best cumulative predictive value in the SVM model (miR-423-5p, miR-126-3p, miR-151a-3p, miR-125a-5p, miR-199a-3p, miR-142-5p, Ro-associated RNA, miR-185-5p, miR-30d-5p, miR-92a-3p, let-7a-5p, let-7f-5p and miR-26b-5p) for total plasma samples of responders *vs.* progressors were further statistically tested together by logistic regression analysis using maximum likelihood estimation, and their number was reduced to five (miR-423-5p, miR-126-3p, miR-151a-3p, miR-125a-5p, and miR-199a-3p), which was in agreement with the SVM method. The following predictive formula was calculated with these five miRNAs (Figure 19D):

Prediction score = 2.629 × ln(level of miR-423-5p) - 2.471 × ln(level of miR-126-3p) + 0.427 x ln(level of miR-151a-3p) – 0.203 x ln(level of miR-125a-5p) – 0.1 x ln(level of miR-199a-3p) + 0.808.

A score > 0 predicted future response to AZA, whereas a score ≤ 0 predicted progression of the disease despite AZA treatment. The quality of the prediction was 88.9 % (16 out of 18 patients).

Figure 19. Combined prediction model for response to AZA treatment in MDS/AML-MRC patients. (A) Results of the SVM-RFE regression model determining the optimal number of sncRNAs whose combined expression could be predictive of the likelihood of response. (B) ROC curve for the five sncRNA predictors. (C) Total plasma levels of the best/most common predictors (miR-423-5p, miR-126-3p, miR-151a-3p, miR-125a-5p, and miR-199a-3p). (D) Coefficients for calculation of predictive formula for AZA treatment response. AUC – area under the ROC curve, Acc – accuracy



D

SncRNA	Coefficient
miR-125a-5p	-0.203
miR-126-3p	-2.471
miR-151a-3p	0.427
miR-199a-3p	-0.100
miR-423-5p	2.629
Constant	**0.808**

10.3 Publication 3

Krejcik et al., MicroRNA profiles as predictive markers of response to azacitidine therapy in myelodysplastic syndromes and acute myeloid leukaemia, Cancer Biomarkers, 2018

In this study, we focused on intracellular miRNA profiles in BM samples in order to search for potential miRNA biomarkers that would predict patient responsiveness to AZA therapy. We found that responders and nonresponders show distinct miRNA profiles and that the pretreatment levels of specific miRNAs may predict patient response to AZA therapy.

10.3.1 Patient cohort

The study was conducted on a cohort of 27 patients (19 MDS and 8 AML-MRC patients) and 11 healthy controls. Based on the WHO classification criteria from 2008 (23), the diagnoses of MDS patients are as follows: 1 MDS patient with RCMD, 2 MDS patients with RAEB-1, and 16 MDS patients with RAEB-2. The detailed clinical characteristics of all patients are summarized in Table 22.

Altogether, the study included 50 samples from 27 patients with MDS/AML-MRC at baseline (i.e. patients before AZA administration) and during AZA therapy (collected at the time of the best response, between cycles 4 and 11). Serial samples (i.e. samples taken from one patient at baseline and during the therapy) were available for 23 patients, four patients were screened only before AZA therapy. The microarray profiling was performed on a discovery cohort that comprised of 28 samples including serial samples from 12 patients prior to and during AZA therapy and four control samples. The validation cohort analysed by RT-qPCR included all 50 patient samples and 11 control samples.

The median number of administered AZA cycles was eight (range, 3-34 cycles), the median time to the best response in the responder cohort was five months (range, 3.2-11.0 months), and the median duration of response was 16.8 months (range, 8.0-29.1 months). ORR that included rates for all responders was 40 % within the cohort. The responses were complete remission (CR) in four cases (15 %), partial remission (PR) in three cases (11 %), marrow CR (mCR) in two cases (7 %), and haematologic improvement (HI) in two cases (7 %). The nonresponders (60 %) consisted of eight patients with stable disease (SD, 30 %) and eight patients with progressed disease (PD, 30 %). The median patient follow-up from the initiation of AZA treatment was 21.1 months (range, 3.4-47.1 months), 25 patients died and two patients were censored at the time of last follow-up. The OS was defined as the time from the beginning of treatment until death from any cause or the last follow-up.

Based on Cox-regression analysis, the responders and nonresponders showed significantly different OS (hazard ratio [HR] = 0.195, 95 % confidence interval [CI], 0.070 to 0.542, p < 0.01).

Table 22. Characteristics of the patients. The data are presented as the median and range for all continuous variables.

Patients	**19 MDS** **8 AML-MRC**
Age	68 (63-82)
Sex (male/female)	15/12
Diagnosis RCMD/RAEB-1/RAEB-2/AML-MRC	1/2/16/8
BM blasts	18 (4-27)
Karyotype	
Isolated del(5q) / del(5q) + any other abnormality	5/10
Isolated trisomy 8 / trisomy 8 + any other abnormality	2/5
Normal	8
Cytogenetics by IPSS-R* good/intermediate/poor/very poor/n.a.	17/6/1/2/1
IPSS-R (MDS patients only) intermediate/high/very high	5/9/5
AZA cycles administered	8 (3-34)
Follow-up (months)	21.1 (3.4-47.1)
Alive	2 (7 %)
Response	
complete remission	4 (15 %)
partial remission	3 (11 %)
marrow complete remission	2 (7 %)
haematologic improvement	2 (7 %)
stable disease	8 (30 %)
progressed disease	8 (30 %)
Overall response rate	11 (40 %)

n.a. – not available
* The IPSS-R cytogenetic risk score has been established for patients with MDS and is not commonly used to stratify AML patients. However, we used this score for both MDS and AML patients, with the aim to compare frequencies of karyotypes across these patient groups.

10.3.2 miRNA profiling in samples at baseline

miRNA profiling was performed on selected samples from 12 patients at baseline and 4 controls using microarrays. ANOVA identified 64 differentially expressed miRNAs (p < 0.05) in samples at baseline, which were stratified according to their later response status (three CR, two PR, four SD, and three PD patient samples) as shown in Figure 20. Hierarchical clustering of these miRNAs clearly defined four sample clusters with different miRNA profiles: i) controls, ii) patients with CR,

iii) patients with PR, and iv) a mixed cluster of nonresponders (SD and PD). The baseline samples obtained from patients who later achieved CR exhibited the most distinct profile with the downregulation of let-7c, miR-27b-3p, miR-100-5p, mir-140-3p, and miR-423-5p and upregulation of miR-21-5p, miR-211-3p, miR-1246, miR-5739, miR-6085, miR-6124, miR-6132, and miR-6165. In nonresponders, significant downregulation of miR-10a/b-5p and upregulation of miR-1 and miR-133b were observed compared to patients with CR and PR.

