Human platelet lysate-derived extracellular vesicles enhance angiogenesis through miR-126

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Abstract

Objectives: Extracellular vesicles (EVs) are key biological mediators of several physiological functions within the cell microenvironment. Platelets are the most abundant source of EVs in the blood. Similarly, platelet lysate (PL), the best platelet derivative and angiogenic performer for regenerative purposes, is enriched of EVs, but their role is still too poorly discovered to be suitably exploited. Here, we explored the contribution of the EVs in PL, by investigating the angiogenic features extrapolated from that possessed by PL.

Methods: We tested angiogenic ability and molecular cargo in 3D bioprinted models and by RNA sequencing analysis of PL-derived EVs.

Results: A subset of small vesicles is highly represented in PL. The EVs do not retain aggregation ability, preserving a low redox state in human umbilical vein endothelial cells (HUVECs) and increasing the angiogenic tubularly-like structures in 3D endothelial bioprinted constructs. EVs resembled the miRNome profile of PL, mainly enriched...
with small RNAs and a high amount of miR-126, the most abundant angiogenic miRNA in platelets. The transfer of miR-126 by EVs in HUVEC after the in vitro inhibition of the endogenous form, restored angiogenesis, without involving VEGF as a downstream target in this system.

**Conclusion:** PL is a biological source of available EVs with angiogenic effects involving a miRNAs-based cargo. These properties can be exploited for targeted molecular/biological manipulation of PL, by potentially developing a product exclusively manufactured of EVs.

## 1 | INTRODUCTION

Beyond haemostasis and thrombosis, platelets have been also described as the main regulators of angiogenesis, a key process for tissue regeneration and repair outcome of vascular insults or wound healing and based on the activation of endothelial proliferation, sprouting and organization into functional tubules. The emerging role of platelets to act as inflammatory/immune effectors and to enhance angiogenesis, stems from their intrinsic physiological role to interact with the endothelium during vascular damage, preserving the integrity and vessel homeostasis.

Platelets exhibit a unique secretory profile of multiple combined factors with a dual pro- and anti-angiogenic role. Among them, we could list growth factors, cytokines, microRNAs, small soluble molecules and proteins, including those related to cytoskeleton, adhesion, inflammation, and extracellular matrix interaction. This balanced combination of mediators, is mainly contained in plasma membranes delimited nanoparticles, named extracellular vesicles (EVs). These later, now conceived as signalosomes and biological vectors of heterogeneous size and composition, are released upon platelets activation, interacting with the microenvironment.

Platelets represent the most abundant source of EVs of different dimensions and quantities in the systemic circulation depending on multiple variables including age, physiological states and lifestyle habits. EVs mirror the haemostatic properties of platelets by exerting both anti- and pro-coagulant effects according to the subpopulation of EVs involved. Hence, their circulating levels can act as predictive biomarkers of haemostatic and inflammatory disorders. Patients with metabolic syndrome, myocardial infarction, atherosclerosis, ischemia or inflammatory diseases exhibit higher levels of circulating EVs, because of activated platelets, suggesting their relevance to mediate pathogenetic effects beyond their physiological role. On the other hand, platelet-derived EVs have been also demonstrated to regulate angiogenesis when released at the site of endothelial sprouts and secretion of VEGF, or to transfer proliferative and survival biological information to the endothelium. Platelet-derived EVs can modulate the vascular tone as shown in rabbit models, or even attenuate blood pressure in preeclampsia women, by stimulating the inducible nitric oxide synthase in endothelial cells, consistently with a beneficial functional role on the vasculature.

Similarly to their parental cells, EVs would also act indirectly in a paracrine fashion on the intercellular network as cargos of cytokines and decay proteins are locally released in the microenvironment. For instance, endothelial progenitor cell-mediated angiogenesis and tissue engraftment is enhanced by EVs of platelets origin through the activation of specific targets (MMP-2, MMP-9, PI3K, ERK) or after the transfer of specific soluble platelet receptors and activation of integrins on the endothelial surface. The preconditioning of bone marrow mesenchymal stem cells (MSCs) with platelet-derived EVs has demonstrated their effective capacity of boosting the biological potency and vascular effects of the stromal fraction. Strong evidence of their ability to support angiogenesis has been also observed in myocardial infarction and cerebral ischemia after in vivo direct injection, when platelets, activated by thrombin, release EVs. Moreover, the key contribution of platelet-derived EVs in supporting the angiogenic profile of cancer invasion and metastasis parallel to clinical thrombotic complications has been strongly highlighted.

