

## RESEARCH PAPER

# Extracellular vesicles and microRNAs are altered in response to exercise, insulin sensitivity and overweight

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## Abstract

Extracellular vesicles induced by exercise have emerged as potential mediators of tissue crosstalk. Extracellular vesicles and their cargo miRNAs have been linked to dysglycemia and obesity in animal models, but their role in humans is unclear.

**Aim:** The aim of the study was to characterize the miRNA content in plasma extracellular vesicle isolates after acute and long-term exercise and to study associations between extracellular vesicle miRNAs, mRNA expression in skeletal muscle and adipose tissue, and cardiometabolic risk factors.

**Methods:** Sedentary men with or without dysglycemia and overweight underwent an acute bicycle test and a 12-week exercise intervention with extensive metabolic phenotyping. Gene expression in *m. vastus lateralis* and subcutaneous adipose tissue was measured with RNA sequencing. Extracellular vesicles were purified from plasma with membrane affinity columns or size exclusion chromatography.

**Results:** Extracellular vesicle miRNA profiling revealed a transient increase in the number of miRNAs after acute exercise. We identified miRNAs, such as miR-652-3p, that were associated to insulin sensitivity and adiposity. By performing explorative association analyses, we identified two miRNAs, miR-32-5p and miR-339-3p, that were strongly correlated to an adipose tissue macrophage signature.

**Conclusion:** Numerous miRNAs in plasma extracellular vesicle isolates were increased by exercise, and several miRNAs correlated to insulin sensitivity and adiposity. Our findings warrant future studies to characterize exercise-induced extracellular vesicles and cargo miRNA to clarify where exercise-induced extracellular vesicles originate from, and to determine whether they influence metabolic health or exercise adaptation.

## KEYWORDS

dysglycemia, exercise, extracellular vesicles, miRNA, overweight

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## 1 | INTRODUCTION

A sedentary lifestyle is one of the leading contributors to premature death worldwide.<sup>1</sup> Physical activity promotes numerous health benefits and is part of the WHO guidelines for preventing and managing noncommunicable diseases.<sup>2</sup> The underlying mechanisms by which physical activity improves health are not fully understood. Tissue crosstalk induced by a single exercise bout (acute exercise) or long-term exercise may play an important role, for instance via myokines secreted from skeletal muscle.<sup>3</sup> More recently, extracellular vesicles (EVs) induced by exercise have emerged as potential mediators of tissue crosstalk.<sup>4</sup> However, carefully designed experiments are needed to understand how these EVs are released, where they come from, and what their biological functions are.

EVs are heterogeneous, lipid bilayer-bound particles released by cells to the extracellular environment. There are discrepancies in the classification of different EV subsets, but it is common to divide EVs released by living cells into exosomes (50–150 nm) and microvesicles (50–1000 nm).<sup>5</sup> It was also recently suggested to divide EVs into small (<200 nm) and large EVs.<sup>6</sup> EVs are mainly released by direct budding from the plasma membrane (microvesicles) or by fusion of multivesicular bodies with the plasma membrane (exosomes). It is currently difficult to purify specific subsets of EVs, and there is a lack of specific markers to distinguish subsets of EVs. Thus, the International Society for Extracellular Vesicles recommends using ‘*extracellular vesicle*’ as the generic term.<sup>6</sup>

All cells are likely capable of producing EVs.<sup>5</sup> During the last five decades, EVs have been found in different body fluids and their concentration has been shown to change in some pathological conditions such as obesity, metabolic syndrome,<sup>7</sup> and cardiovascular disease.<sup>8</sup> Thus, they may have potential use as biomarkers.

EVs are well suited as players in cell–cell and tissue–tissue communication.<sup>5,9</sup> They have surface molecules that allow them to target specific cells,<sup>10</sup> and they carry molecules with signaling abilities, such as proteins, metabolites, and nucleotides like miRNAs. Once attached to recipient cells, EVs can influence biological processes by inducing cell signaling at the plasma membrane or after internalization and delivery of cargo. EVs are implicated in a wide range of biological or pathological processes such as coagulation,<sup>11</sup> immune responses,<sup>12</sup> cancer metastasis,<sup>10</sup> and insulin resistance.<sup>13</sup>

Circulating levels of EVs are increased in mice and humans with obesity,<sup>7</sup> and EV miRNAs may influence metabolic processes in distant tissues. For instance, miRNAs originating from adipose tissue can influence hepatic gene expression.<sup>14</sup> Furthermore, EVs secreted from macrophages in adipose tissue of obese mice can reduce insulin

sensitivity in liver and skeletal muscle.<sup>13</sup> This effect was attributed to the content of miR-155 and its ability to regulate PPAR $\gamma$  expression.

In this study, we characterized the miRNA content in plasma EVs from dysglycemic and normoglycemic men with or without a high BMI undergoing acute and long-term exercise. Importantly, there is no agreement regarding the optimal method for EV purification and a challenge in the EV field is the poor reproducibility across studies due to the use of different EV purification methods.<sup>6</sup> Thus, we used two different purification workflows utilizing membrane affinity (MEM) columns or size exclusion chromatography (SEC). We demonstrate that many miRNAs are upregulated in plasma by acute exercise. Some of these miRNAs correlate with insulin sensitivity, whereas others correlate in the resting state with an adipose tissue macrophage signature.

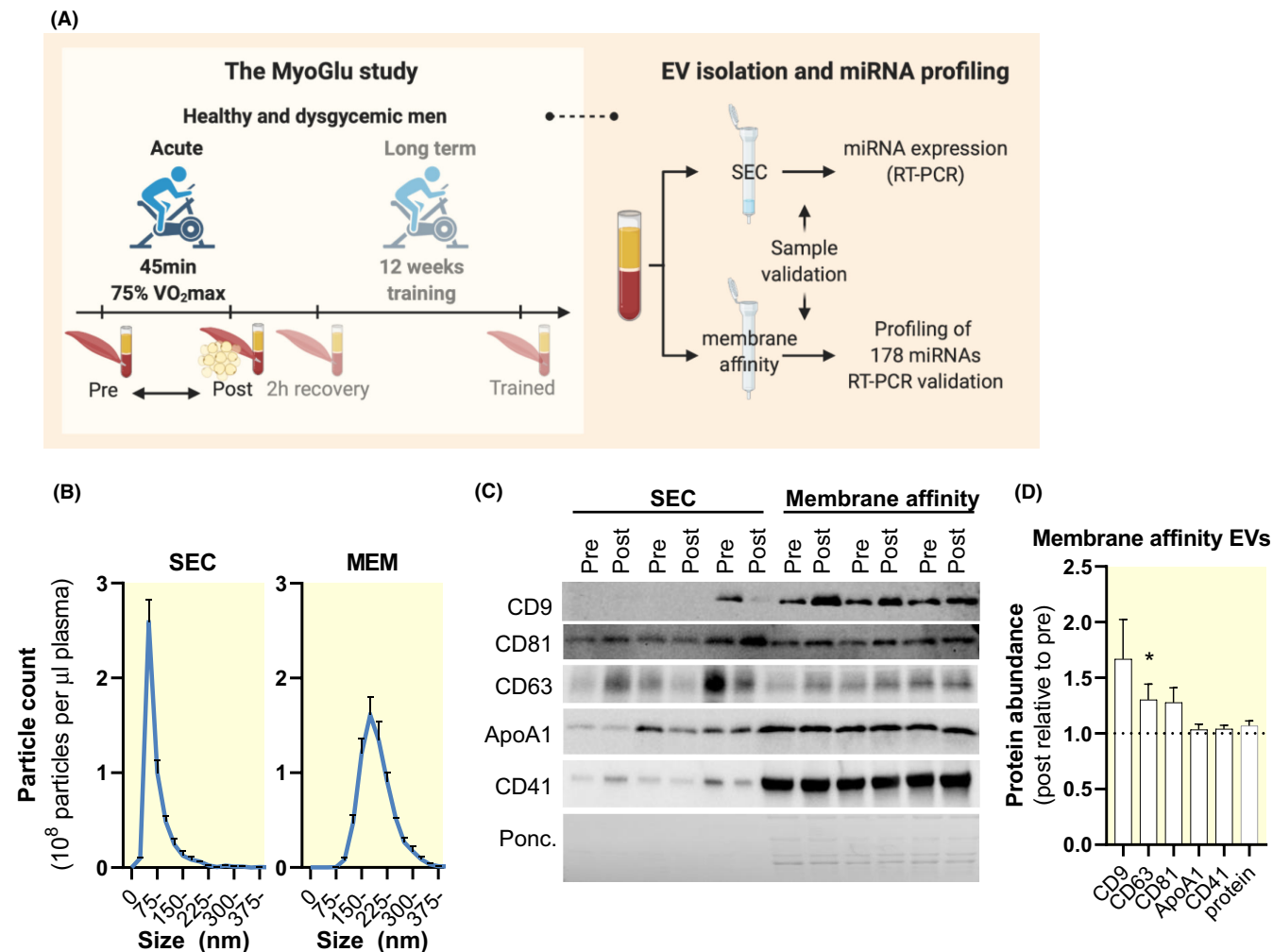
## 2 | RESULTS

### 2.1 | Purification of plasma EVs with SEC or membrane affinity columns

We utilized plasma samples from the MyoGlu exercise intervention study (Figure 1A). Normo- or dysglycemic men underwent an acute exercise bout or 12 weeks of combined endurance and strength training. EVs were isolated from plasma with either SEC or MEM columns, preceded by different pre-clearing protocols.

