Molecular and functional characterization of circulating extracellular vesicles from diabetic patients with and without retinopathy and healthy subjects

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Abstract

Diabetic retinopathy is a sight-threatening complication of diabetes, characterized by loss of retinal pericytes and abnormal angiogenesis. We previously demonstrated that extracellular vesicles (EVs) derived from mesenchymal stem cells cultured in diabetic-like conditions are able to enter the pericytes, causing their detachment and migration, and stimulating angiogenesis *in vitro*. The purpose of this work was the molecular and functional characterization of EVs derived from diabetic subjects with or without diabetic retinopathy, compared with healthy controls. Characterization of EVs extracted from serum/plasma of diabetic patients with or without retinopathy, and healthy controls, was performed by FACS and microarray analysis of microRNA (miRNA) content. Relevant miRNA expression was validated through qRT-PCR. EV influence on pericyte detachment, angiogenesis and permeability of the blood-retinal barrier was also investigated. Diabetic subjects had a 2.5 fold higher EV concentration than controls, while expression of surface molecules was unchanged. Microarray analysis revealed 11 differentially expressed miRNAs. Three of them (miR-150-5p, miR-21-3p and miR-30b-5p) were confirmed by qRT-PCR. Plasma EVs from subjects with diabetic retinopathy induced pericyte detachment and pericyte/endothelial cell migration, increased the permeability of pericyte/endothelial cell bilayers and the formation of vessel-like structures, when compared with EVs from controls. In conclusion, circulating EVs show differences between diabetic patients and healthy subjects. EVs extracted from plasma of diabetic retinopathy patients are able to induce features of retinopathy in *in vitro* models of retinal microvasculature. Our data suggest a role for miR-150-5p, miR-21-3p and miR-30b-5p as potential biomarkers of the onset of diabetic retinopathy.

1. Introduction

The pathogenesis of diabetic microvascular complications is closely linked to vessel abnormalities, due to altered interactions between pericytes and endothelial cells (ECs) (Raza et al., 2010). ECs are primarily exposed to the complex signalling from the blood flow and can influence pericytes, while pericytes transmit to the endothelium signals from neighbouring tissues (Armulik et al., 2005). This is further complicated by the *angiogenesis paradox* in diabetes: while diabetic retinopathy eventually leads to increasing hypoxia which stimulates abnormal neovascularization in the retina, potentially useful angiogenesis is inhibited in other ischaemic organs, such as the heart and limbs (Costa and Soares, 2013).

Therapeutic use of autologous/donor material is considered a potential option for the treatment of patients with multifactorial diseases. Extracellular vesicles (EVs) are released by different cell types in the vascular environment. EV surface antigens are specific of the donor cells and can help identify their origin. EVs have a regenerative potential, since they contain lipids, proteins, RNA and microRNAs (miRNA), and shuttle information that regulate the functions of target cells (Camussi et al., 2010). On the other hand, important pathophysiologic mechanisms associated with endothelial dysfunction in vascular disease (diabetes, atherosclerosis and hypertension), could be orchestrated by circulating EVs, or EVs from surrounding tissues acting in a paracrine way. As EVs can modulate vascular permeability, tone and angiogenesis, they might contribute to vascular complications, in particular diabetic retinopathy (Müller, 2012). EV secretion depends on the status of donor cells; therefore they could represent promising biomarkers in patients with metabolic diseases, such as type 2 diabetes(Müller, 2012). An increased concentration of circulating EVs has been reported in diabetic animals (Müller, 2012) and type 2 diabetic individuals (Koga et al., 2005; Feng et al., 2010; Helal et al., 2010).

MicroRNAs (miRNAs) are small non-coding sequences of 18-24 nucleotides, which interfere with stability and translation of target mRNAs by coupling with complementary sequences and thus exerting a negative regulatory effect (Fabbri et al., 2008). They can circulate freely in the blood flow or be embedded inside EVs, which can transfer them from cell to cell (Valadi et al., 2007). Circulating miRNAs are correlated with the disease states and are currently studied as putative biomarkers of cancer and chronic diseases, such as type 1 and 2 diabetes (Guay and Regazzi, 2013; Snowhite et al., 2017), and diabetic retinopathy (Mastropasqua et al., 2014).

We have recently demonstrated that EVs derived from the mesenchymal stem cells (MScs) enter human retinal pericytes (HRPs), causing their detachment from substrate and migration. They also induce angiogenesis *in vitro* and increase blood-retinal barrier permeability. These effects are worsened by culturing MSCs in high glucose and hypoxia, conditions similar to those described in the diabetic microvasculature (Beltramo et al., 2014). Subsequently, we showed that the expression of miR-126, which plays a prominent role in angiogenesis (Mastropasqua et al., 2014) and is involved in diabetic retinopathy (Bai et l., 2011; Ye et al., 2013), is down-regulated in HRPs exposed to MSC-derived EVs obtained in hyperglycaemic/hypoxic conditions, leading to increased expression of angiogenic molecules, such as vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1 α (HIF-1α) (Mazzeo et al., 2015). Therefore, we concluded that diabetic-like conditions may influence vessel stability through EV paracrine signalling.

Our present hypothesis is that circulating EVs, affected by the diabetic condition, may influence small vessel homeostasis, and that identification of molecular differences in EVs from healthy controls and diabetic subjects, with and without microvascular complications, could represent a predictive option for diagnostic

purposes. The objectives of this work are therefore the molecular and functional characterization of circulating EVs from serum and plasma of diabetic patients with or without retinopathy and healthy controls, and to investigate their role in the regulation of small vessel homeostasis and angiogenesis.

2. Materials and methods

2.1 Subjects

Seven type 1 diabetic subjects with proliferative diabetic retinopathy, but without other diabetic complications, systemic diseases limiting life expectancy (eg cancer, cirrhosis), or other autoimmune diseases, were included in the DR group. They were age- and gender-matched with 7 healthy controls (CTR group) and 7 diabetic subjects without retinopathy (noDR group) (**Table 1**). All diabetic patients were on multiple daily insulin injections and performed self-blood glucose monitoring, but took no other medication. Participants were asked to sign an informed consent. Ethical clearance for involvement of human subjects in research was obtained from the *Comitato Etico Interaziendale A.O.U. Città della Salute e della Scienza di Torino - A.O. Ordine Mauriziano - A.S.L. TO1*.

Overnight fasting venous blood samples were collected in tubes containing EDTA for plasma separation and clot activator for serum.

