Title:

Transcriptomic profiling of platelet senescence and platelet extracellular vesicles

Running head: Platelet extracellular vesicles enrich miRNA

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ABBREVIATIONS

ABC	ATP-binding cassette transporter
AD	Alzheimer´s disease
Ago	Argonaute
ALS	Amyotrophic lateral sclerosis
Apo	Apolipoprotein
APP	Amyloid precursor protein
aSyn	Alpha-synuclein
BACE1	Beta-site APP-cleaving enzyme1
CD	Cluster of differentiation
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FC	Fold change
FKBP4	FK506 binding protein 4
GABA	Gamma-aminobutyric acid
GP	Glycoprotein
HD	Huntington´s disease
LPA	Lysophosphatidic acid
MI	Myocardial infarction
MMP	Matrix metallopeptidase
MS	Multiple sclerosis
NAV3	Neuron navigator 3
OCS	Open canalicular system
PAGE	polyacrylamide gel electrophoresis
PCA	principal component analysis
PD	Parkinson´s disease
PLC	Platelet concentrate
PL-EV	Platelet extracellular vesicle
PLT	Platelet
RBC	Red blood cell
SPHK1	Sphingosine kinase 1
T2D	Type 2 diabetes
TOMM40	Translocase of outer mitochondrial membrane 40 homolog

Abstract

BACKGROUND: Platelets are derived from megakaryocytes during platelet shedding. Senescent or activated platelets are expanded in vascular and neurological diseases and release platelet extracellular vesicles (PL-EVs). A systematic analysis of regular mRNA and small RNA composition in platelets and PL-EVs during *in vitro* platelet senescence has not yet been published.

STUDY DESIGN AND METHODS: We isolated platelets, total PL-EVs and PL-EV subsets on days 0 and 5 from human stored donor platelet concentrates. Isolated mRNA- and miRNA-species were analyzed by microarrays and deep sequencing. Correlation of mRNA- and miRNA-species, and miRNA target analysis were performed by bioinformatics.

RESULTS: During *in vitro* platelet senescence, residual platelet mRNA-species were decreased and partially converted to miRNA-species. Residual mRNAs included encoded genes relevant for atherosclerosis, inflammation (MMP-14, GRN, ANGPTL2) and neurotransmission (DRD2, GABRR3). In comparison to senescent platelets, PL-EVs have up-regulated their miRNA-species, involved in 'diabesity', vascular and metabolic disease (miR-144-3p, miR-486-5p, miR-142-5p, miR-451a, miR-25-3p, miR-145-5p, let-7f-5p). The 100 highest expressed PL-EV miRNA species determined by microarrays were compared with the 100 highest expressed PL-EV miRNA species detected by deep sequencing. This approach resulted in 66 overlaps. The regulated miRNAs (assessed by both methods) were related to neurological disorders, including targets for Alzheimer´s disease (e.g. BACE1, TOMM40, NAV3).

CONCLUSION: During *in vitro* senescence platelets degrade large RNA-species. Concomitantly, they up-regulate a distinct set of known small RNA-species involved in atherosclerosis, inflammation and neurodegeneration. PL-EVs enrich miRNA-

species, likely supporting the role of platelets and PL-EVs in vascular homeostasis and as carriers of neurodegenerative disease-related miRNA-cargo.

Key words: Deep-sequencing, microarray, mRNA, miRNA, neurodegenerative disease, platelet concentrate storage, platelet extracellular vesicle, vascular disease

INTRODUCTION

The anucleated, short-living platelets (PLTs) originate from megakaryocytes by fragmentation of cytoplasm, whereby granules and organelles are transported into pro-PLTs along microtubules that line the shaft of extending PLT-podia [1]. Micro-RNAs (miRNAs) regulate megakaryopoiesis [2]. Platelets possess the whole set of miRNA production machinery, whereby >490 individual mature miRNA-species are detected in human PLTs [2]. Platelet miRNA-species regulate PLT function [3] and apoptosis [4], and in many cases validated target genes correlate to specific miRNA-species expression [5, 6]. Towards atherosclerosis, miRNA-species released from PLTs are reported to regulate vascular endothelial cell gene expression [7] and apoptosis [8-10].

Senescence and/or activation of PLTs induce release of a heterogeneous group of platelet extracellular vesicles (PL-EVs) [11-13], making up about 90% of all circulating EVs [13]. Extracellular miRNAs are largely protected from degradation through association with EVs and formation of protein complexes such as argonaute 2 (Ago2) or high-density lipoprotein (HDL)-associated proteins [14 and references therein]. Several studies have discussed changes in PL-EV-counts in association with atherosclerosis and thrombus formation [14 and references therein], and higher counts of circulating PL-EVs were found in patients with severe peripheral atherosclerosis compared to controls [15]. Atherosclerosis, hypercholesterolemia, hypertension, vascular and metabolic diseases (including ‘diabesity’) are risk factors for neurodegenerative diseases, particularly Alzheimer’s disease (AD) [16-19 and references therein]. Abnormal PLT function [19], increased PLT activation [20] and inflammation [21] are also linked to AD. In blood, PLTs are the major cellular carriers (>75%) of the amyloid precursor protein (APP) [22]. Platelets process APP into amyloidogenic peptides, including the plaque-forming amyloid beta $A\beta_{42}$, secreted

from PLT granules upon PLT activation [23, 24]. MiRNAs regulate APP alternative splicing [25-27].

PL-EVs, implicated in neurodegeneration, carry circulating Aβ [28-30]. Furthermore, EVs in human body fluids transport Parkinson´s disease (PD)-related protein α-synuclein (aSyn) for delivery to recipient cells [31, 32]. Red blood cells (RBCs) are the major cellular source (>90%) of circulating aSyn [31]. In PLTs, aSyn regulates vesicle transfer and granule release [32]. Our proteomic analysis of platelet concentrate (PLC)-derived EVs showed that PL-EVs, beyond RBC-EVs, are also carriers for aSyn [12].

Previously we investigated lipidomic and proteomic changes in stored PLCs, a model for *in vivo* PLT senescence, including PLTs, plasma [33] and PL-EVs [34]. In comparison to PLTs, PL-EVs showed a lipid-raft like composition and enriched lipid-species and proteins, regulating vascular and neurodegenerative diseases [34, 12]. PL-EVs are heterogenous [11], and AD- and PD-related molecules segregate into specific PL-EV density fractions [12].

In order to extend our previous lipidomic and proteomic characterization of *in vitro* PLT senescence [33, 34, 12], the current study analyzes transcriptomic changes during 5 days PLC storage in PLTs and PL-EVs. The regular RNA and miRNA profiles and their changes were extensively investigated during *in vitro* PLT storage. The potential impact of diverse RNAs in PLTs and PL-EVs for the pathogenesis of vascular and neurodegenerative diseases is discussed.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless otherwise stated.