Nanoparticle tracking analysis (NTA) of EV isolates revealed that the SEC purification workflow resulted in particles that were 50–125 nm in diameter (mode size = 62 nm; Figure 1B). We also measured particle concentration pre and post acute exercise but did not find any significant differences (Figure S1). However, it is important to keep in mind that nanoparticle tracking does not distinguish between EVs and other types of particles like lipoprotein particles or protein aggregates that may be present in the sample.<sup>15,16</sup> Thus, the use of NTA alone for the quantification of EVs in plasma has limitations.

To investigate the presence of common proteins of small EVs purified pre- and post-acute exercise, we measured the abundance of CD9, CD63, and CD81<sup>17</sup> with immunoblotting (Figure 1C,D). EV isolates generated with SEC contained CD63 and CD81, while CD9 was only detected in samples from one of the three participants. Furthermore, SEC-generated samples contained CD41 and ApoA1, indicating the presence of platelet-derived particles and high-density lipoprotein particles. This was expected, as it is difficult to avoid contamination with other types of particles with currently available technology.



**FIGURE 1** Purification of plasma EVs before and after acute exercise. (A) Blood samples were taken pre and post an acute exercise bout (45 min, 70% VO<sub>2</sub>max), at 2 h recovery and after 12 weeks of combined endurance and strength training. Biopsies from adipose tissue and skeletal muscle were taken at indicated time points. Plasma EVs were isolated with size exclusion columns (SEC; qEV 35 nm) or membrane affinity columns (MEM). We profiled the expression of 178 miRNAs in MEM-purified EVs with a RT-PCR-based panel pre- and post-acute exercise ( $n = 13$ ). We further measured the expression of selected miRNAs in EVs isolated with MEM in a larger sample subset at all timepoints ( $n = 19$ ). Lastly, we measured miRNA expression in EVs isolated with SEC pre- and post-acute exercise ( $n = 13$ ). (B) Nanoparticle tracking analysis (NTA) of EVs purified with MEM and SEC. (C) Immunoblotting of EV samples pre- and post-acute exercise, isolated with SEC or MEM. (D) Quantification of CD9, CD63, CD81, ApoA1, CD41, and total protein in MEM-purified EV samples, expressed as post relative to pre acute exercise. \* $p$ -value < 0.05, paired, one-sided  $t$ -test.

EV isolates generated with MEM columns contained particles between 150 and 250 nm (mode size = 190 nm) and were positive for CD9, CD81, and CD63 (Figure 1C). Furthermore, the three tetraspanins tended to increase after acute exercise (Figure 1D), but only CD63 reached statistical significance ( $p = 0.04$ ).

EV isolates from MEM columns had a high content of CD41 and ApoA1. Importantly, neither ApoA1 nor CD41 was increased after exercise (Figure 1D), indicating an exercise response that was specific to tetraspanin-containing vesicles. Lastly, MEM samples had a high protein abundance (Figure 1C), as estimated by the Ponceau protein stain of membranes. The strongest protein band had a

molecular weight of approximately 70 kDa, possibly corresponding to serum albumin. Although plasma EVs contain several different proteins,<sup>4</sup> the high protein content is probably due to the presence of plasma proteins or other particles.

## 2.2 | EV miRNAs are transiently increased in plasma after acute exercise

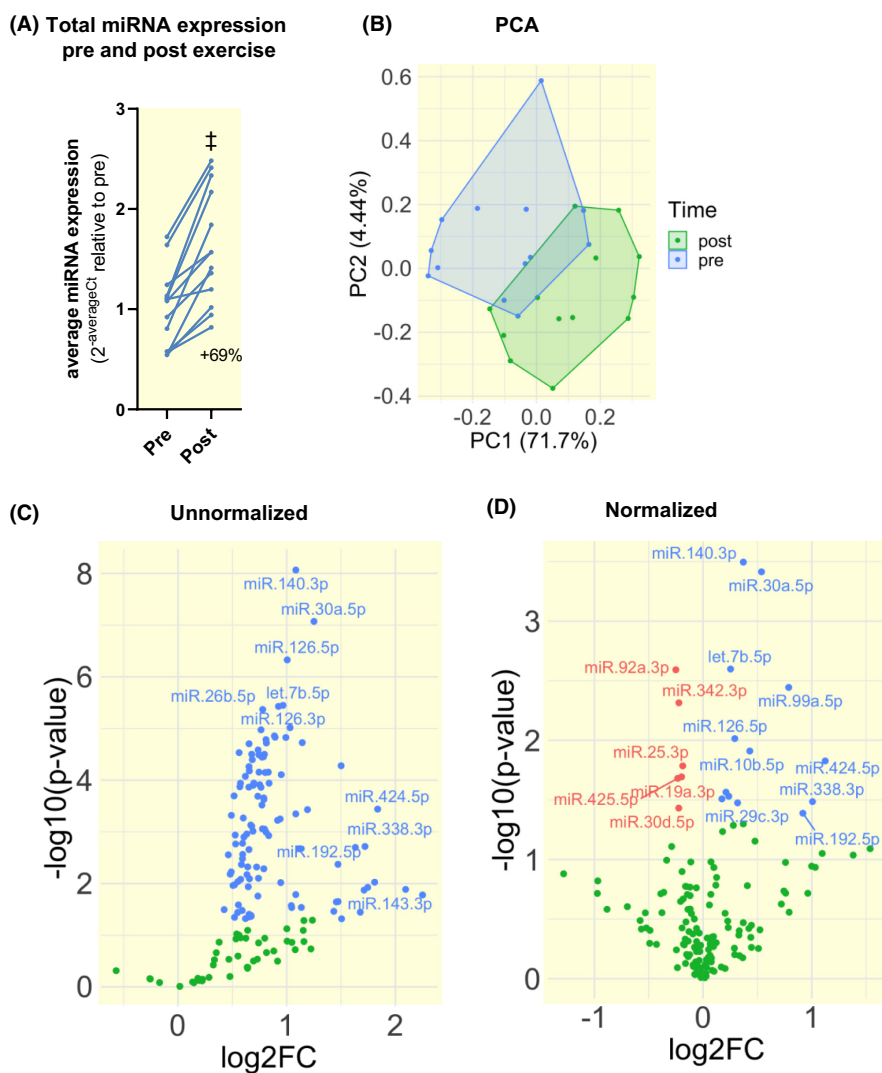
As exercise seems to promote the release of EVs into the circulation, we profiled the miRNA content of EV isolates before (pre) and immediately after (post) an acute exercise

bout. Exercise increased the content of tetraspanins in MEM-purified EVs (Figure 1C,D), and we, therefore, profiled expression levels of 178 miRNAs with an RT-PCR-based panel in these samples. On average, 143 miRNAs were detected per sample, whereof 86 miRNAs were detected in all samples.

The overall miRNA content in plasma EV isolates increased by almost 70% after acute exercise (Figure 2A), calculated as average expression levels of the 86 miRNAs detected in all samples. Furthermore, principal component analysis (PCA) revealed a separation of pre- and post EV samples, clearly demonstrating altered miRNA expression after exercise (Figure 2B). To identify specific miRNAs affected by exercise, we performed differential gene expression analyses on both unnormalized (Figure 2C) and normalized data (Figure 2D). An acute exercise bout induced the overall expression of miRNAs in plasma EVs, with a large proportion of miRNAs being significantly increased (Figure 2C). To identify miRNAs that were enriched post-exercise, we normalized the miRNA expression values by adjusting for the global average expression

of the 86 miRNAs expressed in all samples. In total, 13 miRNAs were significantly enriched by exercise, including miR-30a-5p, miR-140-3p, let-7b-5p, miR-99a-5p, miR-126-5p, miR-10b-5p, miR-424-5p, miR-338-3p, miR-29c-3p, miR-222-3p, miR-126-3p, miR-145-5p, and miR-192-5p (Figure 2D). When correcting for multiple testing, only miR-30a-5p and miR-140-3p had a false discovery rate (fdr) below 0.05. Nevertheless, the normalized data do not reflect absolute abundance, as the samples were normalized to a total miRNA expression that increased after exercise.