2.2 Cell cultures

Human retinal pericytes (HRPs) were stabilized in our laboratory, as previously described (Berrone et al., 2009). Human bone marrow MSCs, and human microvascular ECs (HMECs) were purchased from Lonza (Basel, Switzerland). HRPs and MSCs were maintained in DMEM + 10%FCS, while HMECs in EBM-2 growth medium (Lonza) supplemented with angiogenic factors, according to the instructions. When subcultured for the experiments they were grown in DMEM + 10% FCS. Reagents for cell cultures were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.3 EV isolation and characterization

Collection of EVs from serum, plasma and MSCs was performed by centrifugation at 3000 *g* for 30 minutes to remove debris, apoptotic bodies and platelets, followed by ultracentrifugation at 100,000 *g* for 3 hrs at 4°C of the cell-free supernatants (ultracentrifuge: Optima L-100K, Beckman Coulter, Brea, CA, USA; rotor: 90 Ti, 90000 rpm, fixed angle, Beckman Coulter). EVs were either used immediately or stored at −80°C in DMEM + 5% dimethyl-sulfoxide. No differences in biological activity were observed between fresh and stored EVs. EV size, distribution and number were assessed using a NanoSight LM10 (NanoSight Ltd, Minton Park, UK), running the Nanoparticle Tracking Analysis 2.3 software. For all *in vitro* experiments we used an EV concentration similar to the one measured in peripheral blood of CTR group, according to our preliminary data (8-10×10^8 EV/ml), in order to rule out possible dose-dependent effects on microvascular cells.

2.4 Expression of surface molecules

EV expression of surface molecules was measured by FACS analysis using Guava easyCyte™ Flow Cytometer (Millipore, Burlington, MA, USA) with a panel of antibodies (Abcam, Cambridge, UK) against adhesion molecules (CD29, CD44, CD81), VEGF-receptor 1 and 2 (VEGFR-1 and VEGFR-2), marker proteins for: MSCs (CD73, CD29, CD90, CD105, CD44), exosomes (CD63, CD81), ECs (CD105, CD31), platelets (CD42), monocytes (CD14), lymphocytes (CD3, CD4, CD20, CD45, CD81). Staining with FITC-conjugated annexin-V was used as a marker for phosphatidyl-serine (PS).

2.5 Cell survival parameters

We evaluated the ability of EVs from the 3 groups to influence viability and proliferation of microvascular cells. HMECs and HRPs cultured in serum-deprived DMEM were exposed for 24 hrs to serum/plasma EVs from the different subjects and from MSCs, when appropriate. To evaluate HRP detachment, cells remained attached to wells after washing were trypsinized and counted by 2 individual operators in Bürker chambers after Trypan blue staining. Proliferation was measured as DNA synthesis (*Cell Proliferation ELISA BrdU* kit, Roche Diagnostics, Basel, Switzerland) and apoptosis as DNA fragmentation (*Cell Death Detection ELISA^PLUS* kit, Roche), according to the manufacturer's instructions.

2.6 Permeability

40,000 ECs/well were seeded on the inner surface of 0.45-µm pore-transwell inserts (Corning, New York, USA) and let adhere for 24 hrs. 40,000 HRPs were subsequently added into the same insert. After further 24 hrs, inserts were washed and moved to clean wells. 600 µl DMEM without red phenol and FCS were added in the lower chamber, while 200 µl of the same medium supplemented with serum/plasma EVs were added into the inserts. After 2 hrs, FITC-dextran (100 µg/ml final concentration) was added into the upper chamber, and fluorescence measured in the lower chamber after further 30', 1, 2, 3 and 4hrs, through a Victor-3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA).

2.7 Cell migration

HRP and HMEC migration rate following 24hr exposure to plasma EVs from the different groups was evaluated using the colorimetric *QCM Chemotaxis Cell Migration Assay* (Merck-Millipore, Darmstadt, Germany), according to the instructions. Briefly, cells were seeded inside 8µm pore polycarbonate membranes and exposed to EVs. Cells still inside the insert were removed and those migrated through the membrane stained. The stain was subsequently extracted and transferred to a 96-well ELISA plate for colorimetric reading at 560 nm.

2.8 Vessel-like formation assay

15,000 HRPs and 15,000 HMECs were seeded together onto Matrigel-coated 24-well plates and cultured in serum-deprived DMEM added with plasma EVs from all subjects of the 3 groups. Control cultures were obtained seeding 30,000 HRPs or 30,000 ECs alone in Matrigel-coated wells. After 24 and 48 hr incubation, phase-contrast images at 200x magnification of five random fields per each well were recorded, and the total length of the network structures in each field measured using the MicroImage analysis system (Casti Imaging, Venice, Italy), as previously described (Bussolati et al., 2003). Mean of the 5 fields of each well was calculated. Each measure was performed in duplicate wells and expressed as ratio of control without EVs.

2.9 MicroRNA profiling and bioinformatics analysis

Preliminary microRNA profiling was performed on plasma EVs extracted from the 4 subjects in each group, who best matched with their correspondent subjects in the other two groups, as regards physical and clinical characteristics, in order to reduce as much as possible individual variations.
Total RNA was extracted from EVs using *mirVana RNA Isolation kit* (Thermo Fisher Scientific, Waltham, MA, USA), which also allows for isolation of small RNAs. 50 ng of total RNA were reverse-transcribed to cDNA using *TaqMan® MicroRNA Reverse Transcription Kit* (Thermo Fisher Scientific). Subsequently, cDNA was pre-amplified with *Megaplex™ RT Primers*, *Human Pool Set v3.0* and *TaqMan® PreAmp Master Mix* (Thermo Fisher Scientific). The expression profile of a panel of 754 human microRNAs was evaluated by *TaqMan® Human MicroRNA Array, Card Set A and B, v3.0* (Thermo Fisher Scientific), according to the manufacturer's instructions. qRT-PCR was performed using the *QuantStudioTM 12K Flex Real-Time PCR*

System (Applied Biosystems). Raw Ct values were calculated using the QuantStudio 12k Flex Software, while the Expression Suite software was used to compare miRNA expression. Relative quantification (RQ) was obtained using the $2^{-\Delta\Delta Ct}$ method. Data were normalized using global normalization (Mestdagh et al., 2009). MiRNAs expressed in all groups (i.e. at least 3 samples out of 4 in each group with Ct < 35) were selected for the statistical analysis. Fold increases in DR and noDR were expressed as ratio against the values of the correspondent CTR sample.

2.10 Quantitative Real Time PCR (qRT-PCR)
On the basis of data obtained with arrays, qRT-PCR was performed on plasma EVs from all individuals (7 subjects per each group) to validate the 11 miRNAs found to be differentially expressed. Total RNA was extracted using *mirVana RNA Isolation Kit* and quantified spectrophotometrically (Nanodrop ND-1000, Wilmington, DE, USA). 50 ng of RNA were reverse-transcribed using *TaqMan® microRNA Reverse Transcription kit*. qRT-PCR was performed through *TaqMan® microRNA assay kits* specific for the 11 miRNAs, using the *QuantStudioTM 12K Flex Real-Time PCR System*. miRNA expression was normalized against the small nuclear RNA RNU6B.