For all subsequent analyses, patients were divided in the two groups of responders and nonresponders, because the study comprised limited number of samples and no significant differences in miRNA profiles between SD and PD samples were found.

10.3.3 miRNA profiling in samples before and during AZA therpy

To identify the miRNAs affected by AZA therapy, miRNA expression profiles in samples at baseline and during AZA treatment from 12 patients were compared by paired t-tests. miRNAs with median expression level change of 30 % and more ($p < 0.05$) compared to their level at baseline included 20 downregulated (i.e. let-7f-5p/7g-5p, miR-10a-5p, miR-142-3p, miR-146a-5p, miR-148a-3p, miR-181a-3p/b-5p/c-5p, miR-196b-5p, and miR-451a) and 14 upregulated miRNAs (i.e. miR-1202, miR-1260a, and miR-3656) in all tested patients irrespective of their response status (Figure 21).

To determine the miRNAs involved in response to AZA therapy, miRNA expression levels after AZA treatment in responders and nonresponders were compared. 30 miRNAs with significantly different expression levels ($p < 0.05$) in responders along with unchanged levels in nonresponders were found (Figure 22). Among these, miR-10b-5p, miR-15a-5p/b-5p, miR-24-3p, miR-148b-3p, and miR-199a-3p were downregulated and miR-1202 or miR-1260a were upregulated in responders after AZA treatment compared to the baseline samples.

Figure 20. Differentially expressed miRNAs in the baseline samples. The heatmap shows altered miRNA expression ($p < 0.05$) between sample groups with different response status. Color gradient intensity scale indicates logFC of signal intensities compared with the mean signal intensity of controls. Red indicates an increased level of RNA, blue indicates a decreased level of RNA. Each column represents a patient, and each row represents a miRNA. CTR – control, CR – complete response, PR – partial response, SD – stable disease, PD – progressed disease

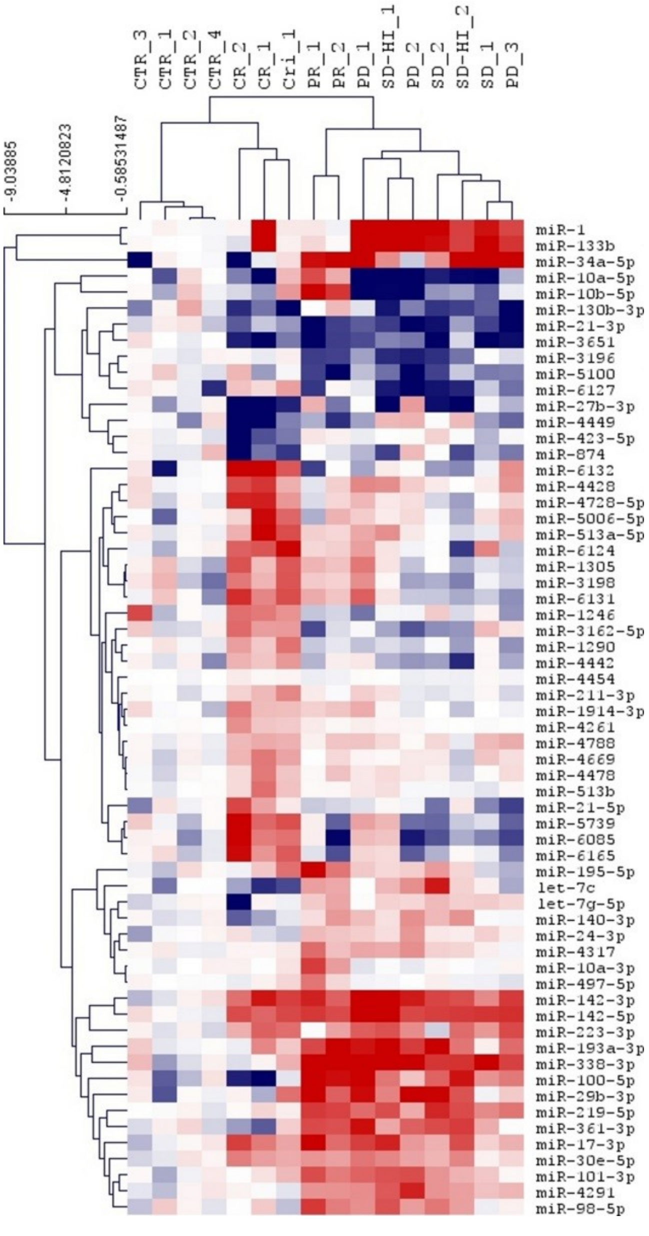


Figure 21. Differentially expressed miRNAs after AZA treatment irrespective to patient response status. The heatmap shows altered miRNA expression in samples after AZA treatment compared to paired samples at baseline. Color gradient intensity scale indicates logFC of signal intensities compared with the mean signal intensity of controls. Red indicates an increased level of RNA, blue indicates a decreased level of RNA. Each column represents a patient, and each row represents a miRNA. CR – complete remission, PR – partial remission, SD – stable disease, and PD – progressed disease

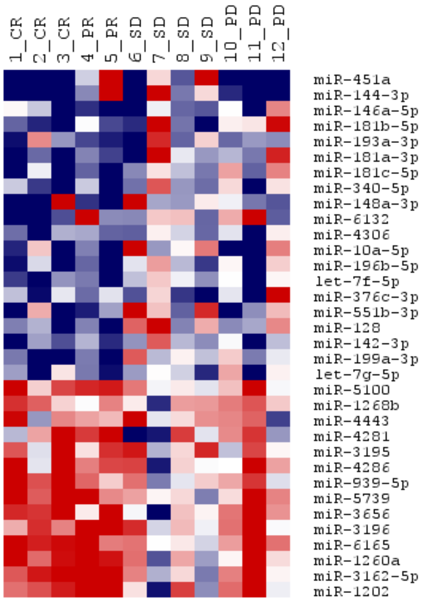
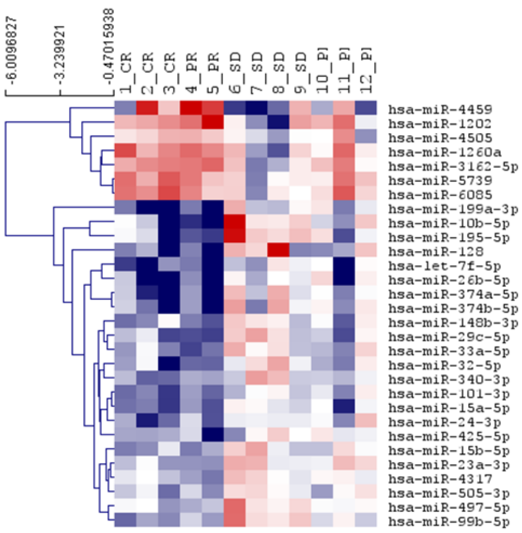