Characteristics of PLT donors, PLT apheresis and PLC storage

PLCs were collected from healthy normo-lipidemic volunteers as described [35]. In brief, hemapheresis donations for PLCs were obtained according to the German regulations for blood donation. All volunteers underwent a physical examination and *in vitro* diagnostic laboratory screening to exclude infections and major metabolic diseases. Donors did not take any drug within 4 weeks before blood drawing. Informed consent and approval of the hospital's Ethical Committee (Approval-Nr. 08/119) were obtained. For single-donor apheresis cell separators Trima Accel (Terumo BCT, Lakewood, CO; single-needle system) and Amicus (Fenwal Inc., Lake Zurich, IL; double-needle system) were used according to standard procedures of blood donation centers. PLCs from a single donation contained 2.0×10^{11} to 4.0×10^{11} PLTs/unit in total volume varying from 200 to 300 mL, the white blood cell (WBC) content was below 1×10^{6}/unit, and the RBC content below 3×10^{9}/unit. Further components of PLCs were autologous plasma (0.74-0.85 mL/mL) and ACD-A (Fenwal Inc.; 0.15-0.26 mL/mL). PLCs were stored for up to 5 days under standard blood banking conditions, i.e. constant agitation at 60 cycles/min, incubated at 22 ± 2°C (PLT agitator PF96i, Helmer Laboratories, Noblesville, IN).

Isolation of PLTs and PL-EVs from PLC

5 mL PLC was taken aseptically from stored PLCs on days 0 and 5 and centrifuged 15 min, 145*g* at room temperature. The pellet was carefully washed twice in 20 mL Tyrode-Hepes buffer containing 10 mM ethylenediaminetetraacetic acid (EDTA) to prevent PLT activation and immediately frozen at -80°C until RNA isolation, as previously described [33]. In brief, In brief, 2 mL of platelet-rich-plasma (PRP) is layered onto a column (column volume, 13.5 mL; GE Healthcare, Munich, Germany) filled with Sepharose 2B in calcium-free Tyrode-Hepes buffer (138 mmol/L NaCl, 3 mmol/L KCl, 12 mmol/L $NaHCO_3$, 0.4 mmol/L NaH_2PO_4, 1 mmol/L $MgCl_2$, 5 mmol/L glucose, 10 mmol/L Hepes, 10 mmol/L EDTA, 0.5% [wt/vol] bovine serum albumin [BSA]), pH 7.4, sterile-filtered. A dead volume of 2.5 mL is discarded and Fractions 3 to 5 (elution volume, 2.5-5.0 mL) are collected and pooled together. This filtration step is repeated 2-3 times until the requested amount of platelets in a given volume is collected.

PL-EVs were isolated from PLCs by differential centrifugation as described [12]. In brief, PLTs were eliminated by centrifugation at 1500*g* for 15 minutes, followed by centrifugation of the supernatant at 13.000*g* for 2 minutes. In order to pellet PL-EVs the final supernatant was centrifuged at 30.000*g* for 45 minutes, for pelleting circulating EVs. The pellet was washed twice in Tyrode-Hepes buffer and stored at −80°C until analysis.

RNA isolation

Total RNA was isolated from pelleted PLTs and PL-EVs by TRIzol according to standard protocols and with addition of 20 µg RNase-free glycogen in the precipitation step (Life Technologies, Darmstadt, Germany). Total RNA concentration was measured spectrophotometrically with the Nanodrop ND-1000 (Peqlab, Erlangen, Germany). Total RNA was freezed at -80°C until analysis.

RNA Analysis

For mRNA and miRNA analysis by microarray, total RNA was labelled by Low Input Quick Amp Labeling Kit (50 ng) and miRNA Complete Labeling and Hybridization Kit (100 ng), respectively, according to the manufacturer's descriptions (Agilent Technologies, Böblingen, Germany). Hybridization and analysis was carried out on an Agilent microarray platform with data processing by Agilent feature extraction software version 10.7.3.1.

For deep sequencing, small RNA cloning and preparation was performed as described previously [36]. In brief, small RNAs were isolated from total RNA samples by PAGE purification. A gel slice containing RNAs between 18-25 nucleotides was excised and the RNA was extracted using TRIzol. In subsequent steps, the RNA was ligated to an adenylated 3' adaptor by a truncated T4 RNA ligase. The 5' adaptor was ligated in a second step. The corresponding product was reverse transcribed using specific primers and amplified. Finally, the samples were separated on a 6% urea PAGE and the ligation products were purified and used for deep sequencing. The libraries were measured on a Genome Analyzer GA*IIx* (Illumina) by Fasteris SA (Geneva, Switzerland) in 1 × 50 bp single-end runs. For search for miRNA targets, a list of experimentally validated micro RNA targets was taken from miRTarBase, release 4.5. [37].

Data Analysis

We used "R" software environment for data processing and visualization (www.r-project.org). Functions of the basic Stats package were used to perform Pearson's correlation and principal component analysis. Normalization and identification of significantly changed miRNA were done with the help of AgiMicroRna package

(version 2.16.0) (www.bioconductor.org) using quantile normalization [38] and Benjamini Hochberg correction [39]. Microarray mRNA data were processed with ChipInspector software (www.genomatix.de). For both mRNA and miRNA arrays only probes with signal intensities above 100 relative units and changes more than 2-fold were taken into account.

To identify potential targets of the regulated miRNA we used experimentally validated data collected in miRTarBase (http://mirtarbase.mbc.nctu.edu.tw) [37].

RESULTS

Gene expression during *in vitro* PLT senescence

Analysis of PLT RNA integrity by Bioanalyzer testing showed decreased 18S and 28S ribosomal RNA from day 0 to day 5 (Fig. 1A, B). Obvious was the high content of small ribosomal RNA-species (5S, 5.9S). Also total PLT RNA concentration decreased from day 0 to day 5, being only approximately one third on day 5 of that isolated on day 0 (Fig. 1C).

Comparison of PLT mRNA-species between days 0 and 5 showed that out of 2210 regulated mRNAs, 2092 (95%) were down-regulated, whereas only 118 mRNAs (5%) increased significantly (Supplementary Table 1). The extent of down-regulation for mRNAs were much higher (77-fold) than the extent of up-regulation (6-fold).

Among down-regulated mRNAs we detected mRNAs encoding proteins important for PLT function and homeostasis, like cytoskeletal and signal cascade proteins, enzymes involved in lipid metabolism (e.g. SPHK1, ABCA3, ABCB6, ABCC1, ABCC3, ABCF1), Rabs, vacuolar sorting proteins, ALIX, clusterin/ApoJ, and proteins required for ubiquitinylation (Supplementary Table 1). We also detected down-regulation of mRNAs important for PLT function, like CD61, CD41, CD42d, GP1A/B, GP5, GP6, thromboxane-A2, LPA, sphingosine-1-phosphate (S1P), estrogen and platelet-activating factor receptors, coagulation factors and CD62P (Supplementary Table 1).