Based on these studies, evidence that platelets and platelet-derived biological products can trigger angiogenic programs in endothelial cells has encouraged a better understanding of their potential therapeutic use for those regenerative-based applications where the restoration or the enhancement of angiogenesis represents the clinical goal. Accordingly, parallel to the investigations regarding the key involvement of platelets in regulating angiogenesis, it has been demonstrated that platelet-derived clinical preparations (i.e., platelet-rich plasma and gels) are similarly able to boost and reflect the angiogenic properties of platelets. Particular attention has been dedicated to platelet lysate (PL), considered the gold preparation concentrate manufactured, platelets are repeatedly lysed, therefore enriching the population of highly concentrated factors in this hemoderivative. PL is a biological source of available EVs with angiogenic effects involving a miRNAs-based cargo. These properties can be exploited for targeted molecular/biological manipulation of PL, by potentially developing a product exclusively manufactured of EVs.
intact and activated platelets. Only a couple of studies have described the presence of exosomes in platelet-derived clinical formulations but as effectors of the osteogenic differentiation on MSCs or with neuroregenerative capacities. Thus, the wide range of mechanisms by which PL-derived EVs might regulate angiogenesis still needs to be fully addressed.

This study investigates from a biological and molecular standpoint the role of EVs in relation to PL formulations regarding the ability to mediate angiogenesis by endothelial cells.

2 | METHODS

2.1 | Isolation of EVs from PL-based preparations

EVs were isolated from PL-based preparations by sequentially ultracentrifugation (500 rcf for 10 min; 2000 rcf for 10 min; 100,000 rcf for 1 h). EV pellet was then resuspended to reconstitute the initial PL volume and subsequently used at 5%, 10% or 20% in the medium for the experiments. For FACS analysis and uptake assay in human umbilical vein endothelial cell (HUVEC), 100 μl of EVs were stained for 10 min at 37°C with 5 μM S(6)-CFDA-SE [5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester] (CFSE; Invitrogen/Thermo Fischer Scientific) according to the manufacturer’s instructions. Excess dye was removed using Exosome Spin Columns (Thermo Fischer Scientific) following the manufacturer’s recommendations.

2.2 | Nanotracking analysis of EVs in PL

Nanoparticles tracking analysis in terms of size distribution and concentration was performed on PL using a NanoSight NS300 instrument (Malvern Panalytical). Five 30-s videos were recorded for each sample with a camera level set at 15 of 16 and a detection threshold set at 5% (wt/vol) milk and the membranes were incubated at 4°C overnight with rabbit Anti-Annexin A1/ANXA1 antibody monoclonal antibody [EPR19342]-BSA and Azide free (1:25,000; Abcam; Cat. N. ab222398), ALIX (1:1000; Biorybt; Cat. N. orb235075), CD9 (1:500; Abcam; ab186429), calnexin (1:1000; Santa Cruz; Cat. N. sc-46669), TSG101 (4A10) (1:500; Invitrogen; Cat. N. ab222398), ALIX (1:1000; Biorbyt; Cat. N. orb235075), CD9 (1:500; Abcam; ab186429), calnexin (1:1000; Santa Cruz; Cat. N. sc-46669), TSG101 (4A10) (1:500; Invitrogen; Cat. N. ab222398).

2.3 | Western blot

EVs were resuspended in a radioimmunoprecipitation buffer and phosphate inhibitor cocktail. Proteins (10 μl solubilized in 2X Laemmli/20% of 2-mercaptoethanol) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 10% polyacrylamide gel (Bio-Rad). Subsequently, the membrane was blocked with 5% (wt/vol) milk and the membranes were incubated at 4°C overnight with rabbit Anti-Annexin A1/ANXA1 antibody monoclonal antibody [EPR19342]-BSA and Azide free (1:25,000; Abcam; Cat. N. ab222398), ALIX (1:1000; Biorybt; Cat. N. orb235075), CD9 (1:500; Abcam; ab186429), calnexin (1:1000; Santa Cruz; Cat. N. sc-46669), TSG101 (4A10) (1:500; Invitrogen; Cat. N. ab222398).