Figure 22. Differentially expressed miRNAs ($p < 0.05$) between responders and nonresponders after AZA treatment. The heatmap shows altered miRNA expression in AZA treated samples from responders and nonresponders. Color gradient intensity scale indicates logFC of miRNA signal intensities compared to the signal intensity of a given miRNA in a paired sample at baseline. Red indicates an increased level of RNA, blue indicates a decreased level of RNA. Each column represents a patient, and each row represents a miRNA. CR – complete remission, PR – partial remission, SD – stable disease, and PD – progressed disease

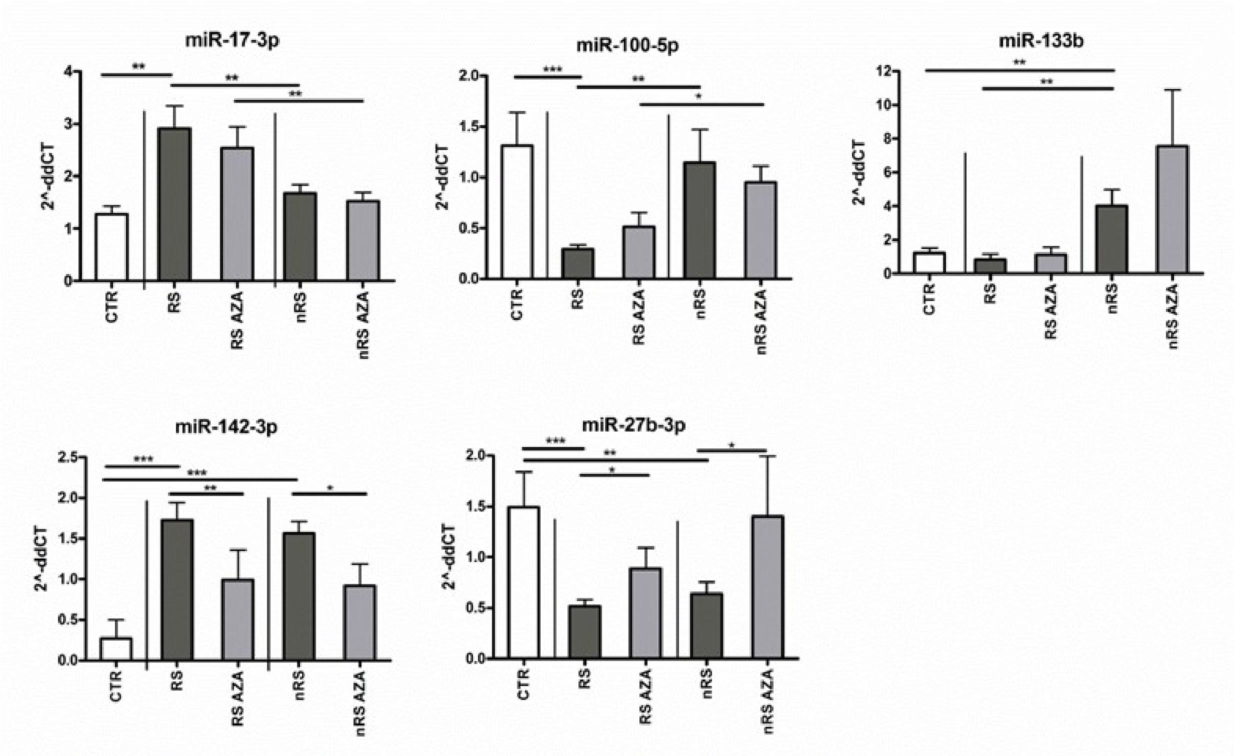


10.3.4 Quantification of individual miRNA levels by RT-qPCR

To verify the microarray results, the levels of miR-17-3p, miR-27b-3p, miR-100-5p, miR-133b, and miR-142-3p were quantified in the validation cohort by RT-qPCR (Figure 23). These miRNAs were selected based on their significant differential expression in microarray profiling. In concordance with the microarray data, miR-17-3p, miR-100-5p, and miR-133b were differentially expressed between the responders and nonresponders. The level of miR-17-3p was significantly increased ($p < 0.05$), and the levels of miR-100-5p and miR-133b were significantly reduced ($p < 0.01$) in responders compared to nonresponders.

Comparison of the baseline samples with those collected during AZA treatment revealed increased level of miR-27b-3p and decreased level of miR-142-3p ($p < 0.01$) during AZA treatment. Levels of both miRNAs during AZA treatment were approaching closer to their levels in control samples.

Figure 23. Relative expression of selected miRNAs measured by RT-qPCR. CTR – controls, RS – responders at baseline, RS AZA – AZA treated responders, nRS – nonresponders at baseline, nRS AZA – AZA treated nonresponders, *p < 0.05, **p < 0.01, ***p < 0.001



10.3.5 Correlation of clinical parameters with miRNA levels

Further, miR-17-3p, miR-100-5p, and miR-133b, which were significantly deregulated between responders and nonresponders, were tested as potential predictive biomarkers of AZA treatment response. Based on the ROC curve analysis, the cut-off values for miR-17-3p, miR-100-5p, and miR-133b were defined (Table 23) and according to them, patients were divided in 'high-miR' and 'low-miR' groups. A series of statistical tests were performed to compare these two groups.

The ORR was significantly higher in patient groups with high-miR-17-3p ($p < 0.05$), low-miR-100-5p ($p < 0.05$), and low-miR-133b ($p < 0.01$) (Table 24). There were no differences in patient age, sex, percentage of BM blasts, cytogenetics, and diagnosis between the groups divided according to the levels of these miRNAs. Similarly, the number of AZA cycles, follow-up period, and the proportion of patients who died during this period were comparable between the groups.

Further statistical testing examined an impact of clinical and molecular variables on the OS after AZA treatment initiation. Univariate analysis revealed that OS was not significantly associated with age, sex, percentage of BM blasts, WHO-based diagnosis, miR-17-3p or miR-133b level. The only variables significantly associated ($p < 0.05$) with OS were the IPSS-R cytogenetics and the level of miR-100-5p (Table 24 and Figure 24). The patients with good karyotype had significantly longer OS than those with unfavourable karyotype. Stratification by miR-100-5p expression showed prolonged OS in the low-miR-100-5p group compared to the high-miR-100-5p group. Multivariate Cox regression analysis confirmed that both the IPSS-R cytogenetics and the level of miR-100-5p represented prognostic factors for the OS (Table 25).

Table 23. Cut-off values (miRNA expression fold change compared to mean miRNA level in healthy controls) for selected miRNAs based on ROC curve analysis.