2.11 Statistical analysis
Statistical comparisons as regard subject characteristics, functional studies, and miRNAs expressed in all groups were carried out by one-way ANOVA with Bonferroni *post-hoc* correction and/or two-tailed Student's t-test for paired data, as appropriate. Results were considered significant for $p \leq 0.05$. SPSS software version 24.0 (IBM) was used for statistical analysis.

3. Results

3.1 EV characterization
EVs collected from serum and plasma of subjects belonging to the 3 groups (CTR, noDR and DR) by ultracentrifugation were analyzed by NanoSight. They showed similar mean size among groups, and between serum and plasma. The number of EV/ml was 2.5 fold higher in both diabetic groups in comparison with healthy controls, but inside each group there were no differences between serum and plasma (**Table 1**). Among diabetic patients, there was no correlation between HbA1$_c$ levels and EV concentrations in serum (r=0.137, p=0.673) or in plasma (r=0.269, p=0.541).

3.2 Expression of surface molecules
EVs from serum and plasma of all groups, analyzed by Guava FACS analysis, expressed surface markers for MSCs (CD73, CD29, CD90, CD105, CD44), ECs (CD105, CD31), exosomes (CD63, CD81), monocytes (CD14), lymphocytes (CD3, CD4, CD20, CD45, CD81) and platelets (CD42), as well as adhesion molecules (CD29, CD44, CD81), VEGFR-1 and VEGFR-2. The expression of all the above did not change among groups or between serum/plasma (**Table 2**). Instead, we found a higher PS$^+$ EV concentration in serum than in plasma in noDR and DR groups.

3.3 EV effects on HRP detachment and survival parameters
Sub-confluent HRP cultures were exposed for 24 hrs to EVs extracted from serum and plasma of the 3 groups and to EVs derived from MSC, as we previously showed that MSC-EVs provoke HRP detachment from the substrate (Beltramo et al., 2014). We found that EVs from blood induce a 20-30% HRP

detachment, similar to that induced by MSC-EVs (-18% vs HRPs cultured without EVs, p<0.05 in all cases vs control cultures without EVs), without significant differences between serum and plasma (**Fig. 1**). Proliferation and apoptosis measured in HRPs which had remained attached to wells were unchanged by EV exposure. As regards HMECs, no direct effects of EV exposure to survival/proliferation/apoptosis parameters were found (data not shown).

3.4 EV effects on retinal blood-barrier permeability
FITC filtration through EC/HRP bilayers mimicking the inner blood retinal barrier was increased in a time-dependent manner by EV exposure (**Fig. 2a**). EVs derived from both serum and plasma of noDR and DR patients increased permeability significantly more than EVs from healthy subjects (CTR group), but DR-EVs further enhanced permeability in comparison with noDR-EVs (**Fig. 2b**). Again, no differences were found between serum and plasma EVs inside each group, consistently with previous observations in the literature (Witwer et al., 2013). Therefore, we chose to use plasma-derived EVs only for the subsequent experiments.

3.5 Retinal cell migration following EV exposure
Migration of HMECs and HRPs through Transwell membranes was measured after 24 hr exposure to plasma EVs from the 3 different groups of subjects. noDR and DR-EVs were able to increase of a 20-40% both HMEC and HRP migration rate, in comparison with CTR-EVs and control cultures without EVs (p<0.05 vs both) (**Fig. 3**).

3.6 In vitro formation of vessel-like structures by HRP/EC co-cultures
Plasma EVs from the 3 groups added to HRP/EC co-cultures on Matrigel promoted the formation of vessel-like structures. While in control wells ECs and HRPs seeded separately remained in a sub-confluent shape, in wells where they had been seeded together, tubular structures were present. EV exposure of EC/HRP co-cultures enhanced the number of these newly-formed structures in all cases. New vessel number was further increased by EVs extracted by noDR subjects (+ 47% in comparison with CTR) and, most of all, by DR patients (+111%, p=0.001 vs CTR and p<0.05 vs noDR) (**Fig. 4**).

3.7 MicroRNA profiling
We assessed the expression of 754 miRNAs in plasma EVs extracted from 4 subjects per each group. MiRNAs considered as expressed (at least 3 samples out of 4 in each group with Ct < 35) were analyzed individually. Microarray analysis revealed 11 miRNAs to be differentially expressed in the 3 groups, especially as regards DR group in comparison with healthy controls (CTR). In particular, 6 miRNAs were upregulated in DR vs CTR (p<0.05): miR-17-5p, miR-21-3p, miR-30b-5p, miR-106a, miR-139-5p, and miR-484. Among these, miR-21-3p was significantly upregulated also in noDR vs CTR (p<0.001), and miR30b-5p in DR vs noDR (p<0.05).
Conversely, 5 miRNAs were downregulated in DR vs CTR (p<0.05): miR-24-3p, miR-150-5p, miR-155-5p, miR-342-3p, and miR-1243. miR-24-3p, miR-150-5p, and miR-1243 were decreased also in DR vs noDR (p<0.05) (**Fig. 5**). The complete list of miRNAs expressed in the 3 groups without significant differences is shown in **Suppl. Mat. 1**.

Subsequently, the expression of the differentially expressed miRNAs was checked by qRT-PCR and 3 out of 11 were confirmed to change significantly among groups. In particular, miR-150-5p was strongly decreased in the DR group (-77.5%, p=0.000 vs CTR and p<0.05 vs noDR) (**Fig. 6a**), while miR-21-3p was 3-fold upregulated in noDR (p<0.05) and 5-fold in DR (p=0.001), as compared with CTR (**Fig. 6b**). Finally, miR-30b-5p was unchanged in noDR group and 5-fold increased in the DR group (p=0.001 vs CTR and p<0.05 vs

noDR) (**Fig. 6c**). miRNA expressions did no correlate with HbA1$_c$ levels in diabetic subjects: miR-150-5p (r=-0.378, p=0.281), miR-21-3p (r=0.520, p=0.123), or miR-30b-5p (r=0.283, p=0.428).