2.4 | Cell culture, transfection and treatment with antagomiR-126

HUVECs were cultured between passages 3 and 6 in an EGM-2 complete medium (Lonza). Fluorescein-conjugated antagonirs were used for quantification of antagomir or control incorporation and detected by flow cytometry. For antagomiR-126 transfection, cells were plated at a density of 3.5 × 10⁴/24 wells with EGM-2 without gentamicin. A mix composed of 25 pmol LNA_126 (miRCURY LNA miRNA Inhibitor (5) - 3’ Fam; Cat. N. 339121; Qiagen) or 25 pmol control (miRCURY LNA miRNA Inhibitor Control (5)-No Modification Fam; Cat. N. 339126; Qiagen) in Opti-MEM Reduced-Serum Media and lipofectamine (1 μl/100 μl OptiMem; RNAiMAX; Invitrogen; Cat. N. 56531) was added to the HUVEC and incubated overnight. The next day, the medium was removed, and new EGM-2 was added to the cells for up to 24 and 48 h of total transfection. To verify transfection, HUVEC were analysed by flow cytometry detecting FAM fluorescence. See Supporting Information Methods S1, for cytometric analysis, transmission electron microscopy, Matrigel assay and immunofluorescence on HUVEC in 3D-bioprinting constructs, platelets aggregation, determination of soluble human prothrombin fragment 1 + 2 and quantification of peroxide hydrogen and molecular biology.

2.5 | Statistics

Statistical analysis was performed by GraphPad PRISM 5 software. Student’s t-test and one-way analysis of variance (Bonferroni correction) were used to compare the difference between the control and groups. A p < 0.05 was considered significant. Data were presented as mean ± standard error unless specified. Additional information on statistics and confidence intervals have been reported in the corresponding sections above and in figure legends.

3 | RESULTS

We investigated in detail the EV content and characteristics of human PL preparations. To assess the concentration and absolute size distribution, we measured the EVs by Nanoparticle Tracking Analysis (NTA) in eight different batches of PL. Results showed that PL-based preparations contain a very high concentration of EVs with a mean of 1.84 × 10¹³ particles/ml, with the EV size mode of 123.37 ± 7.02 nm (Figure 1A,B). Among all distinctive subclasses of EVs, the most representative group in terms of concentration was the 50–200 nm subset, corresponding to small microvesicles including exosomes (Figure 1C,D, p < 0.001 vs. all subsets). Occasionally, EVs of <50 nm size were found, but not in all batches.
FIGURE 1  Characterization of extracellular vesicles (EVs) in platelet lysate (PL). (A) Concentration measurement and (B) size mode from Nanoparticle Tracking Analysis (NTA) of EVs derived by nine batches of PL. (C) NTA graph and (D) pie chart display the EVs size distributions. *p < 0.001. (E) Representative transmission electron microscopy images of EVs within PL-based preparations. Scale bar (200 nm) and magnification (~20,000) are displayed. (F) Western blot analysis of ALIX, calnexin, TSG101, Annexin A1 and CD9 of three EV batches isolated by ultracentrifugation from PL. (G) Representative histogram of the flow cytometry displaying the relative percentage of EVs in the graph below for CD41, platelet marker. N = 3 lots of EVs were tested.
Following international guidelines, EVs were further characterized by evaluating their morphology and phenotype by transmission electronic microscopy (TEM), and Western blot for the recommended universal markers. TEM analysis confirmed that PL preparations contained EVs with heterogeneous but small dimensions, roundish morphology and electron-dense features, suggesting a significant cargo function (Figure 1E). According to the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines, TEM was qualitatively implemented by both Western blot showing the positive expression of the proteins ALIX, CD9, Annexin A1, TSG101 (cytosolic, membrane and marker of biogenesis of EV) in PL-derived EVs and negative expression for calnexin, therefore suggesting the absence of non-EV structures in the preparation of EVs (Figure 1F). The characterization of EVs was further verified by cytofluorimetry. The FACS analysis confirmed the expression of CD41, the main marker of platelet origin of EVs (49.92 ± 5.22%, also known as glycoprotein IIb possessing a critical role in modulating platelet aggregation), but also the negative expression for calnexin (Figure 1G).

To discriminate the biological effects of EVs from the whole PL, we isolated the EVs according to methodological standardized guidelines by high-speed ultracentrifugation. Afterwards, we investigated whether EVs may convey haemostatic properties, such as aggregation and pro-coagulant abilities, which are two key physiological properties exerted by platelets but also reported for EVs. We stimulated platelets of healthy subjects with increasing percentages of EVs (5%, 10% and 20%), PL (10% and 20%) and collagen were used as biological and positive references, respectively. In addition, the quantification of the soluble fragment 1 + 2 of prothrombin (F1 + 2) was employed to test the coagulation property of EVs. Results showed that neither the increasing concentration of EVs nor PL were able to induce aggregation compared to collagen (Figure 2A,B). A similar amount of F1 + 2 among samples was detected (comparable to physiological soluble levels in the human plasma), with no statistically significant differences (Figure 2C).