A single bout of exercise leads to acute metabolic adaptations, whereas in the long run, several repeated bursts of exercise will induce sustained adaptations and result in the reduced risk of chronic diseases.<sup>18,19</sup> Some of the 13 miRNAs significantly enriched by acute exercise, and a few that were not regulated, but highly expressed in muscle,<sup>20</sup> were investigated further. In total, we measured the expression of 12 different miRNAs (miR-206, miR-222-3p, miR-23a-3p, miR-30a-5p, miR-32-5p, miR-339-p, miR-484, miR-486-3p, miR-652-3p, miR-92a-3p,



**FIGURE 2** Acute exercise leads to the release of EV-associated miRNAs. Profiling of 178 miRNAs in plasma EVs purified with MEM. Expression was measured with an RT-PCR-based panel. (A) Average miRNA expression, calculated as  $2^{-\text{Ct}}$ , pre- and post 45 min cycling,  $^{\ddagger}p < 0.001$ . (B) Principal component analysis of the miRNA expression in plasma EVs pre- and post-exercise. (C, D) Volcano plot of differential miRNA expression post-versus pre-acute exercise. Differential expression was either calculated from unnormalized Ct values (C), or from Ct values normalized to the expression of the 86 miRNA that was expressed in all samples. miRNAs that were significantly increased (blue) or decreased (red) are indicated ( $p < 0.05$ ).  $N = 13$ .

miR-99a-5p, and miR-99b-3p) in EVs after both acute exercise and long-term exercise with RT-qPCR. Expression of these miRNAs was measured in a larger subset of samples ( $n = 19$ ), both pre- and post-exercise, after 2 h recovery, and after 12 weeks of long-term exercise. First, we confirmed that acute exercise increases the expression of several miRNAs in plasma EVs (miR-10b-5p, miR-222-3p, miR-23a-3p, miR30a-5p, miR484, miR-652-3p, and miR-92a-3p, miR991-5p; **Figure 3A–K**), with levels returning back to baseline after 2 h of recovery. However, we did not observe an increase in the same selected miRNAs after the 12-week exercise intervention (**Figure 3M**), underlining that these miRNAs are transiently increased in the circulation in response to acute exercise, but not after a 2 h recovery or long-term training.

### 2.3 | Analysis of selected miRNAs in EVs isolated by SEC

There are known problems with poor reproducibility between different EV isolation methods,<sup>6</sup> and we, therefore, used two different EV purification workflows in this study (SEC and MEM; **Figure 1**). Thus, the expression of the 12 miRNAs that were investigated pre- and post-acute exercise in MEM isolates was also analyzed in EVs purified with SEC (**Figure 4A–J**).

Acute exercise significantly increased the expression of 5 miRNAs (miR-10b-5p, miR-222-3p, miR-30a-5p, miR-339-3p and miR-99a-5p) in SEC-purified EVs (**Figure 4A,B,D,F and G**, respectively). Thus, there was some agreement in measured expression levels between the two methods used for EV isolation. However, miR-339-3p, miR-486-3p, and miR-99b-3p miRNAs were only detected in 12, 9, or 2 out of the total 26 SEC-isolated samples, respectively.

### 2.4 | Exercise-induced EV miRNAs probably originate from multiple cell types

Next, we focused our analysis on the 13 miRNAs particularly enriched in exercise-induced EVs isolated with MEM. To investigate the tissue- and cell-specific expression of these miRNAs, we utilized cell ontology data from the Fantom5 miRNA expression atlas.<sup>20–22</sup> Four of the miRNAs (miR-338-3p, miR-29c-3p, miR-99a-5p, and miR-140-3p) are highly expressed in leukocytes, mast cells, and hematopoietic cells (**Table S1**). Another four miRNAs (miR-126-3p, miR-30a-5p, miR-126-5p, and miR-222-3p) are enriched in endothelial cells, and two miRNAs (miR-10b-5p, miR-192-5p) are highly expressed in various epithelial cell types. Lastly, miR-145-5p and miR-424-5p are

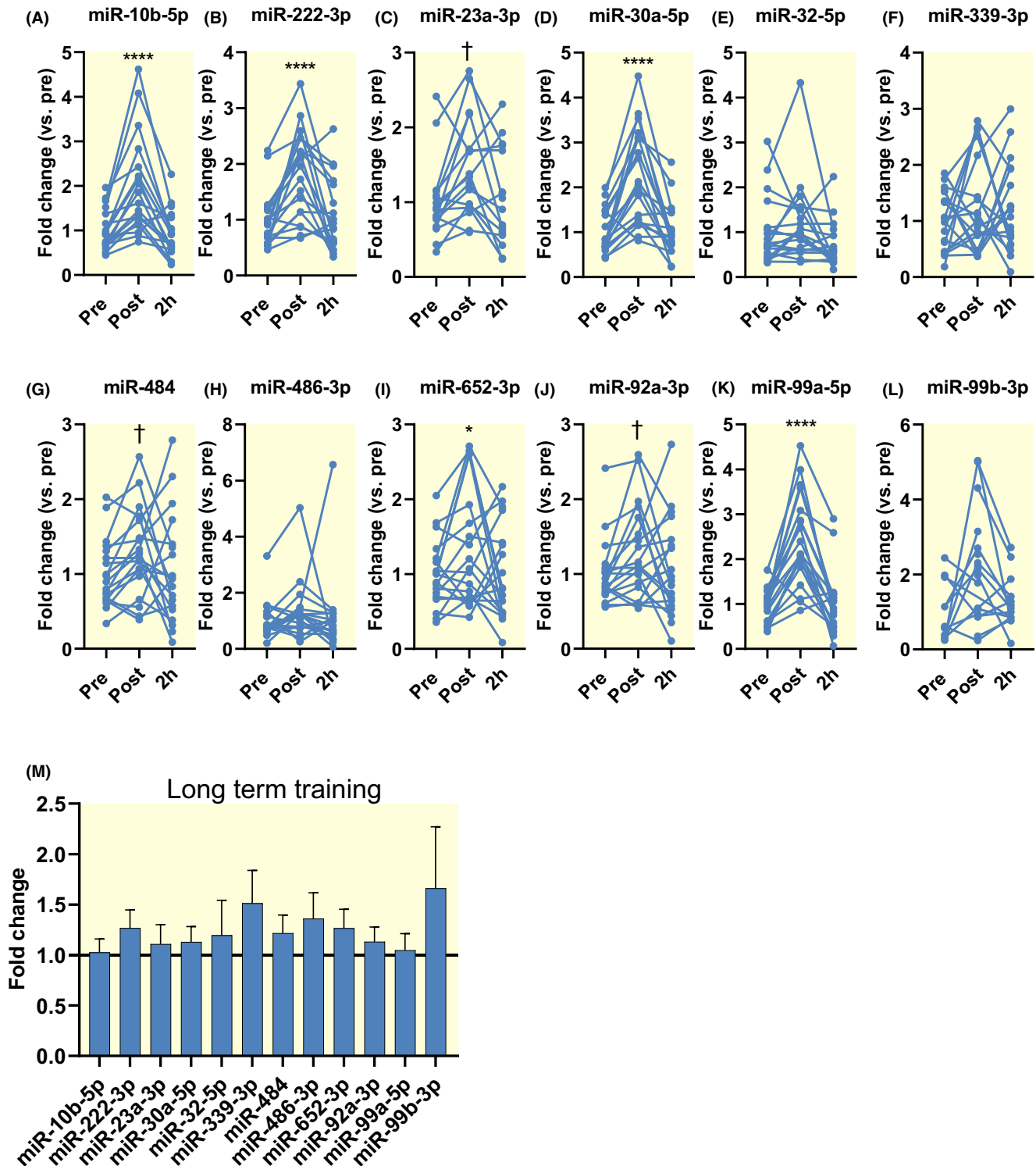
highly expressed in muscle cells. It is, therefore, possible that circulating exercise-induced EVs originate from various cell types, such as immune cells, endothelial cells, epithelial cells, and muscle cells. In addition, extracellular miRNA are also known to originate from platelets.<sup>23</sup>

### 2.5 | Exercise-induced EV miRNA and skeletal muscle

Skeletal muscle has emerged as an important secretory organ, especially in response to acute exercise,<sup>3,24</sup> inducing numerous adaptation processes in muscle tissue.<sup>19</sup> To study the relationship between exercise-induced EV-miRNA responses (post vs. pre) and skeletal muscle mRNA response (post vs. pre), we performed a partial least squares (PLS) regression analysis (**Figure 5**). In particular, two miRNA clusters (indicated in blue and red; cluster miRNAs are listed in the figure legend) displayed correlations to alterations in the skeletal muscle transcriptome after exercise. The miRNA clusters correlated positively or negatively with two mRNA clusters in skeletal muscle. To identify biological pathways associated with these mRNA clusters we performed a KEGG pathway enrichment analysis. The blue mRNA cluster (**Figure 5B**) was enriched in genes related to ubiquitin-mediated proteolysis, branched-chain amino acid degradation, and several metabolic pathways. The red cluster (**Figure 5C**) was enriched in transcripts related to the ribosome and focal adhesion. This suggests that some of the exercise-induced alterations in EV-miRNA content are related to metabolic processes in skeletal muscle or ribosomal or focal adhesion pathways. The detailed results of the KEGG enrichment analysis, including mRNAs in each cluster, are displayed in **Table S2A,B**.