Among the 118 up-regulated mRNAs, we detected genes relevant for vascular remodeling, inflammation, autophagy and vesicle trafficking, e.g. MMP-14, ANGPTL2, GRN, immunophilins, FKBP3/4, RUN and FYVE domain containing-2, GM2 ganglioside activator, sphingolipid activator protein-3, acyl-CoA synthase family

member-4, RNA-binding protein Musashi homolog-1, D(2) dopamine receptor (DRD2), GABRR3 (Supplementary Table 1).

miRNA expression during *in vitro* PLT senescence

Principal Component Analysis (PCA) showed separation of normalized miRNA-species signal intensities between fresh (day 0) and *in vitro* senescent (day 5) PLTs, and a still more prevalent separation between PLTs and PL-EVs (Fig. 2). This reflects significant differences in signal intensities of individual miRNAs between the groups, with a general increase in total normalized miRNA signal intensity during *in vitro* PLT senescence and PL-EV release (Supplementary Fig. 1). Heat-map analysis of the 50 miRNA-species with the highest variance between groups supported increasing signal intensities from PLTs day 0 to day 5 to PL-EVs (Supplementary Fig. 2).

Filtering by 0.05 cut-off for Benjamini-Hochberg (BH) adjusted p-values revealed 3 significantly up- (miR-923, miR-574-3p, miR-328) and 3 significantly down-regulated miRs (miR-1260a, miR-1274b, miR-720) in *in vitro* senescent vs. fresh PLTs (Table 1). According to the current literature, miR-923, miR-1274 and miR-720 are described as not actively produced miRs, but rather degradation products of 28s rRNA (miR-923) and tRNA-species (miR-1274, miR-720) [38]. In our target analysis approaches we therefore focused on miR-328, miR-574-3p and miR-1260a.

Regulated PLT miRNA-species target regulated mRNAs during *in vitro* PLT senescence

mRNA target analysis showed that miR-328 targets 13 (12 down- and one up-regulated) and miR-574-3p targets 3 (two down- and one up-regulated) regulated PLT mRNAs during *in vitro* PLT senescence (Table 2). The down-regulated miR-

1260a targets one down-regulated PLT mRNA (Table 2). Among down-regulated mRNAs we detected species encoding proteins of the ubiquitination pathway, regulators of transcription and maintaining cellular metabolism, as well as structure and protein sorting (Table 2). Interestingly, the miR-328 targets FKBP4, a co-chaperone that regulates cellular transport and is part of steroid receptor complexes, was up-regulated in senescent PLTs (Table 2).

PL-EVs enrich miR-species that bind mRNA potentially targets regulating lipid metabolism, PLT function and vascular-, metabolic- and neurodegenerative disease

Comparison of regulated miRNAs in PL-EVs vs. senescent PLTs revealed 70 regulated miRNAs, and 21 of them were up- and 49 down-regulated (Supplementary Table 2). Among up-regulated miRs, 14 were detected by deep sequencing (Table 3), whereby 13 can bind experimentally validated target sequences [37] (http://mirtarbase.mbc.nctu.edu.tw/) in 1892 human mRNAs (Supplementary Table 3). According the database, up-regulated PL-EV miRs potentially target a number of mRNAs involved in lipid metabolism (e.g. ABCB1, ABCD4, ABCF1 (miR-451a), LPL (miR-29a-3p), LYPLA2 (miR-145-5p), SREBF1 (miR-25-3p, miR-484), SPHK1 (mir-484), ELOVL1 (miR-484)), inflammation and vascular disease (e.g. MIF and MMP2 (miR-451a), complement factors (miR-484 and miR-30b-5p), interleukins (miR-484, let-7f-5p), TIRAP (miR-145-5p), CD40 (miR-486-5p), VEGFA (miR-145-5p)), PLT adhesion and activation (e.g. CD61 (let-7a-5p), GPV (let-7a-5p), F2R (let-7a-5p), COX-2 (let-7a-5p, let-7c-5p)), coagulation (e.g. fibrinogen (miR-144-3p, miR-29a-3p), collagen (miR-29a-3p), SERBP1 (miR-374a-5p, miR-30b-5p)), endocytosis and vesicle trafficking (e.g. CLTC (miR-484), Rab-proteins (miR-484, miR-451a, miR-30b-5p, let-7a-5p, let-7c-5p, let-7f-5p), TMED1 (miR- 486-5p)), autophagy (e.g. ATG4C

(let-7a-5p), ATG12 (miR 30b-5p)) and generation and binding of small RNA (e.g. DICER1 (let-7a-5p, let-7c-5p, miR-29a-3p, miR-374a-5p), AGO1 (miR-25-3p, miR-29a-3p, miR-484, let-7c-5p)) (Supplementary Table 3).

PL-EVs enrich miR-species potentially involved in neurodegenerative disease

Compared to senescent PLTs, PL-EV showed up-regulation of miRNA-species involved in multiple sclerosis (MS) (miR-145-5p) [40], PD and AD, with experimentally validated targets such as fibrinogen (miR-144-3p) [41], BACE, NAV3 (miR-29a-3p) [42, 43], and TOMM40 [44, 45] (miR-484) (Supplementary Table 3).

Comparison of normalized PL-EV miRNA data obtained by microarray and deep sequencing, revealed a significant (p<0.01) correlation according to Pearson (R=0.49) (Fig. 3). Overlap of the 100 most expressed miRNAs from both platforms (i.e. microarray: highest intensity after normalization, deep sequencing: total reads) showed 66 overlapping miRNA-species (Fig. 4), supporting above correlation data, validating a set of highly expressed PL-EV miRNAs.

Comparison of the overlapping 66 PL-EV miRNAs to the published data revealed that 39 miRNA-species (58%) are described to be regulated in human neurodegenerative diseases, with 35 miRs showing relevance in AD and a minor portion in ALS, HD and prion diseases [29, 46, 47] (Table 4). Matching of the above 39 miRNA to experimentally validated functional human targets [37] revealed that 33 miRNA-species target AD-related genes (e.g. BACE1 (miR-107, miR-26b-5p), CFH (miR-146a-5p), TLR4 (let-7i-5p, miR-146a-5p, miR-21-5p), CLU (miR-425-5p), TOMM70 (hsa-miR-148b-3p, hsa-miR-342-3p), TOM22 (hsa-miR-24-3p), TOMM40 (hsa-miR-484)) (Supplementary Tables 3 and 4).

DISCUSSION

Despite PLTs are devoid of a nucleus, they are suggested to be able to transcribe mRNA [48] and translate this into protein [49].