4. Discussion

In this paper, we address the molecular and functional characterization of circulating EVs from diabetic subjects with or without retinopathy, as compared with healthy age- and gender-matched controls, to evaluate if they can be considered as potential biomarkers of the disease. Our results demonstrate, for the first time in our knowledge, that EVs extracted from patients with diabetic retinopathy have a different miRNA profiling pattern in comparison with both patients without complications and healthy controls. Moreover, EVs derived from diabetic patients, and especially from DR ones, are able to increase permeability of an *in vitro* model of the retinal blood-barrier, as well as migration of microvascular cells, and to enhance the formation of vessel-like structures in pericyte-endothelial cell co-cultures.

Novel non-invasive and reliable biomarkers are needed to predict the risk of developing diabetes and its complications. To date, the prediction of the systemic disease is generally based on the measurements of traditional serum biomarkers (carbohydrate and lipid metabolites, glycated hemoglobin), which, together with physical characteristics (BMI, gender), familiarity and life styles, allow to reach a level of probability of prediction that still remains quite low (Herder et al., 2011; Müller, 2012). As regards biomarkers for diabetic retinopathy, research has focused on several molecules involved in the pathogenic mechanisms at the basis of the development of the complication (markers of inflammation, adhesion molecules, advanced glycation end products, turnover of the basement membrane and the extracellular matrix) (Kaviarasan et al., 2015; Simó-Servat et al., 2016). None of them, however, is specific for the retinal tissue, which all together is a very small portion of the body, and could rather represent a marker of systemic damage induced by diabetes (Simó-Servat et al., 2016).

Circulating miRNAs have been largely addressed and investigated as non-invasive potential markers, especially in cancer research (Chen X et al., 2008; Kaduthanam et al., 2013; Liu et al., 2013). More recently, they have been proposed as markers of metabolic disorders (Heneghan et al., 2011; Pescador et al, 2013), and miRNA patterns different from healthy controls have been highlighted in type 1 (Snowhite et al., 2017) and type 2 diabetic patients (Pandey et al., 2009; Zampetaki et al., 2010; Pescador et al, 2013; Cui et al., 2018). In the latest years, the importance of modulation of miRNAs in diabetic retinopathy has also been investigated (Mastropasqua et al., 2014).

EVs could represent promising biomarkers for metabolic disease and diabetes, since their secretion is strictly correlated with the status of the donor cells: increased number of circulating EVs has been detected in diabetic animals (Müller, 2012), as well as in diabetic patients (Koga et al., 2005; Feng et al., 2010; Helal et al., 2010). Moreover, as EVs can modulate vascular permeability and angiogenesis, they might play a role in the development of retinopathy (Müller, 2012). Our results confirm that diabetic subjects, with or without complications, have a 2.5 fold higher serum and plasma EV concentration than healthy controls. Nevertheless, the same EV concentration was used for the 3 groups, in order to rule out possible dose-dependent effects on microvascular cells. The finding that EVs extracted from DR patients, even though added to cell cultures in lower concentrations than circulating ones, are equally able to induce features of retinopathy in *in vitro* models of retinal microvasculature, in our opinion strengthens our data.

An univocal consensus in the literature about EV subdivision by size has not yet been reached. A recent review indicates exosome range as 50-150 nm, and microvesicle as 50-500 nm, with a substantial overlapping in size, and similar morphology (van Niel et al., 2018). The size of the EVs we extracted from the three groups ranged between 74-233 nm, with high deviation among cases. Therefore, they can be considered to include both microvesicles and exosomes, this being confirmed by the presence of typical exosome markers (CD63 and CD81, Kowal et al., 2016) on 20-30% EVs. EVs extracted from the three groups own surface markers of platelets, lymphocytes, monocytes, ECs and MSCs, showing their heterogeneous origin. Moreover, their expression of adhesion molecules and VEGF receptors suggest that they could play a role in angiogenesis. However, since no differences were found among groups as regards the expression of surface antigens, we hypothesize that their putative different effects on retinal microvasculature could be rather ascribed to inner molecules. EVs are in fact a reservoir of lipids, cytokines, signaling proteins, receptors, transporters, enzymes, mRNAs and miRNAs that are shuttled from cell-to-cell (Camussi et al., 2010), and can also be transferred across the blood-brain and blood-retinal barriers (Müller, 2012).

The study of the effects of EVs extracted from the 3 groups on *in vitro* models of retinal microvasculature and retinal blood-barrier showed that circulating EVs have the same potential as MSC-derived EVs to induce pericyte detachment from the substrate (Beltramo et al., 2014). No differences were found between EVs extracted from serum or plasma within each group, consistently with previous observations about superimposable behavior of serum and plasma EVs (Witwer et al., 2013). Therefore, we chose to use plasma-derived EVs only for the subsequent experiments, in agreement with the review by Witwer et al. (2013), which indicates plasma as the medium of choice for EV extraction.

The characteristic features of early diabetic retinopathy comprise loss of retinal pericytes, leading to increased permeability through the blood-retinal barrier, neovascularization and angiogenesis (Armulik et al., 2005; Beltramo and Porta, 2013). In this paper, we demonstrate that EVs derived from DR patients induce a significantly higher detachment of pericytes from the substrate, without affecting those remaining attached. Moreover, pericytes and ECs exposed to EVs from both diabetic groups show a greater migration rate. These findings are consistent with previous observations demonstrating that pericytes detached from their substrate following stimulation with EVs derived from MSCs cultured in diabetic-like conditions remain viable, maintaining their capability of adhesion to new substrates and showing no signs of apoptosis (Beltramo et al., 2014). Pericytes play a major role in new vessel stabilization during angiogenesis (Gerhardt and Betsholtz, 2003), so we can speculate that those that detach from vessels following EV exposure migrate to stabilize new vessels, as previously hypothesized (Pfister et al., 2008; Beltramo et al., 2014). Furthermore, our results show an increased permeability through EC/pericyte bilayers exposed to EVs derived from both groups of diabetic subjects, with a greater increase for those derived from DR patients. Consistently, data from the tube formation assay in EC/pericyte co-cultures in Matrigel further underline the role of EVs from DR patients in inducing *in vitro* pathologic dysfunctions characteristic of retinopathy.