### 2.6 | Dysglycemia and adiposity associated with the expression of specific EV miRNAs

The concentration of circulating EVs is increased in metabolic diseases such as type 2 diabetes, hypertension, and obesity.<sup>25</sup> Thus, we investigated whether EV miRNAs were differentially expressed in the dysglycemic versus the normoglycemic group. The participants in the dysglycemic group had significantly increased body weight and BMI, and reduced insulin sensitivity measured as glucose infusion rate (GIR) during euglycemic hyperinsulinemic clamp (**Table S3**). Age and  $VO_2\max$  were similar between the groups (**Table S3**). The groups were compared by analyzing data obtained from miRNA

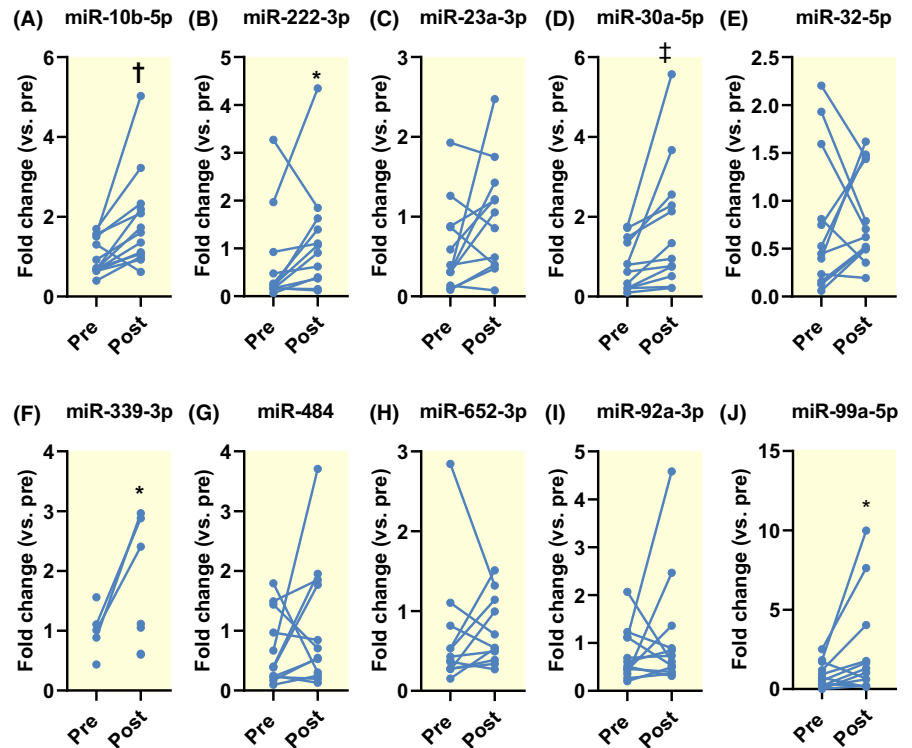


**FIGURE 3** The exercise-induced increase in EV miRNAs is transient. Plasma EVs were purified with membrane affinity (MEM) columns. miRNA expression was measured with RT-qPCR. (A–L) miRNA expression pre- and post acute exercise, and after 2 h recovery, expressed relative to the average expression at pre. (M) miRNA expression after 12 weeks training, relative to before ( $n = 19$ ). Statistical testing was done with a paired  $t$ -test, pre- versus post-exercise. \* $p < 0.05$ , † $p < 0.1$ , \*\*\*\* $p < 0.001$ .

profiling of MEM-purified EVs at pre-exercise (Figure 6). There was no clear separation of the miRNA expression pattern of normo- and dysglycemic participants on the PCA plot (Figure 6A). We further analyzed differential

miRNA expression (Figure 6B) and identified four miRNAs that were decreased and one miRNA that was increased in dysglycemic, as compared to normoglycemic men. However, only miR-652-3p remained significantly

**FIGURE 4** Effect of acute exercise on the miRNA in EVs isolated by SEC. (A–J) Plasma EVs were purified from plasma pre- and post exercise with SEC. miRNA expression was measured with RT-qPCR.  $N = 13$ . Statistical testing was done with a paired  $t$ -test, pre- versus post-exercise. \* $p < 0.05$ , † $p < 0.1$ , ‡ $p < 0.001$ .



reduced in dysglycemic participants after adjustment for multiple testing ( $\text{Log}_2\text{FC} = -0.97$ ,  $p = 1.2 \times 10^{-5}$ ,  $\text{fdr} = 0.0016$ ).

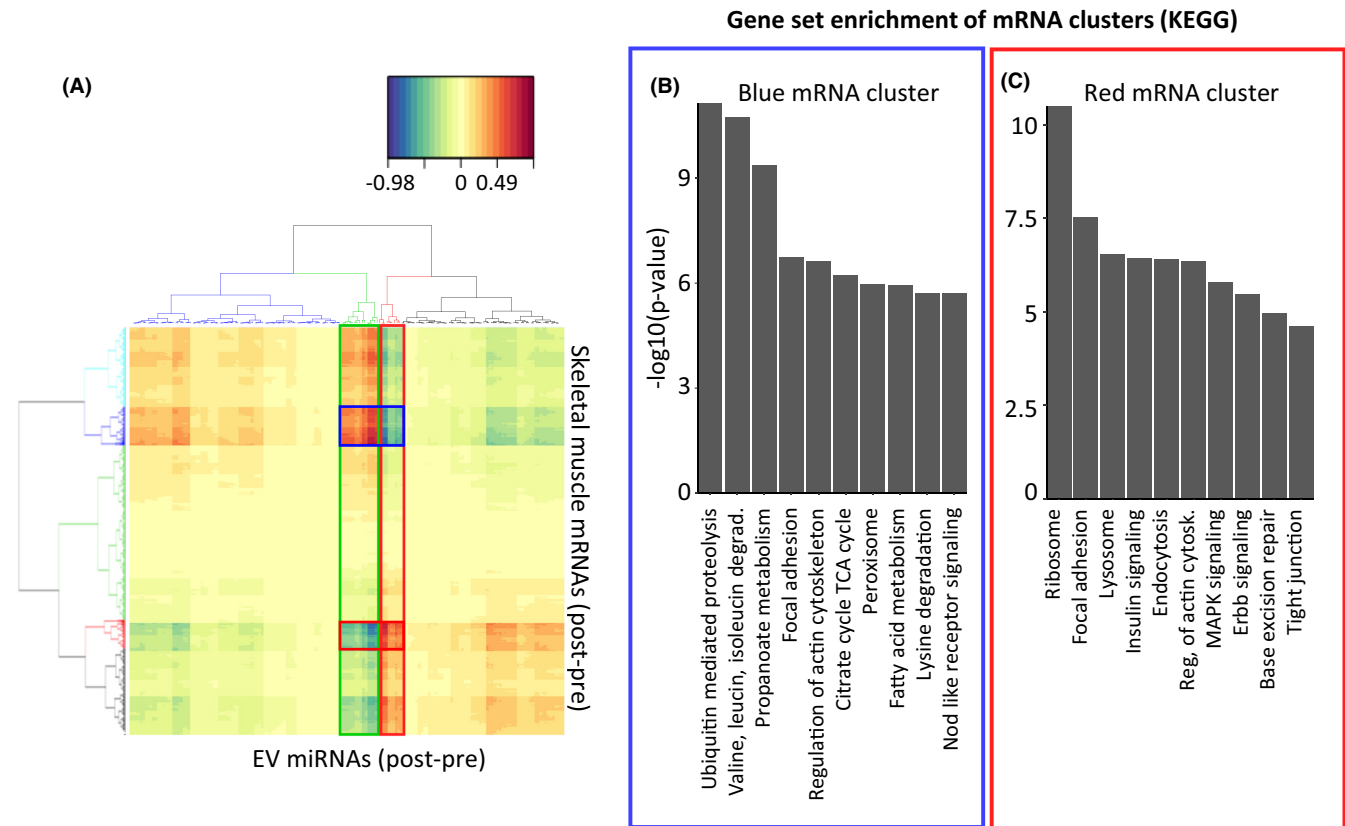
We next performed a PLS regression analysis to identify clusters of miRNAs correlating with metabolic risk factors (Figure 6C). Particularly one cluster (including miR-652-3p, miR-423-3p, miR-23a-3p, miR-23b-3p, and miR-27b-3p) displayed a strong positive correlation with insulin sensitivity, and a negative correlation to adiposity.

## 2.7 | miR-32-5p and miR-339-3p are associated with a macrophage signature in adipose tissue

Thomou et al. reported that a large proportion of miRNAs in EVs in mice originate from adipose tissue and adipocytes.<sup>14</sup> In another study, EVs secreted from macrophages in adipose tissue were shown to influence insulin sensitivity in liver and skeletal muscle.<sup>13</sup> Thus, we investigated the relationship between miRNAs in plasma EVs and the adipose tissue transcriptome. First, we performed a PLS regression analysis on miRNA expression in MEM-purified EVs and adipose tissue mRNA expression at baseline (Figure 7A). We identified several miRNA clusters that correlated to the adipose tissue transcriptome. One miRNA cluster (red cluster containing the following miRNAs: miR-885-5p,

miR-501-3p, miR-200a-3p, miR-32-5p, and miR-375) was positively associated with an adipose tissue cluster enriched in mRNAs involved in valine/isoleucine degradation, citric acid cycle, fatty acid metabolism, and oxidative phosphorylation (Figure 7B). The same miRNA cluster was negatively associated with a mRNA cluster enriched in inflammatory mRNAs (Figure 7C). A second miRNA cluster (a cyan cluster containing the following miRNAs: miR-34a-5p, miR-483-5p, miR-23a-3p, miR-629-5p, miR-24-3p, miR-339-3p, miR-15b-3p, miR-423-3p, miR-23b-3p, miR-26a-5p, miR-2110) was correlated to the same mRNA clusters as mentioned above, but in the opposite direction. The results of the KEGG enrichment analysis, including mRNAs in each cluster, are displayed in Table S2C,D.