In addition to constitutive mRNA expression of PLT cytoskeleton, integrin or coagulation factor proteins [50], platelets also contain mRNA related to cholesterol metabolism [51], oxidative stress [52], and disease states [53]. Abundant rough ER and ribosomes in enlarged, young, reticulated PLTs correlate with their higher protein synthesis potential, compared to aged PLTs [49]. Platelet life-span is similar in length to platelet mRNA life-span [54], and consistent with this observation, we detected a strong reduction in PLT mRNA during 5 days PLC storage, encompassing mRNA-species needed for cellular maintenance, including species relevant for PLT function, such as integrin receptors and coagulation factors. It seems likely that a general "break-down" of PLT residual mRNA takes place during senescence (Fig. 1), and it is tempting to speculate that this break-down may contribute to the short PLT *in vivo* life-span and reduced PLT function during storage. Since no other studies have yet analyzed PLT mRNA behavior during PLC storage, in this context we can only refer to own data.

Assuming that most down-regulated PLT mRNAs are results of a simple degradation (Fig. 1), a more interesting approach is to focus on the 118 mRNAs that increased during *in vitro* PLT senescence. Among these we found genes involved in atherosclerosis and inflammation, possibly relating to the known role of PLTs and PL-EVs in vascular remodeling [55]. The highly up-regulated cell-surface MMP-14 is expressed by PLTs, and is able to activate other MMPs, modulating PLT adhesion/aggregation, PLT-leukocyte interaction [56] and plaque stability [57]. ANGPTL2 activates pro-inflammatory signaling and increases macrophage infiltration, resulting in endothelial dysfunction during atherosclerosis [58]. GRN is

encoded on the same DNA strand, but in opposite 5´ to 3´direction as CD41 [59]. GRN is spliced into several granulin peptides, found in atherosclerotic plaques, where they regulate plaque progression [60].

PLTs possess uptake and storage mechanisms for neurotransmitters, with dedicated roles in psychiatric disorders [61]. Serotonin is transported in PLT dense granules [62], and disturbed activity of serotonin transporters and receptors are found in major depressive disorder [63]. PLTs also express a variety of dopamine receptors, being able to take up, store and release dopamine [62, 63]. In addition, high levels of gamma-aminobutyric acid (GABA) were found in PLTs, but the uptake system for GABA is unknown [64], GABA is released upon PLT stimuli and inhibits collagen-induced aggregation [64]. Interestingly, we detected increased DRD2 and GABRR3 mRNA in senescent PLTs. Platelet DRD2 bind dopamine in migraine patients [65], and increased GABA uptake by PLTs has been recognized in patients with bipolar disorder [66]. Thus, many of the increased miRNAs in *in vitro* PLT senescence have been implicated in atherosclerosis, inflammation, vascular remodeling and different psychiatric disorders in other systems [115, 116]. Although our data have to be further validated, this may indicate a potential involvement of PLT senescence in neurodegenerative diseases.

Few of the up-regulated PLT mRNAs were detected as expressed protein by mass spectrometry (data not shown), also reported by others [67, 68]. The discrepancy between mRNA and protein data might reflect the drawback of protein mass spectrometry to detect membrane or low abundance proteins, resulting from e.g. high turnover [69], the decreased protein translation potential during PLT aging [49], or antagonism of translation by abundant PLT miRNAs. However, detected abundant expression of a set of mRNAs in senescent PLTs might be relevant as transferred cargo into recipient cells, either directly by PLTs [70], or via released PL-EVs [71],

[68], whereby recipient cells are able to translate transferred mRNA into protein [72, 70]. This might in part indicate a mechanism how senescent PLTs and PL-EVs may modulate vascular and neurologic diseases [73, 74].

Increased non-PLT mRNA (e.g. macrophage-derived chemokine CXCL2) in senescent PLTs might rely on the uptake of exogenous plasma components into the PLT endomembraneous open canalicular system (OCS) [75-77], which during PLT storage swells to 25-30% of PLT total volume [75]. The OCS can fuse with intracellular lipid membranes like α-granules [75], which together with exosomes [78, 79], are generated via multivesicular bodies, thus making it possible for PLTs to re-secrete plasma-derived material in form of PL-EVs.

In vitro PLT senescence during PLC storage showed a general trend of increased miRNA expression, especially in PL-EVs (Supplementary Figs. 2, 3). A drawback of this approach, however, is the above discussed fact that PLT mRNA probably degrades during senescence, with less large RNA-species in senescent PLTs (and PL-EVs) than in fresh PLTs, resulting in relatively more small RNA (including miRNA) in the total RNA preparation used for labelling.

The Principal Component Analysis (PCA) is a well-accepted statistical approach that uses an orthogonal transformation to convert a set of data of possibly correlated variables into a set of values of linearly uncorrelated variables, called principal components. By using PCA we were able to separate normalized miRNA-species signal intensities between fresh (day 0) and *in vitro* stored (day 5) PLTs, and a still prevalent separation between PLTs and PL-EVs, despite of generally increased signal intensities during *in vitro* PLT storage. By use of stringent *p*-values [39] for differential miRNA expression, we partly circumvented the above discussed potential bias.

Furthermore, it has been observed by many laboratories that sequencing and

microarray analyses of miRNA expression profiles can differ significantly. PCR or ligation biases can affect cloning and deep sequencing studies. Hybridization issues are often observed for microarray studies. Thus, our results highlight that different approaches should be chosen in order to produce reliable and highly relevant small RNA profiling data.

We detected high up-regulation (11.5-fold change) of 28S rRNA-derived miR-923 in senescent PLTs, which is plausible, as ribosomes and protein synthesis are known to decrease [49]. All three regulated *bona fide* miR-species (miR-574-3p/-328/-1260a) (Table 1) are reported to be expressed in PLTs [80], whereby miR-328 was also detected in megakaryocytes [81]. MiR-328 show overlapping nucleotide sequences in the antisense strands of introns 12 and 13 of the gene ELMO2 (engulfment and cell motility-3), which was strongly down-regulated during PLT storage (Supplementary Table 1). It is thus possible that miR-328 is actively generated from degradation products or spliced out from intronic ELMO2 regions. Circulating miR-328 was up-regulated in myocardial infarction (MI) [82], and distinct PLT miR-species were detected in diverse MI-subgroups [83]. In cell culture experiments, miR-328 directly down-regulated BACE1 by binding to its 3´-UTR, and miR-328 was less expressed in AD mouse model brains [84]. Saba *et al.* showed a 2.7-fold up-regulation of miR-328 in prion-induced neurodegeneration in mice [85].