As one of the most significant bioactive properties of PL is the ability to induce angiogenesis, we investigated the contribution of EVs to the angiogenesis stimuli mediated by PL. We isolated and labelled the EVs with the green fluorescent dye CFSE. Afterwards, HUVECs were stimulated for 24 h with the EV preparation (10% vol/vol, corresponding to the same PL volume in percentage routinely employed in cell culture). HUVECs were able to uptake EVs, as demonstrated by the presence of green fluorescent dots visible in the cytoplasm (Figure 3A). When HUVECs were subjected to the in vitro angiogenesis Matrigel assay at increasing concentrations of EVs (5%, 10%, 20%), we found that 10% EVs was the optimal percentage to significantly enhance the number of closed loops (Figure 3B,C).

**Figure 2** Hemostatic properties of EVs derived from PL. (A) Representative plots of the aggregometer displaying both PL and EVs at different percentage and (B) relative analysis showing no aggregation compared to collagen used as positive reference. (C) Immunoassay of the F1 + 2 assaying the coagulative ability of both PL and EVs compared with human plasma with no significant difference. EV, extracellular vesicle; PL, platelet lysate.
FIGURE 3  Angiogenic effects of PL-derived EVs in endothelial cells. (A) Merged images of optical and fluorescent microscopy showing HUVEC uptaking after 24 h the CSFE-labelled isolated EVs in different experimental conditions (EBM and EGM-2 negative and positive controls, respectively). White scale bar = 50 μm. (B) Representative images of capillary-like structures from Matrigel assay and (C) quantitative analysis of number of formed loops with different percentage of EVs (5%, 10% and 20%). Magnification ×4. White scale bar = 200 μm. *p < 0.05, **p < 0.01, ***p < 0.001. One-way ANOVA test was applied. A range of N = 3–12 experiments was performed. (D) Confocal microscopy images of 3D in vitro bioprinted HUVEC (bright and fluorescent images), displaying the formation of 3D-tubules angiogenic structures. DAPI, CD31 and vWF stain blue, red and green, respectively. White scale bar = 100 μm. (E) Analysis of the immunofluorescence indicating the percentage of the positive 3D endothelial tubules area in the different conditions. *p < 0.05. ANOVA, analysis of variance; EV, extracellular vesicle; HUVEC, human umbilical vein endothelial cell; PL, platelet lysate
compared to the negative control (EBM, non-supplemented endothelial basal media, \( p = 0.0038 \)). Platelets lysate and EGM-2 were used as positive angiogenic inducers. To corroborate this observation, we employed a 3D bioprinting-based approach by encapsulating HUVECs in a gelatin/methacrylamide (GelMA) bioink, to evaluate their ability to induce the generation of vessel-like structures in a more physiologically suitable 3D microenvironment in presence of 10% EVs (the best performer in the Matrigel assay). The confocal microscopy analysis showed that endothelial cells were able to colonize the bioprinted construct after treatment with both PL and PL-derived EVs. Coherent with the observed spatial distribution, the proportion of the endothelial area (defined as CD31\(^+\)/vWF\(^+\)), corresponding to the organization of HUVECs in 3D tubular structures, was significantly higher with PL-derived EV and PL treatments, compared to EBM control (Figure 3D,E; \( p < 0.05 \) vs. 10% EVs and 10% PL).

Several studies have demonstrated the modulation of the redox status in cells exposed to intact platelet-derived EVs.\(^{67}\) Thus, we investigated the levels of hydrogen peroxide in the conditioned media of HUVECs following treatment with different percentages of PL-derived EVs (5%, 10%, 20%). (A) Hydrogen peroxide \((H_2O_2)\) measurement of HUVEC-derived condition media in all experimental conditions. The cartoon on the left of the graph shows the wide concentration range of \( H_2O_2 \) from physiological states \((\leq 100 \mu M)\) to oxidative stress \((\geq 300 \mu M)\). *\( p < 0.05 \), **\( p < 0.01 \). One-way ANOVA test was applied. A range of \( N = 3–16 \) experiments was performed. (B) Relative expression of the NADPH oxidase isoform 4 (NOX4) assayed by real-time PCR and downregulated in HUVEC after treatment with 10% EVs respect to EBM and all other percentage of EVs (5% and 20%). The effect is also and comparable to that exerted by 10% PL and EGM-2. *\( p < 0.05 \). One-way ANOVA test was applied. A range of \( N = 3–16 \) experiments was performed. ANOVA, analysis of variance; EV, extracellular vesicle; HUVEC, human umbilical vein endothelial cell; PL, platelet lysate.