Finally, to identify specific miRNAs in plasma EVs that may function as biomarkers for adipose tissue inflammation or metabolism, we studied the miRNAs identified above in more detail. Interestingly, miR-32-5p (red miRNA cluster) displayed a strong positive correlation to the expression of macrophage markers in adipose tissue (Figure 7D), and a strong negative association with the content of mitochondrial DNA. Furthermore, miR-339-3p (cyan miRNA cluster), was negatively correlated to macrophage mRNA expression (Figure 7F) and positively correlated to mitochondrial mRNA expression (Figure 7G;  $p = 0.07$ ). Thus, EV miR-32-5p and miR-339-3p are candidate biomarkers for adipose tissue inflammation and metabolism.



**FIGURE 5** Associations between acute, exercise-induced changes in EV-miRNA expression and muscle mRNA expression. (A) Partial least squares analysis between changes (post-pre 45 min cycling) in EV miRNA levels and muscle mRNA levels. The column dendrogram depicts the clustering of EV miRNAs (here colored by 4 main clusters) and the row dendrogram depicts clustering of muscle mRNAs (colored by 5 main clusters). Red represents positive correlations and blue signifies negative correlations. The green miRNA cluster contains miR-877-5p, miR-7-5p, miR-362-3p, miR-205-5p, miR-34a-5p, miR-27a-3p, miR-374a-5p, miR-29b-3p, miR-199a-5p, miR-324-5p, miR-27b-3p, miR-26b-5p, miR-335-3p, miR-193a-5p and miR-32-5p. The red miRNA cluster contains miR-141-3p, miR-532-5p, miR-338-3p, miR-92a-3p, miR-339-5p, miR-328-3p, miR-375, miR-629-5p and miR-2110. (B, C) KEGG enrichments in mRNA clusters (blue (B) and red (C)) that correlated to the green and red miRNA cluster.

### 3 | DISCUSSION

In this study, we purified EVs from plasma before and after acute and long-term exercise, in both normo- and dysglycemic men with or without a high BMI. This study has enabled us to perform exploratory analyses of EV miRNA expression, tissue mRNA expression, and various metabolic risk factors. EVs were isolated with SEC and MEM, which are currently two of the most frequently used techniques for EV purification. This allowed us to measure miRNA expression in different EV populations, which to our knowledge, has not been done in relation to exercise.

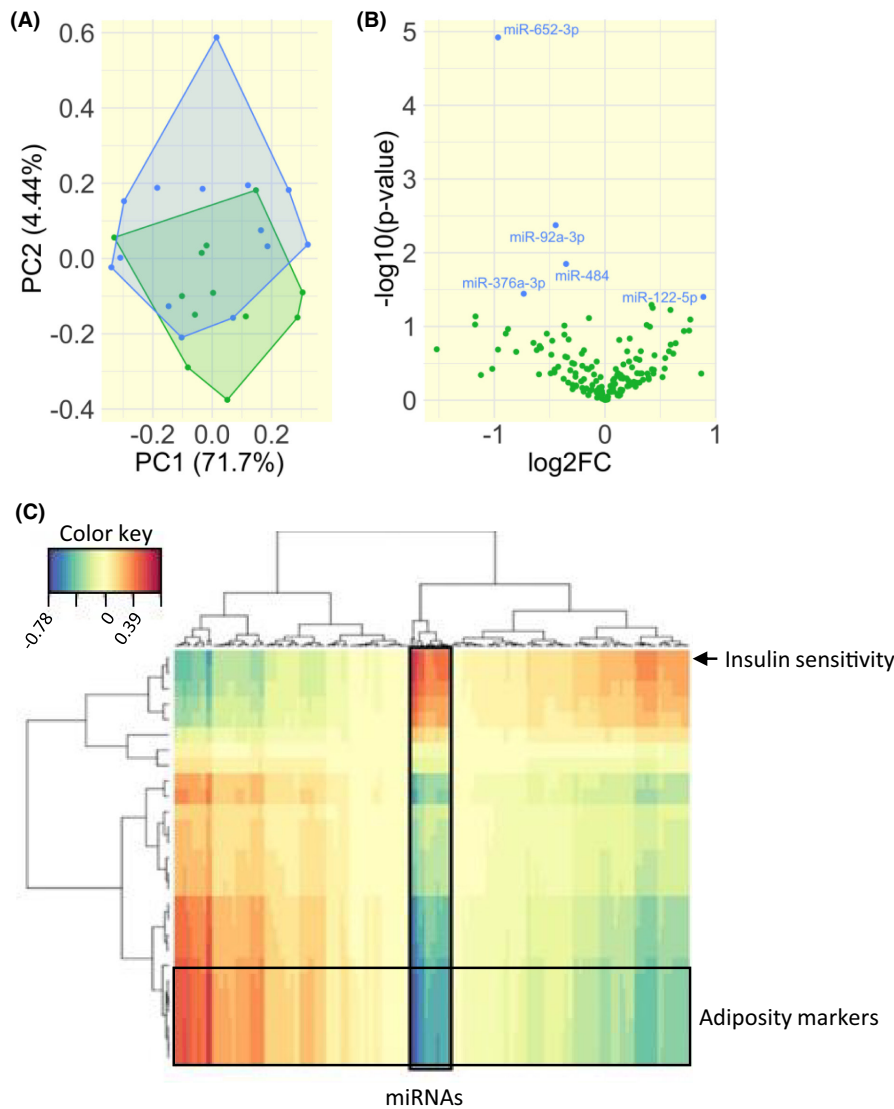
#### 3.1 | Exercise-induced EVs in tissue crosstalk

We first investigated acute exercise-induced alterations in circulating EVs. Several studies have reported

that acute exercise promotes an immediate release of EVs into the circulation.<sup>4,26-31</sup> We observed an exercise-induced increase in the EV tetraspanin CD63 in MEM-purified samples and a tendency for increased CD9 and CD81. However, we did not observe an increased particle concentration with the NTA method, which may be due to the presence of other particles such as protein aggregates and/or lipoprotein particles that mask the EV signal. This is in accordance with a comprehensive investigation by Brahmer et al.<sup>32</sup>, showing that exercise led to the release of EVs when measured by multiple marker-based analyses, but not if measured with nanoparticle tracking.

The cellular origin of exercise-triggered EVs is unclear. Exercise may stimulate the release of microparticles from platelets, possibly due to shear stress and/or activation of coagulation processes.<sup>30,33,34</sup> Such EVs seem to have pro-angiogenic potential.<sup>34</sup> Brahmer et al. detected exercise-induced EVs originating from platelets, endothelial cells, and leukocytes by measuring cell-specific surface