MiR-574-3p was up-regulated in human MI [86], in plasma of specific MI disease subgroups [84], and during sebaceous lipogenesis [87]. In addition, a down-regulation was detected upon treatment of endothelial cells with remnant lipoprotein particles, resulting in senescence [88]. The down-regulated miR-1260a contains the nucleotide sequence that is found antisense in the 3´-UTR part of NGB (neuroglobin) exon-4. NGB was up-regulated in PLTs, however, below the detection cut-off. NGB is

beneficial for neuronal survival [89], protecting against hypoxia-associated apoptosis [90], and it is increased in early phase AD human brains [91].

Among regulated PLT miRNA-species, miR-328 showed most experimentally validated, regulated PLT mRNA targets [37], down-regulating genes affecting all aspects of cellular maintenance (Table 2). Less common, we detected two up-regulated mRNA targets (miR-328-3p for FKBP4 and miR-574-3p for IGLON5) (Table 2). Up-regulation of mRNA-species by miRNAs may occur by binding of miRNA to translation factors, recruiting these to complementary mRNA promoter sequences [92], or by relieving an inhibition by other miRNA-species at the mRNA 3´-UTR [93].

Significant correlation of PL-EV miRNA microarray and deep sequencing data (Fig. 2) show that our novel approach may be used to validate a set of robustly expressed PL-EV miR-species. Up-regulated PL-EV miRs represent circulating miR-species that are regulated in T2D (miR-144-3p, miR-486-5p) [94], miR-142-5p [95], coronary artery disease (miR-451a, miR-25-3p) [84, 96], miR-145-5p [97], pre-eclampsia [98], MI (let-7f-5p) [99], obesity (miR-486-5p) [100, 101], and metabolic risk [102], supporting the potential of these miR-species as biomarkers for vascular and metabolic disease screening. Molecular function analyzes revealed that miR-144-3p targets ABCA1, regulating lipid metabolism and plasma high-density lipoprotein levels [103], and that miR-142-5p targets B cell translocation gene-3, regulating vascular cell proliferation [104]. MiR-484 was shown to target Fis1, regulating mitochondria fission/fusion and apoptosis [105], and let-7c was shown to target Bcl2 antagonist for cell death, regulating apoptosis [106]. Target sequences were also detected within several mRNAs encoding proteins regulating PLT adhesion, activation and coagulation (Supplementary Table 3), connecting the known role of PL-EVs to atherothrombosis [14].

Towards neurodegeneration, PL-EVs enriched miRNA-species involved in MS (miR-145-5p) [40], PD (mir-22-3p/-29a-3p) [107], HD (mir-486-5p) [108], mir-27b-3p/-92a-3p/-22-3p [109], ALS (mir-142-5p/-146b-5p) [110], prion diseases (let-7b-5p, mir-146a-5p/-342-5p/-328) [85], and especially AD (Table 4) [29, 46, 47 and references therein], with several experimentally validated mRNA targets as fibrinogen (miR-144-3p) [41], BACE1 (miR-29a-3p/-19b-3p), APP (miR-101-3p, Let-7i-5p, mir-107) [42, 43], NAV3 (miR-29a-3p) [111], CFH (miR-146a-5p) [112], and TOMM40 (miR-484) [44, 45] (Supplementary Table 3).

The only study that previously analyzed PLT transcriptomic changes during PLC storage focused on 52 apoptosis-inducing miRs using a plate-array system [6]. In our study we provide, for the first time, a more comprehensive view of the transcriptomic changes occuring during *in vitro* PLC storage, including PLT mRNA, miRNA and PL-EV miRNA profiles, with subsequent bioinformatic analysis of potential miRNA targets. We extend our earlier PL-EV proteomic and lipidomic analyses [34, 12] now with RNA data, supporting that PL-EVs enrich vascular remodeling and neurological disease cargo. Vascular- and metabolic diseases are risk factors for neurodegenerative diseases [16], and blood-brain barrier dysfunction connected to vascular disease enable filtration of molecules from the circulation into the perivascular space or the cerebrospinal fluid [113]. Based on our data [34, 12], we propose a role of PL-EVs in endothelial homeostasis, and maybe functional control of targets for dysfunction in aggregate disorders like AD or PD or the BBB. PL-EVs might carry pathologic neurological disease-related cargo from the circulation into the brain, supporting a molecular link of vascular disease to neurological disease progression [16, 114].

This manuscript provides information about changes in different RNAs during *in vitro* PLT senescence, which does not truly reflect the situation of *in vivo* senescence.

Since the total size of the senescent PLT pool *in vivo* is undetermined and likely dynamic, the contribution of senescent PLT to diseases is unknown and it requires further investigation. It is also unclear to what extent diverse pathological states affect PLT and *vice versa*. Thus, the implicated potential link between specific miRNAs and vascular and neurodegenerative diseases has to be further validated in *ex vivo* and *in vitro* approaches.

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Table 1:

Microarray data showing significantly regulated miRNA-species between fresh (day 0: D0) and stored (day 5: D5) PLTs.

miR	FC PLTs D5 vs. D0	adjusted *p*- value*	Normalized signal† D0	Normalized signal† D5	Normalized signal† PL-EVs
hsa-miR-923 v12.0	11,5	0	251	2881	58413
hsa-miR-574-3p	2,4	0,01421	495	1195	337
hsa-miR-328	1,6	0,01421	460	736	193
hsa-miR-1260a	0,4	0,00087	345	142	234
hsa-miR-1274b v16.0	0,4	0,00066	1975	695	2392
hsa-miR-720_v18.0	0,3	0,00002	16331	4775	10653

FC=fold change, *Benjamini Hochberg (BH) adjusted *p* -value, †only normalized signal intensities >100 (PLTs D0, PLTs D5) were taken into account. As a comparison, the signal intensity in PL-EVs is shown.

Table 2:

Correlation of regulated miRNA-species to validated regulated mRNA targets.

miRTarBase* ID	miRNA	FC miR	Target Gene	Gene name	FC§ mRNA
MIRT043807	hsa-miR-328-3p	1,6	DIAPH1†	diaphanous homolog 1 (Drosophila)	-54.6
MIRT043811	hsa-miR-328-3p	1,6	UBE2O†	ubiquitin-conjugating enzyme E2O	-46.2
MIRT040520	hsa-miR-574-3p	2,41	USP31†	ubiquitin specific peptidase 31	-19.6
MIRT043763	hsa-miR-328-3p	1,6	ADNP†	activity-dependent neuroprotector homeobox	-17.8
MIRT006477	hsa-miR-328-3p	1,6	PTPRJ‡	protein tyrosine phosphatase, receptor type, J	-11.5
MIRT043789	hsa-miR-328-3p	1,6	BSG†	basigin (Ok blood group)	-10.1
MIRT043774	hsa-miR-328-3p	1,6	MRFAP1†	Mof4 family associated protein 1	-8.6
MIRT043797	hsa-miR-328-3p	1,6	EZR†	ezrin	-8.1
MIRT043821	hsa-miR-328-3p	1,6	LPHN1†	latrophilin 1	-7.3
MIRT035849	hsa-miR-1260a	0,41	DDX1†	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	-7.1
MIRT043813	hsa-miR-328-3p	1,6	CBX6†	chromobox homolog 6	-5.9
MIRT040519	hsa-miR-574-3p	2,41	MKRN1†	makorin ring finger protein 1	-5.9
MIRT043793	hsa-miR-328-3p	1,6	OTUD5†	OTU domain containing 5	-5.4
MIRT043816	hsa-miR-328-3p	1,6	BCOR†	BCL6 co-repressor	-5.3
MIRT043761	hsa-miR-328-3p	1,6	VPS28†	vacuolar protein sorting 28 homolog (S. cerevisiae)	-5.0
MIRT040521	hsa-miR-574-3p	2,41	IGLON5†	IgLON family member 5	2.6
MIRT043773	hsa-miR-328-3p	1,6	FKBP4†	FK506 binding protein 4, 59kDa	4.0