We chose to investigate EV miRNA content because, as stated above, miRNAs are considered promising biomarkers. Differences in circulating miRNAs in diabetic patients (Pandey et al., 2009; Zampetaki et al., 2010; Guay et al., 2013; Pescador et al, 2013; Snowhite et al., 2017; Cui et al., 2018) and in retinopathy (Mastropasqua et al., 2014) have been described, but the role of miRNAs shuttled by EVs in diabetes and its complications is scarcely addressed. Moreover, circulating miRNAs are subject to possible degradation in the blood flow, while miRNAs shuttled by EVs are protected by the lipid membrane of the EVs itself (Müller, 2012). Using the microarray analysis, 11 miRNAs were found to be differentially expressed in DR patients as compared with healthy controls, while uncomplicated diabetic subjects showed only one of these to be altered. This suggests that miRNAs shuttled by EVs might be involved in the development of diabetic

retinopathy, rather than generically in diabetes. To support this hypothesis, some of the miRNAs we found upregulated have already been described as involved in angiogenesis and inflammation (miR-21-3p) (Ng et al., 2015; Snowhite et al., 2017), migration (miR-21-3p, miR-17-5p) (Otsuka et al., 2008; Ng et al., 2015), increased in the ischemic retina (miR-106a-5p) (Shen et al., 2008), or overexpressed in the retina of diabetic mice (miR-21) (Chen Q et al., 2017). Among those downregulated, miR-150-5p and miR-342-3p are described as anti-angiogenic (Shen et al., 2008; Fayyad-Kazan et al., 2013; Li et al., 2014), miR-150-5p is decreased in type 1 (Estrella et al., 2016) and miR-155-5p in type-2 diabetes (Corral-Fernandez et al., 2013).

Subsequent validation through qRT-PCR using specific primers confirmed 3 out of the 11 miRNAs only to be differentially expressed. miR-150-5p was found to be strongly downregulated in the EVs from DR subjects, consistently with previous findings showing its suppression in pathological neovascularization in mice with oxygen-induced proliferative retinopathy (Liu CH et al., 2015). miR-150 was also demonstrated to decrease EC migration and tubular formation, by inhibiting the expression of several angiogenic mediators (Liu CH et al., 2015). On the contrary, miR-21-3p was confirmed to be upregulated in both noDR and DR groups. This is consistent with observations of its overexpression in the retina of db/db mice, with concomitant decrease in peroxisome proliferator-activated receptor-α levels (Chen Q et al., 2017). miR-21 was also shown to induce angiogenesis through activation of AKT and extracellular-signal-regulated kinase (ERK), and associated increase of HIF-1α and VEGF expression (Liu LZ et al., 2011), while, more recently, an analogue function specific for miR-21-3p has been proposed (Báez-Vega et al., 2016; Snowhite et al., 2017). Finally, our finding of an upregulation of miR-30b-5p in patients with DR is consistent with recent evidence in the literature showing its role as a pro-angio-miRNA, shuttled by EVs inside EC, and promoting EC migration and tube formation *in vitro* (Gong et al., 2017). Thus, we can hypothesize a role for miR-150-5p, miR-21-3p and miR-30b-5p as potential biomarkers for the onset of DR.

The strengths of our study are bound to the description of novel mechanisms involved in the onset and progression of diabetic retinopathy. For the first time in our knowledge we demonstrate that circulating EVs from diabetic patients with retinopathy have miRNA profiling patterns different from both diabetic subjects without complications and healthy controls. In addition, we show that EVs derived from DR patients are able to determine pathological changes in microvascular cells compatible to those characteristics of the disease. Finally, we hypothesize that miR-150-5p, miR-21-3p and miR-30b-5p could be novel potential biomarkers for the onset and progression of diabetic retinopathy. As regards the weaknesses of our work, a limited number of patients have been enrolled. Further subjects are needed to confirm the potential role of these molecules. Moreover, we restrict to the miRNA profiling, but EVs contain several other molecules, such as proteins and mRNAs, which are worthwhile to be investigated.

5. Conclusions

Non-invasive and reliable biomarkers are needed to predict the risk of developing diabetes and its complications. Our results demonstrate that circulating EVs extracted from DR patients are able to induce features of retinopathy in *in vitro* models of retinal microvasculature, such as detachment and migration of pericytes, formation of new vessels and increased retinal blood-barrier permeability. The identification of molecular differences in EVs from healthy controls and diabetic subjects with or without retinopathy could hopefully provide new predictive biomarkers of the onset of diabetic retinopathy or for prevention purposes. We present here a panel of EV-derived miRNAs, involved in angiogenesis and inflammation, which are differently modulated in DR subjects. Among these, miR-150-5p, miR-21-3p and miR-30b-5p

seem to be strongly related to diabetic retinopathy and might be taken into account as potential biomarkers of the onset/development of the disease. In addition, they could be addressed as specific targets for anti-angiogenic strategies aimed at the prevention of this complication.

Author Contributions
Design of the study: AM, EB, TL, MP. Selection of subjects: AM, EB, MT, MP. Experimental work, data collection and analysis: AM, EB, TL, CG. Interpretation of data: AM, EB, MP. Manuscript drafting: AM, EB. Critical revision: TL, CG, MT, MP. All authors have approved the final article.

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Table 1 Group characteristics. CTR: healthy controls; **noDR**: diabetic subjects without retinopathy, **DR**: diabetic patients with severe retinopathy. **a) Subjects clinical characteristics, b) EV characteristics**. Data are expressed as mean±SD *(range)*, as appropriate. HbA_{1c} is expressed as mmol/mol *(%)*. Statistical comparisons among groups were performed by one-way ANOVA with Bonferroni *post-hoc* correction or Student's t-test for paired data. No differences among groups were found as regards age and BMI (3 groups), duration of disease and HbA1c (noDR and DR). noDR and DR had a 2.5 fold increase in EV concentration vs CTR, [a] = $p<0.05$.

	CTR	noDR	DR
a. Subject clinical characteristics			
Gender (F/M)	3/4	3/4	3/4
Age (years)	41.0±10.6 *(33-63)*	46.1±11.7 *(27-67)*	39.3±5.9 *(29-56)*
Bmi (Kg/m^2)	24.3±2.9 *(20.2-29.0)*	23.1±2.0 *(20.3-26.4)*	26.8±2.9 *(22.1-29.0)*
Duration of disease (years)	N/A	27.3±14.2 *(15-47)*	28.0±12.8 *(8-41)*
HbA_{1c} [mmol/mol *(%)*]	N/A	58.5±10.1 *(7.5±1.3)*	68.3±4.1 *(8.4±0.5)*
Diabetic retinopathy Y/N)	N/A	N	Y
b. EV characteristics			
Serum EV size (nm)	181±36	165±28	173±34
Plasma EV size (nm)	166±31	175±24	158±39
Serum EV concentration (EV/ml)	$8.6±1.1 \times 10^8$	$19.8±4.1 \times 10^{8}$ [a]	$18.6±3.1 \times 10^{8}$ [a]
Plasma EV concentration (EV/ml)	$7.3±1.0 \times 10^8$	$19.2±2.7 \times 10^{8}$ [a]	$18.8±4.3 \times 10^{8}$ [a]