	Cut-off value	AUC	95 % CI
miR-17-3p	2.0	0.641	0.427-0.855
miR-100-5p	0.4	0.713	0.518-0.908
miR-133b	0.6	0.715	0.509-0.921

Table 24. Relation of clinical and molecular variables to ORR and OS. Int – intermediate

Variable		**n**	**ORR n (%)**	**p value**	**OS median (mo.)**	**p value**
Age	< 70 years	15	6 (40 %)	.930	19.6	.385
	≥ 70 years	12	5 (42 %)		21.2	
Sex	Male	15	6 (40 %)	.930	19.6	.837
	Female	12	5 (42 %)		24.9	
Blasts	< 10 %	7	3 (43 %)	.895	23.9	.515
	≥ 10 %	20	8 (40 %)		19.4	
Karyotype	Normal	8	3 (38 %)	.330	21.2	.853
	Isolated del(5q)	5	3 (56%)		25.9	
	Other	13	3 (33 %)		13.0	
Cytogenetics by IPSS-R	Good	17	7 (41 %)	.334	25.9	**.033**
	Int/poor/very poor	9	2 (22 %)		11.3	
Diagnosis	MDS	19	8 (42 %)	.824	21.1	.953
	AML-MRC	8	3 (38 %)		20.4	
miR-17-3p	Low	15	3 (20 %)	**.014**	21.2	.215
	High	12	8 (67 %)		19.1	
miR-100-5p	Low	13	8 (62 %)	**.034**	25.9	**.047**
	High	14	3 (21 %)		16.4	
miR-133b	Low	9	7 (78 %)	**.005**	19.1	.915
	High	18	4 (22 %)		22.4	

Figure 24. OS of patients stratified according to IPSS-R cytogenetics and miR-100-5p level ($p < 0.05$).

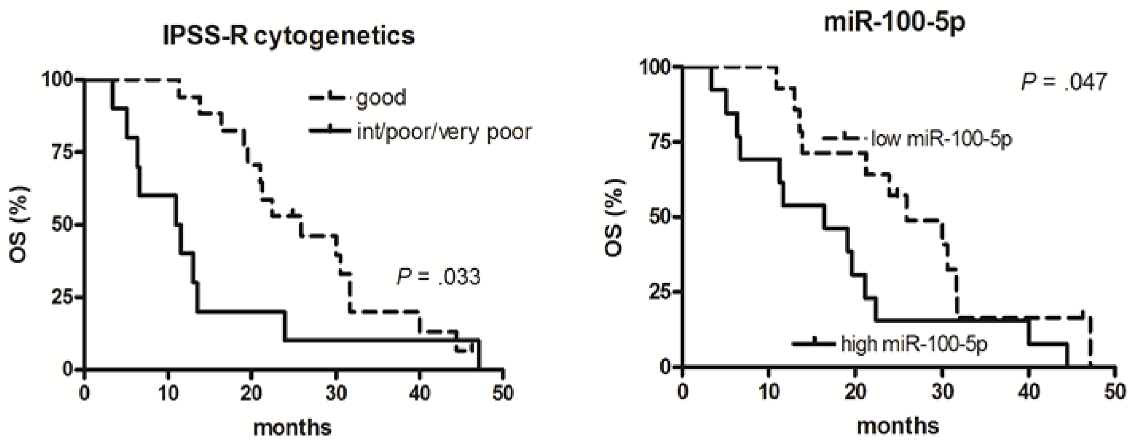


Table 25. Multivariate Cox analysis for OS. Int – intermediate, HR – hazard ratio, CI – confidence interval

Variable	**HR**	**95 % CI**	**p value**
Age (≥ 70 years)	2.540	0.795 to 8.123	.116
Female sex	0.659	0.243 to 1.788	.413
Blasts (≥ 10 %)	4.245	0.930 to 19.372	.062
Cytogenetics by IPSS-R (int/poor/very poor)	10.040	2.649 to 38.058	**.001**
AML-MRC diagnosis	1.289	0.474 to 3.508	.620
High miR-100-5p	4.066	1.444 to 11.445	**.008**

11 DISCUSSION

Because MDS are a heterogeneous group of disorders, the need to investigate further possibilities of the disease classification, prognosis assessment, and treatment responsiveness is still emerging. Since the discovery of sncRNA importance in cellular processes and disease development, there has been a great effort to investigate functions and relevance of these molecules in the pathophysiology of MDS as well. So far, mostly cellular miRNAs have been explored in MDS. Most of these studies associate MDS subtypes with different miRNA profiles, indicating that miRNAs are able to classify MDS. Concerning circulating sncRNAs in MDS, only a few studies have been performed up to now. However, circulating sncRNAs are of a great interest as potential novel noninvasive molecular biomarkers of the disease. The investigation of their profiles, functions, and regulation in plasma is needed to better understand their relevance and find the way of their application as biomarkers.

Thus, we studied profiles of circulating sncRNAs in plasma (P1 and P2) in order to search for individual miRNAs associated with MDS, its progression and AZA therapy. Except for circulating sncRNAs, we also investigated miRNA profiles in BM cells exclusively focusing on specific miRNAs that may predict patient response to AZA therapy (P3).

11.1 Publication 1

Dostalova Merkerova et al., Microarray profiling defines circulating microRNAs associated with myelodysplastic syndromes, Neoplasma, 2017

In our initial study, we aimed to investigate circulating miRNA profiles in total plasma of MDS patients and to determine specific miRNAs that could serve as disease biomarkers. In view of the fact that comprehensive profiling by NGS was not available to us at that time, we used a microarray platform that allowed an investigation of 2,006 predefined miRNAs.

The results showed altered miRNA profiles between MDS patients and controls, and between lower-risk and higher-risk MDS groups. Based on the expression data, we selected several haematopoiesis and/or oncology related miRNAs (miR-16-5p, miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a) to explore their levels and association with MDS in detail. When comparing miRNA levels between lower-risk and higher-risk MDS groups, significantly decreased levels of miR-27a-3p, miR-199a-5p, and miR-223-3p were revealed in higher-risk disease, suggesting that higher levels of these miRNAs are associated with favourable prognosis. Further, we evaluated prognostic values of these miRNAs for PFS and OS. The results showed that high level of miR-451a indicate better PFS and high level of miR-223-3p associates significantly with better OS.