*http://mirtarbase.mbc.nctu.edu.tw/. MiRNA-mRNA interaction was experimentally validated by †cross-linking, ligation, and sequencing of hybrids, ‡quantitative real-time. PCR/Western Blot. §After normalization, mRNAs with a signal intensity >100 and a fold change (FC) of down- or up-regulation >2 were taken into account.

Table 3:

List of up-regulated miR-species in PL-EVs vs. senescent PLTs. Normalized signal intensities in fresh PLTs (day 0: D0) are shown as comparison.

Targets*	miR	FC PL-EVs vs. PLTs D5	adjusted p-value†	Normalized signal‡ PLTs D0	Normalized signal‡ PLTs D5	Normalized signal‡ PL-EVs	Rank order§ deep seq
8	hsa-miR-144-3p	16,0	0,000	398	170	2715	92
13	hsa-miR-451a	15,3	0,000	11008	9834	150458	29
2	hsa-miR-486-5p	2,0	0,027	1401	1886	3720	1
1	hsa-miR-142-5p	1,7	0,020	3920	2089	3507	50
78	hsa-miR-29a-3p	1,5	0,004	2192	1957	2894	118
780	hsa-miR-484	1,4	0,017	897	785	1131	35
164	hsa-let-7c	1,4	0,042	1273	1627	2249	84
9	hsa-miR-374a-5p	1,4	0,035	2677	2415	3334	200
342	hsa-let-7a-5p	1,4	0,017	24847	26485	36007	3
110	hsa-miR-145-5p	1,3	0,026	224	270	354	147
76	hsa-let-7f-5p	1,3	0,046	31330	31330	39321	6
177	hsa-miR-25-3p	1,3	0,023	4153	4964	6221	19
123	hsa-miR-30b-5p	1,2	0,048	6072	6221	7606	115

FC=fold change. *Number of experimentally validated targets according to miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/). Targets in bold contain genes involved in AD regulation. †Benjamini Hochberg (BH) adjusted p-value. ‡only normalized signal intensities >100 (PLTs D5 and PL-EVs) were taken into account. §Rank order (amount of reads) of detected miR-species by deep sequencing.

Table 4:

39 out of 66 overlapping miRNAs show regulation in neurological diseases [115, 116].

miR	Disease
hsa-miR-21-5p	AD
hsa-miR-16-5p	HD
hsa-miR-15b-5p	AD
hsa-let-7f-5p	AD
hsa-miR-19b-3p	AD
hsa-miR-27a-3p	AD, ALS
hsa-miR-22-3p	AD, HD
hsa-miR-26a-5p	AD
hsa-miR-107	AD
hsa-miR-26b-5p	AD
hsa-miR-20a-5p	AD
hsa-let-7i-5p	AD
hsa-let-7d-5p	AD
hsa-let-7b-5p	Prion diseases, AD
hsa-miR-92a-3p	AD, HD
hsa-miR-146a-5p	AD, Prion diseases, ALS
hsa-miR-27b-3p	AD, HD
hsa-miR-30d-5p	AD
hsa-miR-221-3p	AD
hsa-miR-425-5p	AD
hsa-miR-93-5p	AD
hsa-miR-142-5p	AD, ALS
hsa-miR-101-3p	AD
hsa-miR-486-5p	HD
hsa-let-7e-5p	AD
hsa-miR-181a-5p	AD
hsa-miR-30e-5p	AD
hsa-miR-148b-3p	AD
hsa-miR-342-3p	Prion diseases
hsa-miR-148a-3p	AD
hsa-let-7c	AD
hsa-miR-126-5p	AD
hsa-miR-30c-5p	AD
hsa-miR-361-5p	AD
hsa-miR-197-3p	AD
hsa-miR-125a-5p	AD
hsa-miR-409-3p	AD
hsa-miR-146b-5p	AD, ALS
hsa-miR-423-5p	AD

AD=Alzheimer´s disease, HD=Huntington's disease, ALS=Amyotrophic Lateral Sclerosis.

Figure legends

Figure 1: Degradation of RNA during *in vitro* PLT senescence.

Total RNA was isolated on day 0 and on day 5 from PLT obtained from platelet concentrates. Integrity of RNA as electrogram, showing RNA-species over the time on day 0 (A) and day 5 (B), as measured by Bioanalyzer. (C) Concentration of total RNA on day 5, in % as compared to day 0, and measured by Nanodrop (n=4).

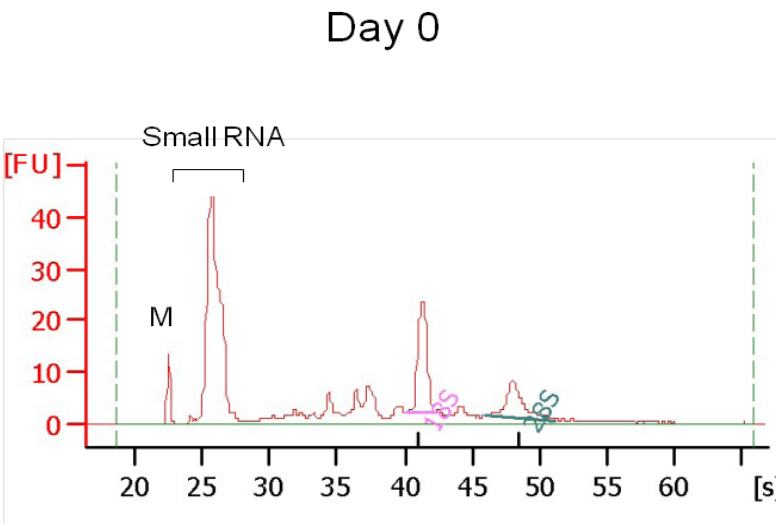
Figure 2: Principal Component Analysis (PCA) of miRNAs during *in vitro* PLT senescence. Normalized miRNA-species intensities obtained by microarray were compared between fresh (day 0) PLTs (circles), 5 days stored PLTs (triangles) and PL-EVs isolated after 5 days (crosses). Each circle represents a PLC replicate.