Table 2 Marker expression on EV surface. CTR: healthy controls; **noDR**: diabetic subjects without retinopathy, **DR**: diabetic patients with severe retinopathy. Data are expressed as mean±SD. Statistical comparisons among groups were performed by one-way ANOVA with Bonferroni *post-hoc* correction. [a)] = p<0.05 vs no DR serum, [b)] = p<0.05 vs DR serum

|	*CTR serum*	*CTR plasma*	*noDR serum*	*noDR plasma*	*DR serum*	*DR plasma*
CD3	10.5±12.0	7.3±9.3	32.1±10.1	30.2±8.3	30.8±15.2	29.7±15.1
CD4	52.0±26.7	54.7±25.7	59.1±7.9	52.9±7.6	65.5±9.5	63.1±11.3
CD14	70.1±7.5	74.0±7.5	70.4±8.2	71.0±6.9	68.8±5.3	69.5±6.3
CD20	25.7±15.0	27.3±15.5	47.2±8.6	50.8±11.3	52.5±7.8	53.8±9.5
CD29	66.7±7.5	70.0±4.3	60.1±5.8	59.0±6.1	60.3±0.4	60.5±4.9
CD31	69.0±3.1	70.2±2.1	72.2±8.5	70.6±6.7	73.8±2.5	72.5±3.5
CD42	49.7±12.7	51.0±10.3	52.3±5.3	58.1±10.4	56.1±9.5	55.3±7.8
CD44	70.3±3.2	72.0±7.5	58.1±7.8	67.1±11.6	66.2±2.8	69.3±8.5
CD45	38.2±6.0	39.1±6.6	28.5±8.7	37.5±7.7	38.8±14.5	42.3±13.8
CD63	21.7±4.9	21.7±6.1	18.8±5.2	17.2±6.8	19.5±9.2	20.7±5.3
CD73	69.7±5.9	72.7±3.2	70.0±5.9	68.4±6.8	68.5±2.2	67.8±6.7
CD81	30.7±2.9	30.6±2.8	35.1±5.8	41±9.9	37.2±14.1	36.9±14.0
CD90	72.5±7.8	73.3±10.7	79.1±8.7	80.3±9.5	82.3±7.4	80.7±6.2
CD105	54.3±4.9	57.7±6.7	49.2±6.7	55.7±5.8	39.5±13.4	47.2±11.3
VEGFR-2	10.8±7.6	12.8±9.8	15.3±2.9	22.0±8.5	22.0±6.2	23.5±5.8
VEGFR-1	62.7±13.7	64.3±12.2	77.0±12.1	74.1±11.0	75.0±13.6	75.2±9.8
PS	39.9±15.2	25.6±7.8	23.5±2.5	3.5±3.2 [a)]	18.3±3.6	10.4±3.8 [b)]
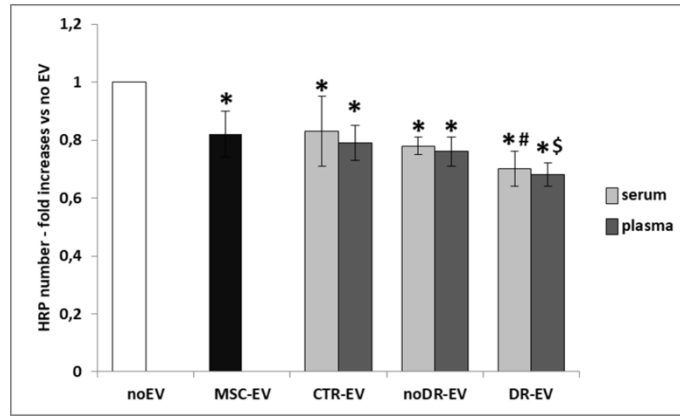
Fig. 1 Comparison of the effects of EV from different sources on HRP detachment after 24 hr exposure to EV derived from serum (*light grey bars*) and plasma (*dark grey bars*) of the 3 groups (CTR, noDR, DR), compared to MSC-derived EV (*black bar*) and control HRP cultures without EV (*white bar*), n=7 per group, * $p < 0.05$ vs noEV, # $p < 0.05$ vs CTR serum EV, \$ $p < 0.05$ vs CTR plasma EV. Same EV concentration ($8\text{-}10 \times 10^8$ EV/ml) was used for all cases.
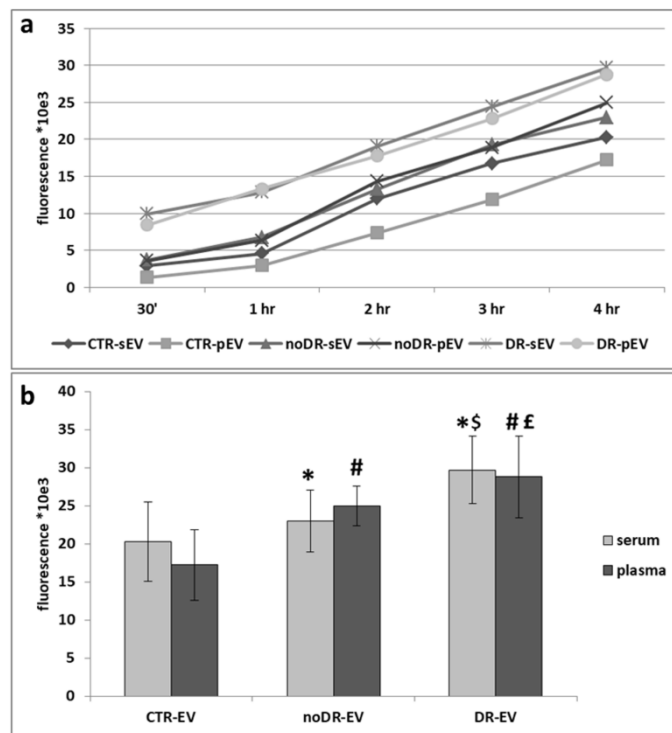