Because patients with different prognoses showed specific levels of plasma miRNAs, it may be anticipated that these miRNAs play a role in the disease progression. It was demonstrated that miR-27 enhances differentiation of myeloblasts *via* posttranscriptional downregulation of RUNX1 (240), which is a key transcription factor in haematopoiesis (241). Level of miR-199a-5p was found to be significantly higher in BM of AML patients who achieved complete remission after chemotherapy compared to refractory/relapsed AML patients. It was demonstrated that miR-199a-5p represses protective autophagy of AML cells and overcomes chemoresistance by directly targeting damage regulator autophagy modulator 1 (DRAM1) (242). miR-451 is a positive regulator of erythroid cell maturation (97) and miR-223 is a key regulator of myeloid lineage (243). Both miR-451 and miR-223 are believed to act as tumour suppressors, and the low plasma levels of these miRNAs in higher-risk MDS patients may contribute to the promotion of leukaemic cell growth. It was shown that miR-223 blocks cell cycle progression in myeloid cells and its expression is suppressed in AML (244,245). Further, AML patients with significantly higher levels of miR-223 in blasts show favourable prognosis, whereas miR-223 levels are low in patients with worse outcomes (246). Most recently, it has been demonstrated that miR-223 inhibits the expression of ribosomal protein S14 (RPS14) in all risk groups and different MDS subtypes (247). RPS14 is commonly deleted in del(5q) MDS (248), but its low expression was also shown to be frequently detected in MDS patients without 5q deletion, and is associated with prolonged survival of these patients (249). This evidence indicates that high level of miR-223 suppressing RPS14 may contribute to better survival of patients, which is in concordance with our results. Notably, we have previously detected overexpression of cellular miR-451 and miR-223 in CD34+ cells in MDS patients with del(5q) (197), further supporting our findings that high levels of miR-451 and miR-223 are associated with a favourable prognosis in MDS.

There is a great interest in revealing the origin of circulating miRNAs in haematopoietic diseases. Pritchard et al. (250) demonstrated that blood cells are major contributors to circulating miRNAs and their levels are strongly influenced by changes in blood cell counts. They showed that plasma levels of miRNAs expressed by myeloid (e.g. miR-223, miR-227, miR-574-3p, and let-7a) and lymphoid (e.g. miR-150) blood cells correlate with corresponding blood cell counts (250). Further, Stamatopoulos et al. (189) demonstrated that high serum level of miR-150 in CLL patients is linked to a high lymphocyte count and cellular miR-150 level might be regulated by its release from leukaemic cells into the extracellular space. However, they could not definitively prove the correlation between cellular and serum levels of miR-150, suggesting that it might be released by multiple cell types (189). We also attempted to trace the origin of circulating miRNAs. As cytopenia is one of the main features of MDS, we examined whether miRNAs enriched in the cytopenic lineages exhibit reduced levels in MDS plasma. We tested levels of miR-223, which is enriched in myeloid cells (250,251), miR-451a and miR-16 enriched in erythrocytes (97,250), miR-27 enriched in granulocytes (240), miR-150 enriched in lymphocytes (250,251), and miR-199 enriched in platelets (252). However, we did not observe any correlation between circulating miRNA levels and blood counts of the tested lineages. Furthermore, we examined the myeloblastic cell population as a source of circulating miRNAs in MDS. To assess the correlation between cellular and circulating miRNA levels, we used our previously measured data on miRNA expression profiles of CD34+ MDS cells (196), which form the majority of MDS myeloblasts (253). The levels of circulating miRNAs, however, did not reflect their levels in CD34+ cells. These results suggest that the release of miRNAs from cells is more likely miRNA specific, not cell specific process. This finding is in agreement with other studies reporting that extracellular miRNA profiles do not reflect profiles of original cells (132,254), suggesting that miRNAs are retained or released selectively and that cellular and extracellular miRNAs should be considered independently when evaluating them as disease biomarkers.

11.2 Publication 2

Hrustincova et al., Circulating small noncoding RNAs have specific patterns in plasma and extracellular vesicles in myelodysplastic syndromes and are predictive of patient outcome, Cells, 2020

In the most recent publication, we have performed small RNA-seq of circulating RNAs, which allowed us to study not only miRNAs, but also other sncRNA species present in MDS plasma. In recent years, investigation of circulating sncRNAs has moved from analysing of their content in total plasma to analyses of separated EVs. Thus, we compared circulating sncRNA profiles of paired samples from total plasma and EVs to find whether their sncRNA contents reflect each other or not and which of these two materials would be a better source of circulating sncRNA biomarkers for MDS.

EVs are lipid bound vesicles secreted by cells into the extracellular space. They contain a cargo of diverse molecules and are considered to play a role in cell-to-cell communication (255), which is of special scientific interest. Moreover, they appear to be more protective of RNA degradation compared to plasma or serum, suggesting that they may provide more reliable profile of sncRNAs (145). It was reported that tumour cells secrete significantly more exosomes into the tumour microenvironment than normal cells, leading to an increased number of exosomes in the circulatory system (256). Szczepanski et al. observed that serum of AML patients contained higher levels of microvesicles compared to that of controls (257). On the contrary, Enjeti et al. (220) found significantly lower levels of microvesicles in MDS plasma than in controls (220). In our study, we did not identify different levels of EVs between MDS and control plasma but rather noticed differences in their sizes, specifically an increase in EVs of larger diameters. Further, we observed a higher content of their RNA cargo, which is in agreement with a study of Enjeti et al. (220) determining doubled RNA content in EVs from MDS plasma compared to controls (220). These results indicate that features and content of EVs are changed in MDS and further exploration is needed.

Importantly, we compared RNA content between total plasma and EVs. Hierarchical clustering of all samples based on their RNA profiles showed that the RNA content of EVs is more homogeneous than that of total plasma. The larger heterogeneity of RNAs in total plasma can be expected because plasma includes not only various EVs but also other RNA carriers, such as proteins, lipoprotein particles, and apoptotic bodies, whereas our isolated EV fraction contained mostly exosomes. The larger homogeneity of RNA content in EV samples may be promoted by a selective packaging of specific RNAs in EVs (132,134,146). Although the numbers of particular miRNAs and their levels detected in exosomes were found to be significantly lower than in plasma (258), specific miRNAs were found to be enriched in exosomes (258,259), indicating exosomes to be the major source for some circulating miRNAs. These results suggest that the contents of the two materials do not completely reflect one another but rather represent independent sources of RNA, which may provide different insights into the biological processes occurring during disease pathogenesis.

Subsequent classification of read sequences into various RNA categories revealed additional differences between the two materials. Samples of total plasma had a substantially higher proportion of miRNA reads (60 and 46 percent of miRNA reads in total plasma and EVs, respectively) and a lower proportion of uncharacterized reads (33 and 48 percent of uncharacterized reads in total plasma and EVs, respectively). Xie et al. (258) measured miRNA levels in total plasma and exosomes and also found significantly higher miRNA levels in total plasma (258). It is still debated whether EVs contain biologically meaningful amount of miRNAs able to provide a reliable source of miRNA biomarkers. Chevillet et al. (260) suggested that most individual exosomes do not carry any biologically significant numbers of miRNAs and are, therefore, unlikely to be of physiologic relevance in miRNA-based cell-to-cell communication (260). A significant underrepresentation of miRNAs over other RNA species in exosomes has been confirmed by other studies (259,261,262). On the contrary, there are studies demonstrating that exosomes provide a sufficient source of miRNAs for disease biomarker detection (145,263). Thus, there is still no definitive consensus on the relevance of EV-mediated cell-to-cell communication and further investigation is needed.