Figure 3: Pearson´s correlation of all PL-EV miRNA data obtained by microarray (normalized signal intensity) and deep sequencing (total reads). A logarithmic scale is shown. *R* = correlation coefficient.

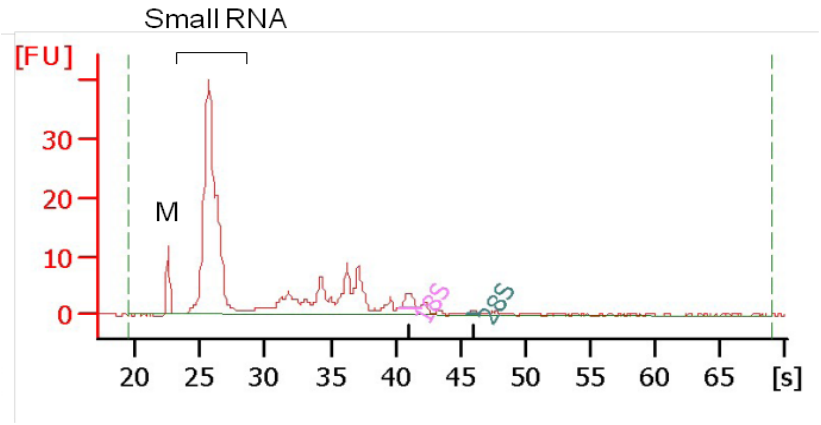
Figure 4: Venn-diagram showing overlap of 100 most expressed miRNA-species detected by microarray (upon normalization of miRNA data) and deep sequencing (total reads).

Fig. 1

(A)


Day 0
Small RNA
[FU]
40
30
20
10
0
M
18S
28S
20
25
30
35
40
45
50
55
60
[s

(B)


Day 5
Small RNA
[FU]
30
20
10
0
M
18S
28S
20
25
30
35
40
45
50
55
60
65
[s]

(C)

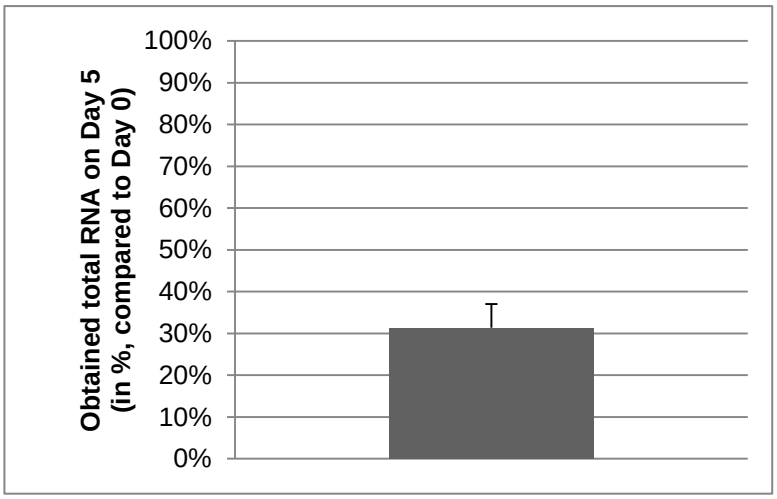

Obtained total RNA on Day 5
(in %, compared to Day 0)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%