Fig. 2 Effects of EVs from serum/plasma on permeability of EC/HRP co-cultures in transwell inserts **a**) time-course permeability, after 2 hr EV stimulation; **b**) permeability at t=4 hrs. n=7 per group, sEV= EVs derived from serum, pEV = EVs derived from plasma, *=$p < 0.05$ vs serum-derived EVs in CTR group, # = $p < 0.05$ vs plasma-derived EVs in CTR group, \$ =$p < 0.05$ vs serum-derived EVs in noDR group, £=$p < 0.05$ vs plasma-derived EVs in noDR group. Same EV concentration ($8\text{-}10 \times 10^8$ EV/ml) was used for all cases.
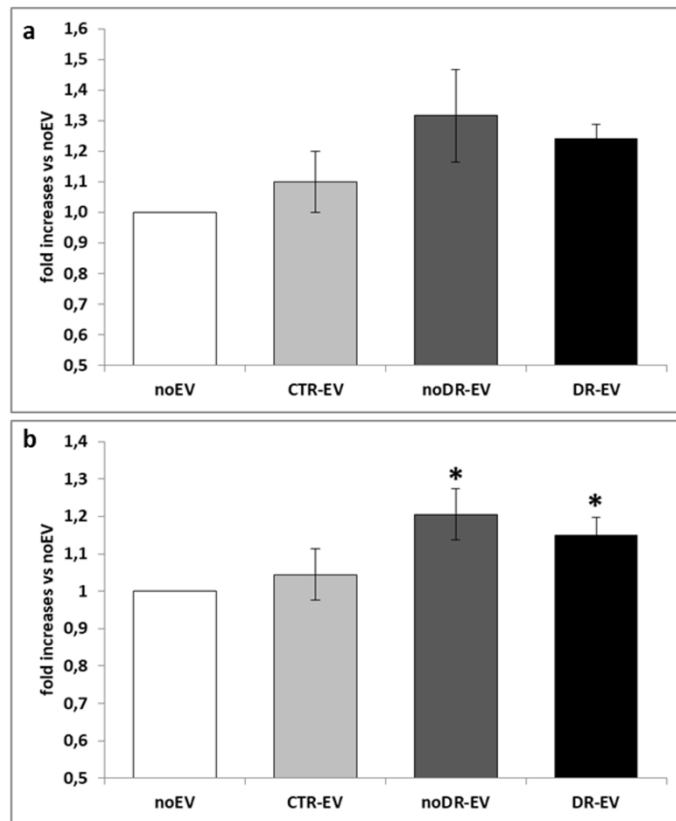
Fig. 3 EVs from plasma of diabetic subjects enhance retinal cell migration. **a**) HMEC, **b**) HRP. N=7 per group, noEV= cell cultures without EV exposure, CTR-EV= cell cultures exposed to EVs from CTR group, noDR-EV cell cultures exposed to EVs from noDR group, DR-EV cell cultures exposed to EVs from DR group. * $p \leq 0.05$ vs noEV and CTR-EV. Same EV concentration (8-10×10^8 EV/ml) was used for all cases.
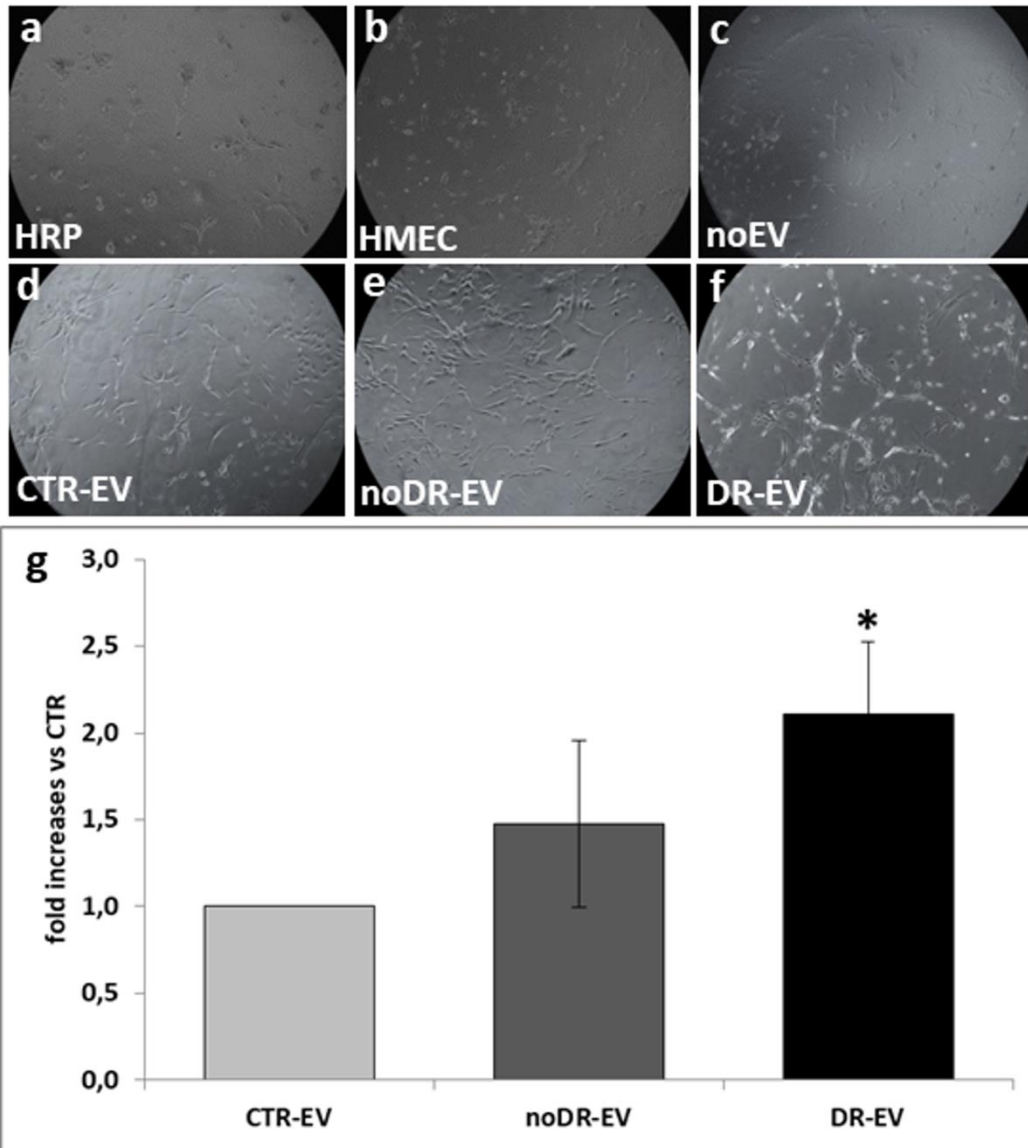
Fig. 4 EVs from plasma of diabetic subjects increase in vitro formation of vessel-like structures by HRP/EC co-cultures. a-f) Vessel-like structure formation by HRPs and HMECs alone, HRP/HMEC co-cultures in Matrigel