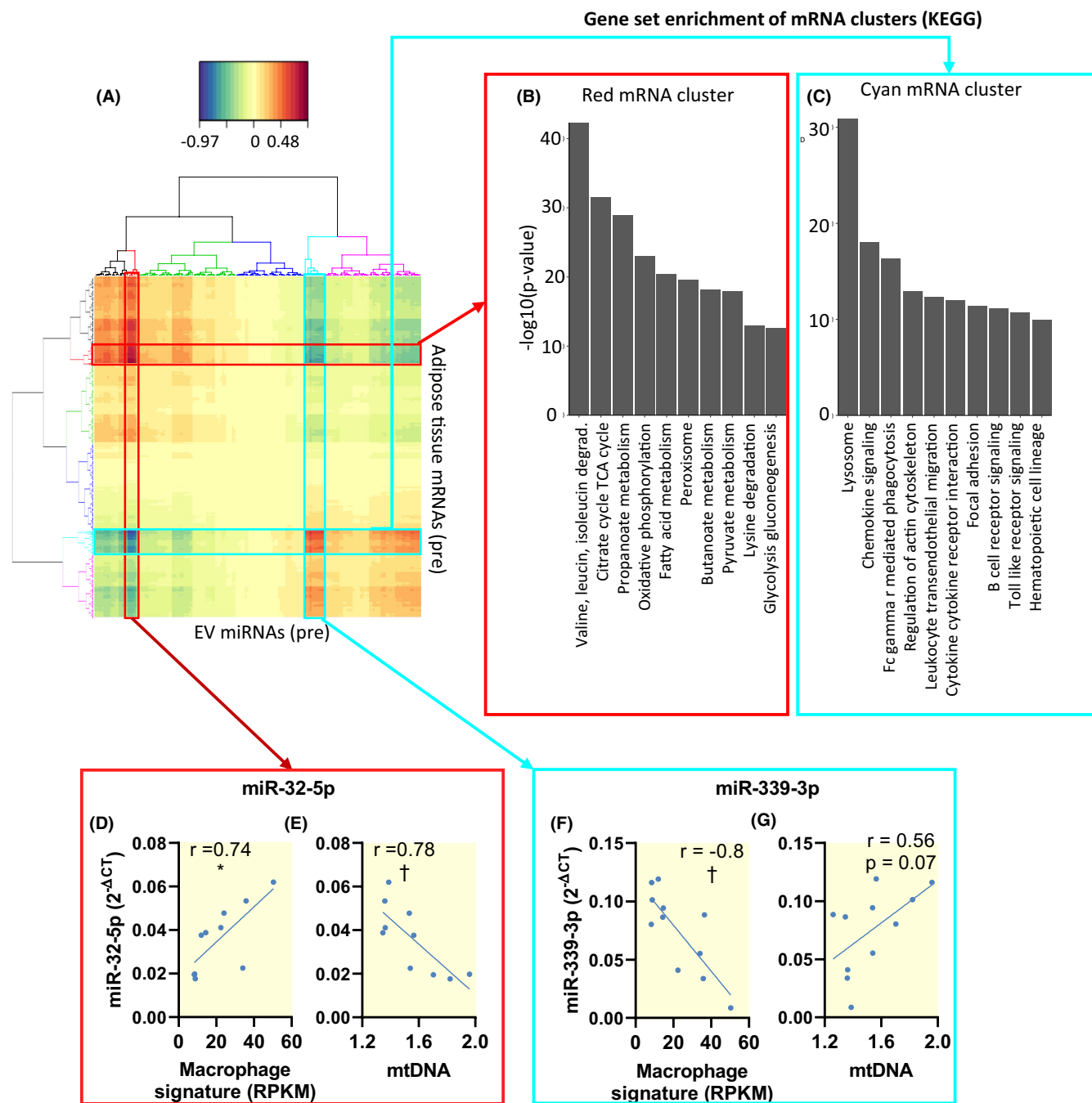


**FIGURE 6** EV miRNAs in the resting state were associated with dysglycemia and adiposity. EV-RNA was isolated from plasma of normoglycemic ( $n = 7$ ) and dysglycemic ( $n = 6$ ) men at the baseline of the study with MEM. The dysglycemic participants also had a higher BMI. (A) Principal component analysis of normalized miRNA expression at baseline (pre) in normoglycemic (blue) and dysglycemic (green) participants. (B) Differential expression of miRNAs in dysglycemic versus normoglycemic participants, at baseline. Significantly altered miRNAs ( $p < 0.05$ ) are indicated in blue. (C) PLS analysis of baseline EV-miRNA expression in relation to several clinical and physiological markers. The indicated miRNA cluster correlated negatively with adiposity markers (subcutaneous adipose tissue, fat volume, total adipose tissue, intra-abdominal adipose tissue, fat volume measured by magnetic resonance imaging, body weight, and body mass index) and positively with insulin sensitivity measured as glucose infusion rate during hyperinsulinemic-euglycemic clamp ( $\text{mg kg}^{-1} \text{min}^{-1}$ ).

markers.<sup>32</sup> Other studies have identified skeletal muscle as a source of exercise-triggered EVs.<sup>4,35</sup> In our study, we measured the platelet marker CD41, which indicates the presence of platelets or platelet-derived material in our samples. Importantly, CD41 was not increased in plasma after exercise. To get an indication of where specific EV miRNAs originate, we investigated cell ontologies of the miRNAs that were most enriched in EVs after exercise (Table S1). These miRNAs are highly expressed in immune cells, endothelial and epithelial cells, and muscle cells suggesting that exercise-induced EVs are produced and released from several cell types. However, whereas

some miRNAs display tissue-specific patterns, others are more widely expressed,<sup>20,36</sup> and the data should therefore be interpreted with caution.

A few previous studies in humans and rodents have investigated miRNA content in circulating EVs before and after exercise.<sup>26,37–41</sup> In the majority of these studies, only a few selected miRNAs have been analyzed,<sup>26,37,39,41</sup> of which some have been described as muscle-specific miRNAs (myo-miRs). One exception is a study by Just et al. sequencing the miRNA content in EVs purified before and 1 h after blood flow-restricted resistance exercise.<sup>38</sup> Unaltered tetraspanin levels and



**FIGURE 7** Associations between EV miRNA and mRNA in adipose tissue. (A) Partial least squares analysis between miRNA content in MEM-purified EVs in plasma and adipose tissue mRNA expression at baseline. The column dendrogram depicts the clustering of EV miRNAs (here colored by 6 main clusters) and the row dendrogram depicts clustering of muscle mRNAs (colored by 6 main clusters). Red represents positive correlations and blue signifies negative correlations, as indicated by the color key. Two miRNA clusters that were particularly correlated to adipose tissue mRNA expression are indicated in red and cyan. The red cluster contains the following miRNAs: miR-885-5p, miR-501-3p, miR-200a-3p, miR-32-5p, and miR-375. The cyan cluster contains the following miRNAs: miR-34a-5p, miR-483-5p, miR-23a-3p, miR-629-5p, miR-24-3p, miR-339-3p, miR-15b-3p, miR-423-3p, miR-23b-3p, miR-26a-5p, and miR-2110. (B, C) KEGG enrichments in adipose tissue mRNA correlated to the red and cyan miRNA clusters. The red miRNA cluster was positively and negatively correlated to the red and cyan mRNA cluster, respectively, whereas the opposite was the case for the cyan miRNA cluster. (D–G) Correlation between the plasma EV content of miR-32-5p and miR-339-3p against adipose tissue macrophage gene expression signature or mitochondrial DNA content at baseline. Statistical testing was done with a Pearson correlation test. \* $p < 0.05$ , † $p < 0.01$ .

particle counts indicated that the EV concentration was not increased in their study, possibly because the EV concentration had already returned to baseline 1-h post-exercise. However, the authors identified 12 miRNAs that were significantly altered. In our study, we detected the expression of 186 different miRNAs in MEM-purified EVs, whereof 86 miRNAs were detected in all samples. We also measured the expression of selected miRNAs in SEC purified EVs. Particularly, three miRNAs were robustly increased by exercise in both EV subsets; miR-222-3p, miR-10b-5p, and miR-30a-5p. Little is known about the function of these miRNAs. In a previous study, miR-222-3p was found to be increased in skeletal muscle, plasma, and plasma EVs after exercise.<sup>37</sup> miR-222-3p may regulate myoblast proliferation and myofiber formation<sup>42,43</sup> or insulin sensitivity in cultured muscle cells.<sup>44</sup> Interestingly, also miR-30a-5p and miR-10b-5p have been implicated in the regulation of muscle cell proliferation and/or differentiation.<sup>45,46</sup> However, miRNAs may influence a large number of different mRNA targets, and may therefore have multiple and context-dependent functions.

Skeletal muscle myofibers may account for around 5% of circulating EVs in mice.<sup>47,48</sup> Furthermore, EVs produced in muscle may have local effects. For instance, EVs from muscle stem cells have been shown to have a regenerative potential on aging muscle cells.<sup>49</sup> To explore whether the exercise-induced response in EV-miRNA expression was associated with skeletal muscle mRNA expression, we performed an exploratory PLS regression analysis. This led to the identification of miRNA clusters correlating with proteolysis and several metabolic pathways, as well as ribosomes and focal adhesion. However, these data should be interpreted with caution due to the low number of participants included in the analysis. Some of the miRNAs in these clusters were present at low levels in EVs, and it is unclear whether they are expressed in muscle, or whether they can influence muscle mRNA expression.

Exercise-induced EVs may also influence biological processes in distant tissues. Whitham et al. performed proteomics profiling of exercise-induced EVs and found an increase in more than 300 different proteins.<sup>4</sup> Furthermore, intravital imaging revealed that exercise-induced EVs have a more pronounced tropism to the liver. In future experiments, it would be valuable to compare circulating EVs and skeletal muscle miRNA expression levels and to investigate this muscle-liver axis. Furthermore, to prove the physiological relevance of miRNAs in EVs it is necessary to confirm cellular uptake; a recent study showed that miRNAs in EVs may not be efficiently taken up by target organs.<sup>50</sup>

### 3.2 | EV miRNAs, long-term training, and metabolic health

In our study, 12 weeks of combined endurance and strength training increased insulin sensitivity by approximately 40% (measured as glucose-infusion rate (GIR) during euglycemic hyperinsulinemic clamp) and reduced fat mass by almost 7%.<sup>51</sup> Studies in mice have suggested that long-term exercise may improve metabolic health via regulation of circulating EVs, with potential effects on adipose tissue browning,<sup>52</sup> improved insulin sensitivity, or a decline in liver steatosis.<sup>53</sup> Of the 12 miRNAs that were investigated in more detail, none were significantly changed compared to baseline after 12 weeks of exercise, arguing against an adaptive change in EV miRNAs in response to long-term exercise.