Fig. 2

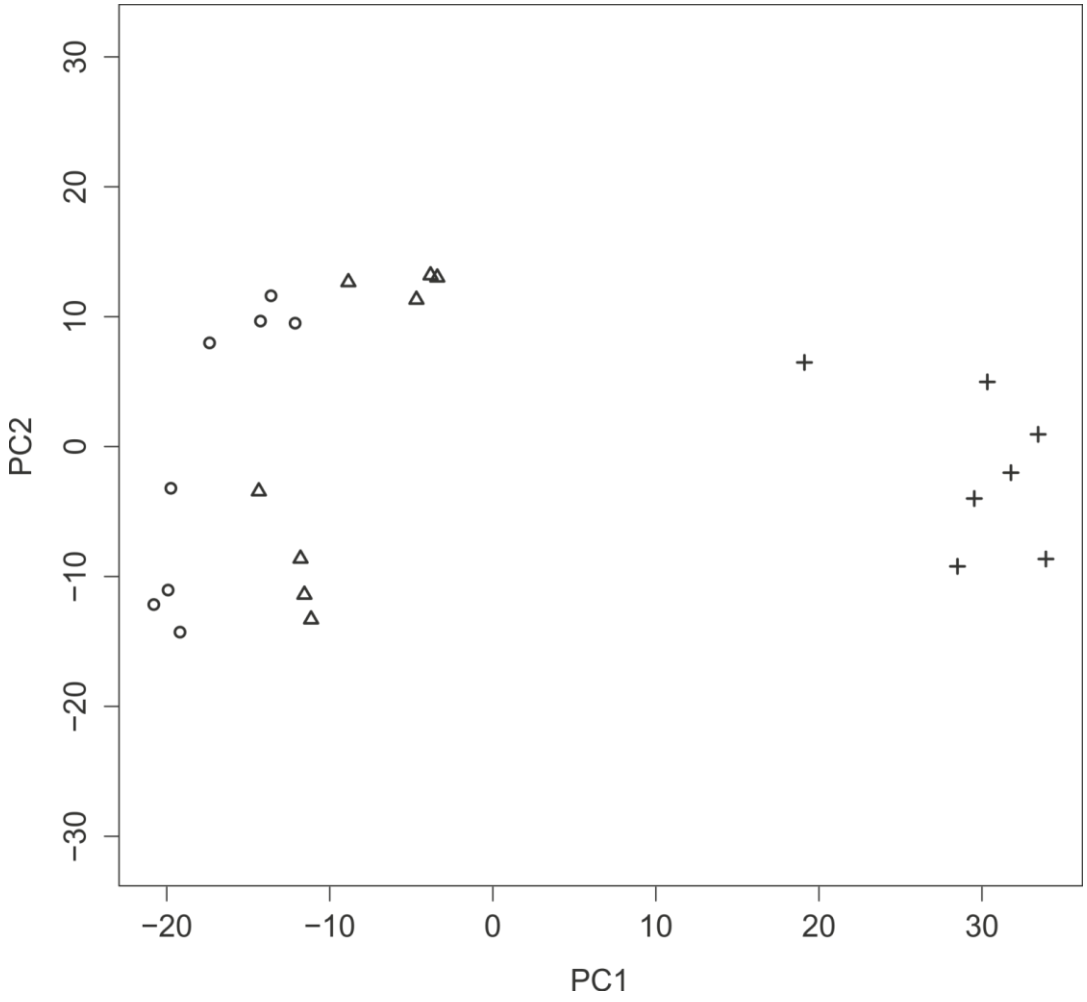


Fig. 3

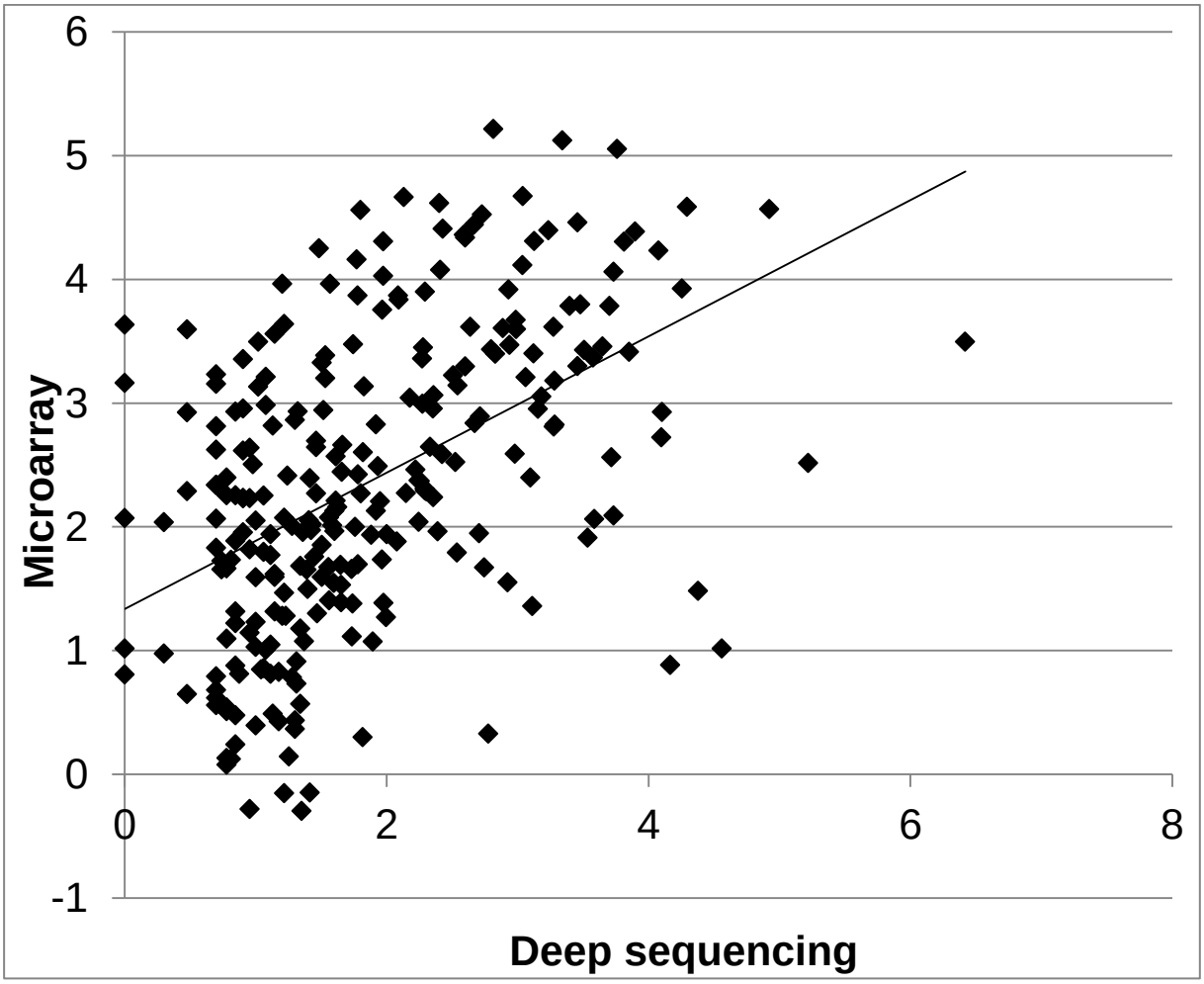
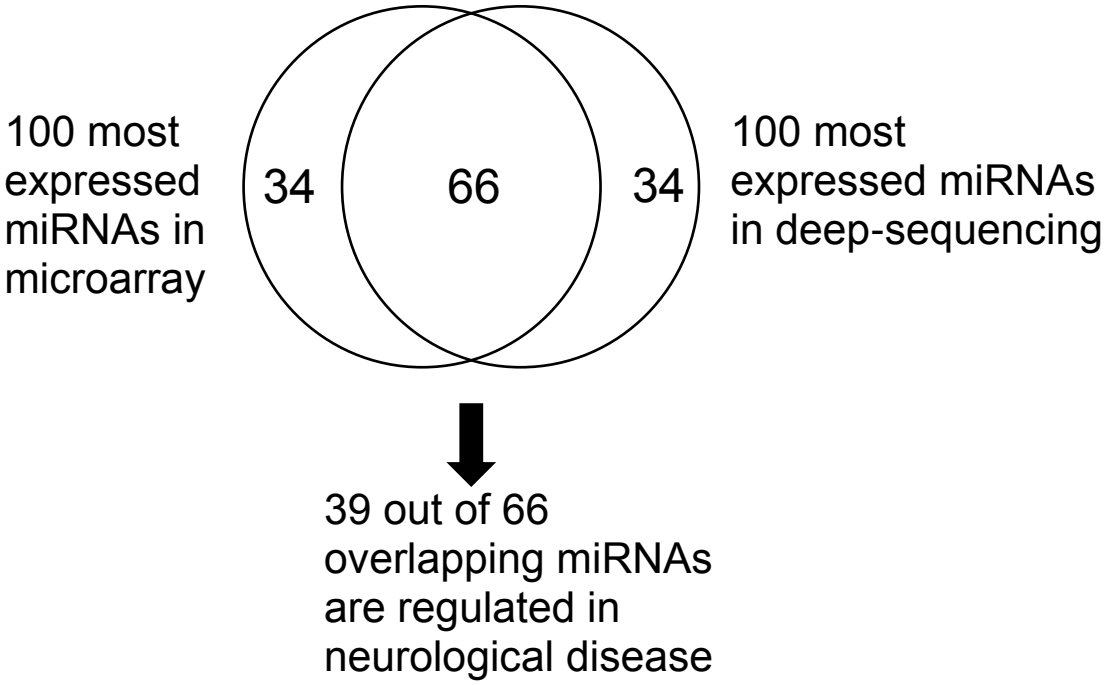


Fig. 4


100 most expressed miRNAs in microarray
34
66
34
100 most expressed miRNAs in deep-sequencing
39 out of 66 overlapping miRNAs are regulated in neurological disease