without addition of EVs (noEV), and after 48 hr addition of EVs from the 3 groups: healthy controls (CTR), diabetic subjects without (noDR) and with retinopathy (DR). Magnification 200x. **g)** Quantitative analysis of newly-formed vessel-like structures after 48 hr EV incubation, fold increases vs CTR group, n=7 per group, * $p=0.001$ vs CTR and $p<0.05$ vs noDR. Same EV concentration ($8-10\times10^{8}$ EV/ml) was used for all cases.
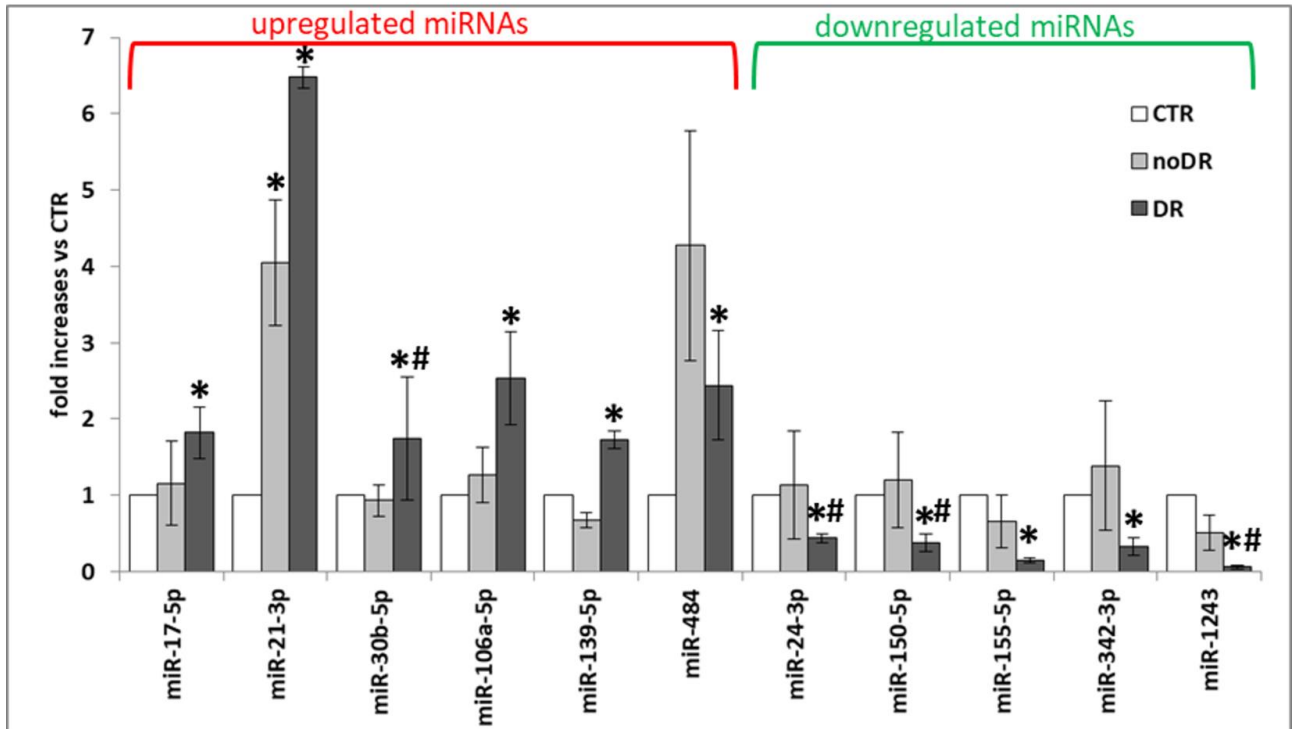
Fig. 5 miRNA profiling in plasma EVs extracted from healthy subjects (**CTR**) and from diabetic patients without (**noDR**) and with retinopathy (**DR**), expression of the 11 differentially expressed miRNAs, fold increases vs the control group (CTR). *White bars*: CTR, *light grey bars*: noDR, *dark grey bars*: DR. N=4 per group, * $p<0.05$ vs CTR, # $p<0.05$ vs noDR.
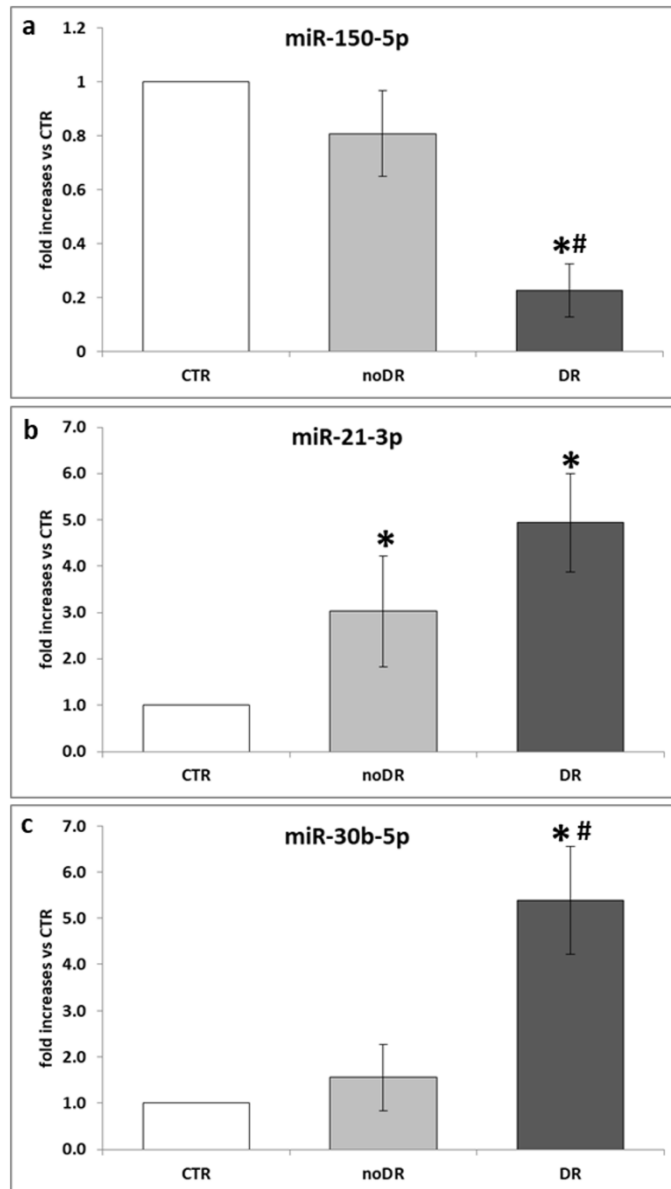
Fig. 6 a) miR-150-5-p, b) miR-21-3-p and **c) miR-30b-5p expression in plasma EVs** extracted from healthy subjects (**CTR**) and from diabetic patients without (**noDR**) and with retinopathy (**DR**), fold increases vs the control group (CTR). *White bars*: CTR, *light grey bars*: noDR, *dark grey bars*: DR. N=7 per group, * p<0.05 vs CTR, # p<0.05 vs noDR.