We next analyzed data generated from the RT-qPCR-based panel of 186 miRNAs to compare miRNA expression at baseline in dysglycemic versus normoglycemic participants with or without a high BMI, respectively. We identified miR-652-3p as a miRNA that was significantly reduced in plasma EVs from dysglycemic participants. miR-652-3p is highly expressed in immune cells, including leukocytes, hematopoietic cells, and myeloid leukocytes.<sup>20</sup> However, a slight correlation between circulating miR-652-3p and platelet count has also been reported, suggesting that circulating miR-652-3p may be of platelet origin and regulate coagulation.<sup>54</sup> Studies have described diverse roles for miR-652-3p in cancer, cardiovascular disease, and neurological disorders.<sup>55</sup> Interestingly, adipose tissue expression of miR-652-3p was associated with insulin sensitivity in subcutaneous adipose tissue of obese women, and overexpression of miR-652-3p in human adipocytes increased insulin sensitivity as measured by insulin-stimulated lipogenesis.<sup>56</sup> These results are in agreement with our study where we find reduced expression of miR-652-3p in dysglycemic compared to normoglycemic participants. In future experiments, it would be interesting to investigate whether miR-652-3p in EVs can have direct effects on insulin sensitivity in recipient tissues.

It has been shown that EV miRNAs originating from adipose tissue are able to alter hepatic mRNA expression.<sup>14</sup> Furthermore, EVs secreted from macrophages in adipose tissue are able to influence insulin sensitivity in the liver and skeletal muscle.<sup>13</sup> EVs from adipose tissue macrophages of obese mice caused insulin resistance when administered to lean mice. This was attributed to the content of miR-155, which may regulate PPAR $\gamma$  expression. Because miRNAs in EVs secreted from adipose tissue seem to influence metabolic health, we explored the relationship between EV miRNA and adipose tissue mRNA expression in our participants (Figure 7). We

identified two miRNAs, miR-32-5p, and miR-339-3p, that strongly correlated with a macrophage mRNA expression signature and mitochondrial DNA content in adipose tissue. miR-32-5p was positively and negatively correlated to macrophage mRNA expression and mitochondrial DNA content, respectively. miR-32-5p is highly expressed in different immune cells, including leukocytes, hematopoietic cells, and lymphocytes,<sup>20</sup> and might be an inflammatory marker. miR-339-3p was negatively associated with macrophage mRNA expression and positively associated with mitochondrial DNA gene expression in adipose tissue. Little is known about the function of this miRNA, but studies suggest that miR-339-3p may have anti-tumourigenic effects.<sup>57,58</sup>

### 3.3 | Methodological considerations

Although EVs were discovered a while ago, the EV research field is still young, and there are considerable methodological challenges. Reproducibility issues across studies seem to involve different EV isolation methods.<sup>6</sup> Furthermore, plasma is a complex biofluid containing different lipoparticles and EV populations, and it is difficult to purify specific EV subsets. The technical challenges and limitations when studying EVs in physical exercise were recently reviewed by Brahmer et al.<sup>33</sup> Different purification methods seem to enrich different EV populations of varying quantity and purity. We used different workflows consisting of different pre-clearing steps and MEM or SEC, which enabled us to study miRNA content in two different EV populations.

Stranska et al. previously compared MEM (Qiagen) and SEC (qEV) for purification of EVs from plasma.<sup>59</sup> In agreement with our study, they reported that MEM-purified samples contained larger particles and had higher levels of protein as compared to SEC-purified samples. The presence of protein aggregates and lipoprotein particles is unavoidable and is a challenge for down-stream applications such as NTA and determination of protein and nucleic acid content.<sup>33</sup> For instance, lipoprotein particles can carry RNA molecules and thereby may confound miRNA expression data. We detected ApoA1 in all MEM-purified samples, suggestive of lipoprotein-particle contamination in our EV samples, but at a concentration that was unaltered by acute exercise. Thus, it is reasonable to assume that the increase in specific miRNAs after acute exercise was not due to the presence of lipoprotein particles in our samples.

Others have used transmission electron microscopy on samples purified with MEM or SEC,<sup>59</sup> demonstrating fewer EVs in MEM-purified samples. This was accompanied with very low or absent levels of EV markers in

MEM-purified samples measured with immunoblotting.<sup>59</sup> In our study, however, we detected high and exercise-responsive levels of EV markers in MEM-purified samples and lower levels in SEC-purified samples. This is illustrative of the above-mentioned reproducibility issues in the field.

### 3.4 | Conclusion and future directions

We identified several miRNAs that were robustly increased after acute exercise in EVs purified with two different EV isolation methods. We also identified EV miRNAs, including miR-652-3p, that were associated with insulin sensitivity and adiposity. Lastly, we identified two EV miRNAs strongly associated with a macrophage mRNA expression profile in adipose tissue. Follow-up studies should investigate whether these miRNAs can influence gene expression, exercise adaptation or metabolism in recipient tissues. Furthermore, future studies should aim to investigate exercise-induced release of specific EV subsets.

## 4 | METHODS

### 4.1 | Exercise intervention study

Plasma samples and data generated from an exercise intervention study (MyoGlu; NCT01803568) were used for new analyses. The exercise intervention has been described in great detail elsewhere.<sup>60</sup> The study was approved by the National Committee for Research Ethics North, Tromsø, Oslo, Norway. All participants gave their written consent and the study adhered to the guidelines in the Declaration of Helsinki. In short, 26 sedentary (<1 bout of exercise per week) men aged 40–65 years with body mass index (BMI)  $26 \pm 4.0 \text{ kg}^{-1} \text{ m}^2$  were recruited. They were either normoglycemic or dysglycemic (fasting glucose  $\geq 5.6 \text{ mmol L}^{-1}$  and/or 2 h glucose  $\geq 7.8 \text{ mmol L}^{-1}$  based on an oral glucose tolerance test [OGTT]). Two of the subjects had normal glucose levels at the OGTT but were later defined as dysglycemic during an euglycemic hyperinsulinemic clamp (GIR  $4.4 \text{ mg min}^{-1} \text{ kg}^{-1}$ ). None of the participants had type 1 diabetes or medically treated type 2 diabetes. Participants with known hypertension, liver or kidney disease, chronic inflammatory disease, or on any medication expected to affect glucose metabolism (lipid-lowering, anti-hypertensive, ASA, corticosteroids, etc.) were excluded.

In this study, we pre-selected a subset of participants and compared dysglycemic ( $n = 6$ ) or normoglycemic ( $n = 7$ ) individuals. The dysglycemic group had a GIR during euglycemic hyperinsulinemic clamp

between 2.4–5 mg kg<sup>-1</sup> min<sup>-1</sup> and BMI 26.5–32.3 kg<sup>-1</sup> m<sup>2</sup>. The normoglycemic subset had a GIR between 5.3–9.8 mg kg<sup>-1</sup> min<sup>-1</sup> and BMI of 21.2–25.4 kg<sup>-1</sup> m<sup>2</sup>. Subject characteristics of these subsets are summarized in Table S3.

The participants underwent 12 weeks of supervised exercise intervention consisting of two whole-body strength training sessions (60 min) and two interval training sessions on an ergometer bicycle (60 min) weekly. An acute exercise test (70% VO<sub>2</sub>max, 45 min, cycling) was performed at baseline and after 12 weeks. The two exercise tests were performed in the morning and the participants consumed a standardized meal 90–120 min prior to exercise.

Biopsies from *m. vastus lateralis* were collected at 6 time points; before (pre), immediately after (post), and after 2 h after the end of both exercise sessions (before and after the 12-week exercise intervention). Biopsies from subcutaneous adipose tissue were collected from the peri-umbilical region 30–60 min after the two bicycle tests.<sup>60</sup> Biopsy procedures are described in detail elsewhere.<sup>60</sup>

Venous blood was collected at all 6 time points in EDTA tubes with standard antecubital venous puncture. Blood samples were centrifuged at 1400 g for 10 min at 4 °C, snap-frozen in liquid nitrogen, and stored at –80 °C. We focused our analyses on the effects of the first acute exercise bout and the 12-week exercise intervention (Figure 1A), meaning that we analyzed samples collected pre, post, and 2 h after the first exercise bout, and after 12 weeks training (in the resting state, before the second acute exercise bout).

Euglycaemic–hyperinsulinaemic clamp was performed as described previously.<sup>60</sup> Insulin sensitivity was reported as GIR (mg kg<sup>-1</sup> min<sup>-1</sup>) during the last 30 min of the clamp. The insulin sensitivity index was calculated as the ratio of GIR relative to the serum insulin level at the end of the clamp. Body composition was measured by magnetic resonance imaging/magnetic resonance spectrometry as described previously.<sup>60</sup>

## 4.2 | RNA isolation and sequencing

RNA from tissue biopsies was isolated and subjected to RNA sequencing as described in more detail elsewhere.<sup>24</sup> Biopsies were homogenized in QIAzol Lysis Reagent (Qiagen, Hilden, Germany), and RNA from muscle or adipose tissue was purified with the miRNeasy Mini or RNeasy Lipid Tissue Mini Kit (Qiagen), respectively. RNA integrity and concentration were determined using Agilent RNA 6000 Nano Chips on a Bioanalyzer 2100 (Agilent Technologies Inc, Santa Clara, CA). Deep sequencing was

performed with the Illumina HiSeq 2000 system. cDNA fragments with 51 bp nucleotides were selected and amplified. Reads were aligned against the UCSC hg19 annotated transcriptome and genome with Tophat 2.0.8 with Bowtie 2.1.0,<sup>61</sup> while EdgeR v3.4.2<sup>62</sup> was used for gene filtering, normalization, and calculation of *p*-values using a negative binomial generalized linear model in R v3.0.3 (R Core Team 2014).