One of the most interesting findings of this study was the discovery that RNA profiles of paired total plasma and EV samples substantially differed in MDS patients, whereas they remained closely similar in healthy individuals. The number of deregulated sncRNAs between paired samples of total plasma and EVs was almost ten-fold higher in MDS compared to controls. Such a great increase suggests that regulation and mechanisms of sncRNA release into the blood circulation are specifically affected in MDS, changing sncRNA profile as a whole. In agreement with our observation, Xie et al. (258) found deregulated miRNA levels between plasma and exosomes in animal models with kidney disease (258), whereas no significant differences in miRNA profiles between plasma and exosomes were found in healthy people (264). Therefore, it seems that the release of sncRNAs into blood circulation is specifically affected under various pathological conditions. As a result, these changes may affect cell-to-cell communication of blood cells, potentially contributing to ineffective haematopoiesis and disease progression.

Regarding other sncRNA species, such as piRNAs and tRNAs, we noticed an interesting pattern in their levels in total plasma and EVs. Whereas differentially represented miRNAs between total plasma and EVs were equally increased and decreased in the two materials, other sncRNA species were almost exclusively increased in plasma compared to EVs. Several studies demonstrated that different sncRNA species are preferentially loaded into different types of carriers (140,262). Moreover, packaging of RNA molecules within individual RNA species into carriers seems to be selective, i.e. specific RNA molecules are preffered to the others (258,265). Thus, sncRNA release appears to be thoroughly regulated process.

To characterize the profile of circulating sncRNAs specific for MDS, we compared sncRNA levels of MDS samples with those of AML-MRC and control samples. We found hundreds of differentially represented sncRNAs between MDS and control samples in both total plasma and EVs, indicating molecular changes related to the disease. However, we found almost no significant differences in sncRNA profiles between MDS and AML-MRC samples. It has been emphasized that MDS should not be considered as an early phase of AML as there are biological and clinical differences between them (266). Still, MDS and AML share important signatures at the molecular level in terms of signalling pathways and genetic markers (267,268). In MDS and AML-MRC, which share myelodysplasia-related features, we observed similar molecular characteristics described here and in our preceding paper (196). The link between MDS and AML-MRC may enable the application of similar therapeutic approaches that specifically address both of these clinical entities.

Further, we investigated changes in levels of individual miRNAs between MDS and controls and observed that the majority of the deregulated miRNAs were increased in MDS in both, total plasma and EVs. Among these miRNAs, we found many miRNAs whose intracellular deregulation has been already associated with various haematopoietic disorders, including MDS, such as miR-34a, miR-125a, miR-99b, miR-10a, miR-221, miR-222, miR-223, miR-29a, and miR-150 (57,193). Although the roles of circulating miRNAs and the impact of their deregulation on (patho)physiological processes are still unclear, there is convincing evidence of their intracellular regulatory functions in haematopoiesis and disease development. Altogether, it appears that these circulating sncRNAs may affect recipient cells and contribute to the development of myelodysplasia.

To obtain an additional look at biological functions that may be affected by deregulated miRNAs in MDS, we performed a miRNA target prediction and pathway enrichment analyses. Although different miRNAs were deregulated in total plasma *versus* EVs, considerable similarities in the affected pathways were observed, pointing to the shared targets of different miRNAs. Several signalling pathways associated with cancer and pluripotency of stem cells were identified as potentially targeted by the deregulated miRNAs (i.e. the Ras, TGF-β, and ErbB pathways). Furthermore, the deregulated miRNAs were also associated with pathways related to the extracellular environment and cell interactions (ECM-receptor interactions, focal adhesion, and proteoglycans in cancer). Extracellular matrix (ECM) is a complex network of extracellular macromolecules that provides support for surrounding cells. Specific interactions between cells and the ECM are mediated by transmembrane and cell-surface-associated components, leading to the control of different cellular activities, such as adhesion, migration, differentiation, proliferation, and apoptosis. Proteoglycans have been shown to be key macromolecules that contribute to the biology of various types of cancers through the abovementioned processes (269). Exosomes can also be considered integral components of the ECM since they modulate the assembly of the molecular network and signalling through the ECM (270). The pathway analysis thus suggest that the miRNAs released into blood circulation in MDS patients may further potentiate dysregulation of biological processes in the extracellular environment. The miRNAs may even act as a kind of regulatory loop affecting the functionality of EVs themselves and/or facilitating changes in the haematopoietic niche microenvironment.

Somatic mutations in multiple genes have been recently described in MDS and are rapidly becoming the most frequently discussed aberrations associated with MDS (8). The most frequently mutated genes in our patient cohort were SF3B1 and DNMT3A. Therefore, we investigated the association between mutational status of these genes and the levels of circulating sncRNAs. Only low numbers of circulating sncRNAs, however, were significantly associated with the mutational status of SF3B1 or DNMT3A genes. This raises the question of whether the effects of a somatic mutation in a single gene on deregulation of circulating sncRNA levels are either negligible or whether the analysis was affected by a bias. Regarding possible bias, a low number of patients with detected mutations were analysed. Moreover, the data were undoubtedly influenced by substantial heterogeneity on multiple levels (i.e., additional presence of various cytogenetic aberrations that are more penetrant, differences in variant allele frequencies, cooccurrence of several mutations in a single patient, or a wide spectrum of variants in one gene that may differentially influence protein activity). Taken together, we observed only slight trends in deregulation of sncRNA levels, suggesting that there is no fundamental association between mutational status of SF3B1 or DNMT3A and levels of circulating sncRNAs. However, with respect to possible significant bias, further investigations would be beneficial.

We also evaluated circulating sncRNAs as potential biomarkers of MDS. To define the sncRNAs that are associated with MDS progression and are able to predict the outcome of MDS patients, we compared sncRNA profiles between early and advanced MDS. Interestingly, in both plasma and EVs, 80 percent of the total amount of deregulated sncRNAs were increased in early MDS compared to advanced MDS. Among these we identified many miRNAs from a large cluster located within the 14q32 locus, e.g. miR-127-3p, miR-154-5p, and miR-323b-3p. These miRNAs are frequently altered in various cancers, and their deregulation has been linked to abnormal induction of apoptosis and suppression of proliferation. They are also involved in HSC differentiation (271). Interestingly, an *in vitro* study showed that miRNAs from 14q32 locus are selectively and massively released *via* exosomes, whereas they are nearly undetectable in lymphoblastoid B-cell lines (272). Moreover, their levels were reported to be stable in serum of healthy individuals in the course of longitudinal study and able to discriminate between healthy individuals and those who had been diagnosed with cancer over the study period (273). In MDS, the upregulation of miRNAs within the 14q32 locus was reported by several studies (197,274,275). In our study (276), we observed increased expression of 14q32 miRNAs in CD34+ cells in advanced stages of MDS and in AML-MRC and this elevation was associated with poor outcome. Moreover, the increased intracellular levels of 14q32 miRNAs were reduced after AZA treatment (276). Altogether with our current data showing the upregulation of miRNAs from 14q32 locus in early MDS plasma, it could be suggested that the levels of 14q32 miRNAs have opposite trends in HSCs and in plasma in different stages of MDS. These miRNAs seem to be released into the extracellular environment in early proapoptotic stages of MDS but appear to be retained intrinsically along with the disease progression.

Besides association of some miRNAs with MDS progression, we intended to identify the sncRNA biomarkers with the highest predictive values of patient outcome throughout various sncRNA species circulating in MDS patient blood. Therefore, we performed additional series of bioinformatic analyses of RNA-seq data with regard to the survival of MDS patients and their response to AZA therapy. At the level of individual molecules, we identified several sncRNAs whose levels were strongly associated with OS of MDS patients in both total plasma and EVs. However, we almost completely failed to predict the response to AZA therapy. The predictive value of circulating sncRNAs for prediction of both patient survival and response to the therapy was substantially increased by the generation of combined panels of specific sncRNAs. More importantly, multivariate analysis proved that the combined sncRNA panel for EV samples was associated with patient OS more significantly than the clinical variables typically used for routine MDS diagnostics, which clearly points to the considerable potential of sncRNA applicability for better stratification of MDS patients.

In addition to miRNAs, we identified a large set of other sncRNA species deregulated in our MDS cohort. To date, only two studies on MDS addressing intracellular non-miRNA sncRNA species have emerged demonstrating the utilization of tsRNAs from BM samples as predictive biomarkers of response to therapy and progression to AML (202,214). However, little is known about non-miRNA sncRNA species in blood circulation. Savelyeva et al. (277) reported that also snRNAs and snoRNAs represent prominent sncRNA species as promising circulating biomarkers of disease (277). Yet, in our results, mostly miRNAs were represented among sncRNAs with the highest predictive values for patient outcome. Therefore, utilizing miRNAs as possible biomarkers seems to be the most meaningful approach under the present circumstances. Further exploration of other sncRNA species is essential to represent real conditions and to bring new insights into sncRNA functions in health and disease.

Altogether, all the evidence suggests the considerable potential of circulating sncRNAs to become auxiliary noninvasive biomarkers in MDS prognostication. However, further validation of the data on larger independent patient cohorts is necessary before application of sncRNA biomarkers into routine clinical practice.

11.3 Publication 3

Krejcik et al., MicroRNA profiles as predictive markers of response to azacitidine therapy in myelodysplastic syndromes and acute myeloid leukaemia, Cancer Biomarkers, 2018

Besides studies on circulating sncRNAs, we also focused on cellular miRNAs as predictive biomarkers of AZA treatment response. Despite the fact that AZA therapy improves clinical outcomes of patients with advanced MDS (37,38), the ORR is only between 40 to 50 percent (42,43). Therefore, the identification of biomarkers predictive of AZA treatment response would greatly contribute to individualized therapy and avoidance of therapy-related burden.

There is an increasing number of studies focusing on search for predicitve biomarkers of response to AZA therapy. A variety of clinical features and molecular markers have been identified as potentially predictive of AZA treatment response. For example, factors such as lower haemoglobin level, low platelet count (278), absence of aberrant myeloid progenitors (279), mRNA expression of PARP1 (280), GATA1, and FLI1 (281) in BM, and mutational status of SF3B1, SETBP1, NPM1, RUNX1 (282), TET2 (283,284), and U2AF1 (278) were reported to predict either favourable or poor response to AZA therapy in MDS. However, existing results are highly inconsistent and favourable response rates do not often associate with improved OS (285). Thus, further search for biomarkers predictive of AZA therapy response is needed.

In this publication, we analysed changes in miRNA profiles of BM CD34+ cells from higher-risk MDS and AML-MRC patients treated with AZA. We aimed to determine miRNA profiles associated with therapy response and to find miRNA biomarkers that could predict response to AZA therapy. For this purpose, we utilized the same microarray platform as in P1, which allowed an investigation of 2,006 predefined miRNAs.

The data from miRNA expression profiling demonstrated distinct miRNA profiles at baseline (i.e. in samples before AZA administration) between patients who later achieved a response to AZA therapy (responders) and patients who exhibited stable disease (SD) or progressed disease (SD) after AZA therapy (patients with SD and PD were considered nonresponders). As expected, miRNA profiles of patients with SD and PD did not exhibit significant differences and clustered together. With respect to this finding and the limited number of patients in the study, we performed subsequent analyses on the two groups, responders and nonresponders.

We focused on individual miRNAs with deregulated levels at baseline between later responders and nonresponders and found that the patients with upregulated miR-17-3p and downregulated miR-100-5p and miR-133b achieved a response to AZA more often. Moreover, low level of miR-100-5p associated with favourable OS of the AZA treated patients. miR-100 has been reported to act as an tumour suppressor and its reduction has been found in solid tumours (286–288). The upregulation of miR-100, however, has been observed in AML (289–291), indicating that it may act differently in various cancers. In haematopoiesis, the upregulation of miR-100 was found to arrest granulocyte and monocyte differentiation and promote cell survival (289). Increased level of miR-100 is associated with advanced clinical features (290) and unfavourable karyotypes (291) of pediatric AML patients and predicts shorter relapse-free survival and OS (290). The upregulation of miR-100 was also observed in AML cell lines (291). The direct target of miR-100 is ATM transcript (291), whose protein product is a protein kinase that acts upstream of p53 and responds to DNA damage (292). Overexpression of ATM protein *in vitro* suppresses cell viability and induces cell apoptosis. miR-100 downregulates ATM expression leading to inhibition of cell apoptosis and increased AML cell viability (291). Taken together, the data indicate that upregulated miR-100 is associated with worse prognosis and may play an important role in the development of AML. Based on this evidence and our results, we propose that the decreased level of miR-100 detected in MDS/AML-MRC patients who later responded to AZA treatment may reflect lower aggressiveness of the disease and may be associated with better responsiveness to AZA therapy and patient survival.

Among the miRNAs deregulated between AZA responders and nonresponders at baseline, we identified other miRNAs that are implicated in cell proliferation and oncogenesis. For example, miR-1 and miR-133b showed increased levels in nonresponders. These miRNAs are encoded in homologous clusters of miR-1/133a and miR-206/133b (293). Although miR-1 is downregulated in many tumours (293), its overexpression was detected in AML (294,295) in which it promotes cell proliferation, suggesting that it may act as an oncogene in haematologic malignancies (295). The HOX-related miRNAs (296), miR-10a and miR-10b, showed decreased levels in nonresponders and increased levels in responders. It has been shown that these miRNAs are aberrantly expressed in myeloid malignancies (196,297,298) and high baseline expression of the miR-10 family in untreated AML patients is associated with complete remission in response to induction chemotherapy (196).