To estimate the number of mitochondria in adipose tissue, we utilized the RNAseq data to calculate the mean RNA expression (RPKM) of the 37 mitochondrial genes. Similarly, to estimate the number of macrophages in adipose tissue, we calculated the mean RPKM of 15 validated gene markers (ACP5, CCL22, CD68, CD163, CHIT1, CRABP2, CSF1R, GLA, GM2A, IL1RN, LILRB4, LIPA, MRC1, MSR1, PLA2G7).<sup>63</sup>

## 4.3 | Isolation of EVs with membrane affinity columns

Non-hemolysed samples were used for EV purification, as measured by absorbance at 414 and 375 nm on a NanoDrop 1000 spectrophotometer (Thermo Fisher, Waltham, MA, US).

Prior to isolation of intact EVs or RNA from plasma EVs with MEMs, 500 µl plasma was thawed at room temperature, immediately mixed 1:1 with PBS, and centrifuged at 3200 g for 20 min to remove cells and large debris. A control sample with only PBS was included from the beginning of the isolation process. The upper 90% fraction of the plasma/PBS mixture was processed for EV isolation using exoEasy Midi spin columns from the exoRNeasy midi kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

To elute intact EVs from the MEMs, the membranes were incubated with 1 ml Buffer XE (Qiagen), centrifuged at 500 g for 5 min, and incubated again with the eluate. Samples were centrifuged on Amicon® Ultra-2 Centrifugal Filter Devices (molecular weight cutoff 10 K) to concentrate EVs.

## 4.4 | Isolation of EVs with size exclusion chromatography (SEC)

Plasma was thawed and immediately centrifuged at 1500 g for 10 min, and then at 10 000 g for 10 min. Plasma (150 µl) was then loaded to qEVsingle 35 nm SEC columns (IZON, Christchurch, New Zealand). After the void volume (1 ml), we collected a fraction of 600 µl for analysis. Isolated EVs were concentrated with Amicon® Ultra-2 Centrifugal Filter Devices (10 K MWCO, Merck Millipore,

Burlington, MA, US) and stored in low-RNA binding tubes.

#### 4.5 | Assessment of protein EV markers with immunoblotting

Concentrated EV samples were solubilized in Laemmli buffer (with DTT or without DTT as required for detecting the different tetraspanins) and separated on criterion TGX™ gels (BioRad, Hercules, CA, US). The sample volume was adjusted to achieve the same plasma volume per lane for SEC and MEM-purified EVs. Proteins were transferred to nitrocellulose membranes with the Trans-Blot Turbo transfer system and the RTA transfer kit (Bio-Rad). Membranes were stained with Ponceau S to visualize proteins and then blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% BSA. Membranes were incubated overnight with primary antibodies: CD63 (BD Biosciences, cat# 556019), CD9 (Abcam, cat# ab92726), CD81 (Adipogen Life Sciences, cat# ANC-302-020), CD41/Integrin  $\alpha$ 2b (Cell Signaling, cat# 13807) and ApoA1 (SantaCruz, cat# sc-376818). Chemiluminescence detection was performed on the ChemiDoc™ Touch Imaging System (Bio-Rad), and band intensities were quantified using ImageJ software (NIH, Bethesda, MD, US).

#### 4.6 | Nanoparticle tracking analysis

The concentration and size distribution of isolated EVs were measured by NTA. Purified plasma EVs were diluted in PBS (filtered through a 0.02  $\mu$ m Anotop 25 filter) to obtain a concentration within the recommended range ( $2 \times 10^8$ – $1 \times 10^9$  particles per ml) for analysis in a NanoSight NS500 instrument (Malvern Instruments, Worcestershire, UK). We also included the following negative controls: PBS, Buffer XE (Qiagen), and a mock control (PBS was loaded on ExoEasy columns as was done for plasma samples). Samples were vortexed for 1 min, then measured where five videos of 60 s were acquired for every sample. Videos were subsequently analyzed with the NTA 3.4 software (Malvern Panalytical, Worcestershire, UK), which identifies and tracks the center of each particle under Brownian motion to measure the average distance the particles move on a frame-by-frame basis.

#### 4.7 | RNA isolation from isolated EVs

To isolate RNA from EVs captured by MEM, we used the exoRNeasy midi kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). As an RNA

isolation control, QIAzol Lysis reagent (Qiagen) for all samples was spiked with synthetic RNA controls (UniSp2, UniSp4, UniSp5 from the Qiagen miRCURY® spike-in kit), according to the manufacturer's instructions. MaXtract High-Density tubes (Qiagen) were used for phase separation. The aqueous phase was mixed 1:2 with 100% ethanol and loaded into RNeasy MinElute spin column from the kit. EV miRNA was eluted from columns in 25  $\mu$ l water.

EVs were purified from plasma with qEV columns, concentrated (as described above), and homogenized in 700  $\mu$ l QIAzol Lysis Reagent spiked with synthetic RNA controls as for the MEM-purified samples. Phase-separation and RNA purification with RNeasy MinElute spin columns from the exoRNeasy midi kit (Qiagen) was done as described above.

#### 4.8 | miRNA profiling

RNA from MEM-purified EVs ( $n = 13$ ) was processed by Qiagen Genomic Services (Hilden, Germany). RNA was transcribed into cDNA and run on the miRCURY LNA miRNA PCR serum/plasma focus panel (Qiagen) including 183 miRNA targets. Additional controls included a RNA spike-in for reverse transcription (UniSp6) and a DNA spike-in for qPCR (UniSp3). As negative controls, samples without templates were included from the RNA isolation step as well as from the reverse transcription step. On average, we detected 143 miRNAs per sample, and 86 miRNAs were detected in all samples. To monitor hemolysis, the red blood cell-specific miRNA miR-451 was measured and expressed relative to miR-23a-3p. Normalization of miRNA expression was done against the global mean of the 86 miRNAs detected in all samples, which had higher stability than any single miRNA in the data set. For differential gene expression analyses, miRNAs that were undetected in more than 8 out of 13 samples, were excluded. Raw expression values for all miRNAs are found in Table S4.

#### 4.9 | miRNA validation with reverse transcriptase quantitative PCR (RT-qPCR)

For miRNA validation with RT-qPCR, RNA was reverse transcribed using a miRCURY LNA RT kit (Qiagen). RNA input was 2 and 4  $\mu$ l for MEM-purified or SEC-purified samples, respectively. Obtained cDNA was diluted at 1:50 with RNase-free water and RT-qPCR was performed using miRCURY SYBR® Green PCR Kit from Qiagen. Pre-designed miRCURY LNA PCR primer mixes (Qiagen) were used for hsa-miR-10b-5p, hsa-miR-155-3p, hsa-miR-222-3p, hsa-miR-23a-3p, hsa-miR-30a-5p,

hsa-miR-339-3p, hsa-miR-484, hsa-miR-486-3p, hsa-miR-652-3p, hsa-miR-652-3p, hsa-miR-92ab-5p, hsa-miR-99b-3p and Unisp2, Unisp4, Unisp5, and Unisp6. miRNA SYBR green signal was detected following the cycling program recommended by the manufacturer. miRNA expression was normalized to the expression UniSp6, and relative miRNA expression was calculated relative to the “pre” timepoint using the  $2^{-\Delta\Delta C_t}$  method.

#### 4.10 | Statistical analyses

Statistical analyses were performed in Graphpad prism version 8 or R version 4.0.3. Differential expression analyses were carried out on log scale values with student's *t*-test for paired or unpaired observations, or with repeated measures ANOVA for more than two timepoints, as indicated in figure legends. False discovery rates were calculated to control for multiple testing (Benjamini Hochberg, cut-off 0.05). Statistical testing of miRNA expression was done on normalized (Ct values normalized to the mean expression of 86 miRNAs detected in all samples) and unnormalized data (Ct values). PCA plots were generated with the `prcomp()` function in R using unnormalized Ct values. As previously described,<sup>64</sup> PLS analyses were performed using partial least squares and subsequent clustering using the `modk-prototypes` algorithm, which simultaneously considers both X and Y parameters.<sup>65</sup> Data were centered and scaled prior to analyses. KEGG enrichment analyses of discovered gene clusters were performed using KEGG pathways and hypergeometric tests.<sup>64,66</sup>

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#### CONFLICT OF INTEREST

The authors have no conflicts of interest to declare regarding the present manuscript.


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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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