In the final part of this study, we determined miRNAs with changed levels after AZA therapy compared to their levels at baseline, indicating that they might be modulated by AZA exposure. Among them, numerous haematopoiesis and oncology related miRNAs, such as let-7f, miR-10, miR-15, and miR-181 families, were found. For example, the expression of miR-181 family members was significantly reduced after the therapy. miR-181 family plays an important role in the regulation of haematopoiesis, including proliferation and differentiation of HSPCs and megakaryocytic lineage development (299). Increased expression of miR-181 family members was previously detected in higher-risk MDS (195), in MDS patients who later transformed to AML (202), and M1 and M2 subtypes of AML (300). The reduction in the level of miR-181 may therefore be one of the downstream effects of the treatment with AZA, modulating the aberrant proliferation and differentiation in myeloid disorders.

With respect to response status, we identified that the level of miR-199a-3p was exclusively decreased in responders after AZA therapy. Interestingly, our results (P2) also determined this miRNA to be one of the most common sncRNAs with the best cumulative predictive values of AZA treatment in total plasma. Increased level of miR-199a-3p was demonstrated to enhance proliferation of myeloid progenitor cells and cause AML in a preleukaemic mouse model (301), indicating its oncogenic role in myeloid disorders. Decreased level of miR-199-3p exclusively in responders after the therapy suggests that AZA may contribute to the improved patient outcome *via* downregulating the expression of miR-199a-3p.

To conclude, our data indicate that AZA responders and nonresponders show distinct miRNA profiles and that specific miRNA levels before therapy initiation may predict the efficacy of AZA therapy.

12 SUMMARY

In this thesis, we focused on profiling of extracellular (total plasma and EVs) and intracellular (CD34+ BM cells) sncRNAs in MDS. In general, our results indicate that sncRNA profiles measured in both plasma and BM samples are specific to different stages of the disease and may predict patient outcomes. We identified several miRNAs and other sncRNAs that are associated with the disease development and may be considered as potential novel biomarkers of MDS progression or responsiveness to the treatment. Moreover, the sncRNA profiling performed on MDS plasma provides new information on the phenomenon of circulating miRNAs in MDS pathophysiology.

One of the important aims of this study was to compare sncRNA profiles in different materials and to define which of them would be more suitable for monitoring of MDS biomarkers. Our results show specific sncRNA profiles to CD34+ BM cells, total plasma, and EVs. Thus, it appears that each material as a source of sncRNAs provide different point of view on the actual condition of an organism. Nowadays, BM cells are considered the most meaningful source of MDS biomarkers. The necessity of BM biopsy, however, leads to an effort to find less invasively accessible source of biomarkers, such as blood plasma. Hornick et al. (181) proposed that exosome miRNAs may serve as biomarkers for early detection of AML recurrence as exosomes derived from aberrant blasts can be detected in blood circulation earlier than the blasts themselves. In addition, they indicated that AML exosomes reflect contributions from both leukaemic blasts and marrow stromal cells modified by the presence of the malignancy and suggested them to be the first potential leukaemia biomarker representing multiple components of the malignant microenvironment (181).

Remarkably, our results indicate that the two extracellular materials, total plasma and EVs, do not show the same sncRNA profiles. It is in agreement with the findings that sncRNAs are released from cells selectively in different ways. A study that compared miRNA content in total plasma and plasma-derived exosomes in kidney disease (258) showed that miRNAs in plasma and in exosomes are differentially regulated and thus, the measurement of exosomal miRNAs cannot be replaced by the measurement of miRNAs in plasma, or vice versa (258). It remains to be elucidated which of the materials would be most useful in particular circumstances. Based on our results, EVs seem to be a better source of sncRNA biomarkers for patient survival, whereas sncRNAs circulating in total plasma might be predictive of AZA treatment response.

Regarding biomarkers predictive of response to AZA treatment, our results show that BM cells and total plasma may be useful sources. In both materials, we identified specific miRNAs that show potential to predict the therapy response. The specific miRNAs were different between BM cells and total plasma except for miR-199a-3p, which was identified as a predictive biomarker in both materials. Importantly, the predictive value of circulating miRNAs substantially increased when the predictive score was generated from the data of multiple miRNAs. Further investigation of expression and functions of these miRNAs (particularly of miR-199a-3p) would clarify the significance of their ability to predict the response to AZA therapy.

Along with MDS progression, sncRNA profiles change, showing alterations at the levels of many haematopoiesis-related miRNAs as well as other sncRNA species. It remains to be clarified whether changes in circulating sncRNA levels are a consequence of deregulated sncRNA release from cells related to a disease without any specific function, when its impact on recipient cells would be just an accident, or if there is a purpose of affecting specific recipient cells in a particular way. Notably, the effects of sncRNAs taken up by recipient cells may lead either towards improvement of disease outcome or disease progression. It may be influenced by the origin of particular EVs, i.e. whether they are derived from the normal or the aberrant cells. It has been shown that tumour cells secrete often increased amount of exosomes than normal cells, carrying molecules specific to the tumour cells from which the EVs were derived (302). EVs in haematologic malignancies have been reported to modify malignant cells themselves and also cells of BM microenvironment, making the microenvironment more supportive of malignancy, suppressing the immune system, and inducing drug resistance (303). For example, Hornick et al. (155) observed that a unique miRNA profile of exosomes released from AML cells has the potential to increase leukaemic fitness by dysregulating other cell types (155). Based on this evidence, it appears that EVs may be implicated in disease development and progression.

With the increasing evidence of EV-mediated intercellular communication, their ability to alter the functions of recipient cells, and their role in disease development and progression, EVs have become considered as novel therapeutic targets. The strategy is to inhibit aberrant cell communication *via* EVs to prevent their growth and spread. Different approaches can be applied, i.e. interfering with EV biogenesis and release from cells, blocking EV uptake by recipient cells, and removing of EVs from the circulation (303,304). Further, EVs have potential to be utilized for therapeutic purposes in terms of both drug delivery and regenerative medicine. They have natural features that make them suitable therapeutic vehicles, such as natural stability in body fluids, protection of their cargo from degradation, inherent targeting ability, low immunogenicity, and finally, the possibility of engineering them (303,305,306). However, despite of recent advances, in order to effectively utilize EVs as therapeutic targets and tools, it would be highly appropriate to improve the understanding of their biology and become fully aware of the intricacy of the targeted therapy application.

To conclude, our data provide not only a rationale for the potential clinically effective application of circulating sncRNAs as prognostic and predictive biomarkers of MDS but also raise new intriguing questions about the pathobiology of release mechanisms and possible consequences of their defects in haematopoietic disorders. Further investigations on sncRNA function, role in cell-to-cell communication, and their release dynamics in health and disease conditions are still needed to gain better insight and draw meaningful application of sncRNAs in diagnostics, prognostics and prediction of treatment response in MDS.

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