Some key functions of platelets, such as aggregation, activation and angiogenesis, are known to be mediated by miRNAs released by platelets in response to a wide range of stimuli, both physiological and pathological.\(^{71,72}\) This ability can also be mediated by EVs, since they are known to transfer information to target cells through miRNAs,\(^{73}\) and therefore to determine diverse biological effects in relation to the cargo within the vesicles. With these premises, we hypothesized the presence of miRNAs in PL-based formulations and assessed this by analysing the miRNA profile of two different batches of PL for a total of four replicates. Results showed that the majority of the small RNA content in PL is represented by miRNAs (43%), followed by Y RNAs (17%), anti-sense RNAs (10%), and lincRNAs (8%) (Figure 5A). A miscellaneous group is also represented (22%). After applying a cut-off of >10 copies in all analysed batches of PL for a total of four replicates. Results showed that the majority of the small RNA content in PL is represented by miRNAs (43%), followed by Y RNAs (17%), anti-sense RNAs (10%), and lincRNAs (8%) (Figure 5A).
FIGURE 5 MiRNome characterization of platelet lysate by small RNA sequencing. (A) Small RNA percentage distribution in PL (a total of four replicates). (B) The heatmap has been obtained by including highly and consistently expressed miRNAs among the four analysed replicates with a cut-off of >10 copies. The red-yellow and the blue range colour indicate miRNAs with high and low average copy count, respectively. (C) Integration of the biological function with the expression of miRNAs in PL using a linkage group intertwined with five GO terms. Both the dimension of the box (circle/triangle) and the colour range reflect the expression levels as in the heatmap. (D) Quantitative PCR of hsa-miR-320a, hsa-miR-25 and hsa-miR-126 have been employed to quantitatively validate the miRNome of PL. These three miRNAs are represented in the triangle box in C. PL, platelet lysate.
| ENSEMBL gene   | miRBase ID   | Avg count (four replicates) |
|---------------|--------------|----------------------------|
| ENSG00000199179 | hsa-let-7i    | 35,420.48575               |
| ENSG00000283450.1 | hsa-miR-486  | 25,404.28176               |
| ENSG00000199085 | hsa-miR-148a | 21,102.40537               |
| ENSG00000207789.1 | hsa-miR-26a  | 16,213.89862               |
| ENSG00000208037 | hsa-miR-320a | 9701.952607                |
| ENSG00000199065.3 | hsa-miR-101  | 9436.507077                |
| ENSG00000199122 | hsa-miR-148b | 7450.524382                |
| ENSG00000199150 | hsa-let-7g   | 7267.479277                |
| ENSG00000207581 | hsa-miR-199b | 6969.810643                |
| ENSG00000208012 | hsa-let-7f   | 6357.87107                 |
| ENSG00000207605 | hsa-miR-191  | 5812.57756                 |
| ENSG00000207547 | hsa-miR-25   | 5236.777868                |
| ENSG00000207948 | hsa-miR-328  | 3040.629801                |
| ENSG00000207714 | hsa-miR-584  | 2989.18177                 |
| ENSG00000266297 | hsa-miR-744  | 2822.249442                |
| ENSG00000254324 | hsa-miR-151a | 2812.521101                |
| ENSG00000199121 | hsa-miR-26b  | 2632.304808                |
| ENSG00000208023 | hsa-miR-185  | 2630.517199                |
| ENSG00000199153 | hsa-miR-30d  | 2122.871247                |
| ENSG00000207550 | hsa-miR-99b  | 2005.789909                |
| ENSG00000198995 | hsa-miR-340  | 1944.070041                |
| ENSG00000199107 | hsa-miR-409  | 1709.570737                |
| ENSG00000207595.1 | hsa-miR-181a | 1664.90131                 |
| ENSG00000207654.4 | hsa-miR-128  | 1615.027686                |
| ENSG00000264297 | hsa-miR-443b | 1526.263215                |
| ENSG00000208036 | hsa-miR-106b | 1475.271861                |
| ENSG00000207608 | hsa-miR-127  | 1461.537107                |
| ENSG00000208004 | hsa-miR-323b | 1379.391397                |
| ENSG00000207651 | hsa-miR-28   | 1345.078854                |
| ENSG00000208017 | hsa-miR-140  | 1321.751562                |
| ENSG00000271886 | hsa-miR-98   | 1222.679492                |
| ENSG00000199030 | hsa-let-7c   | 1214.65648                 |
| ENSG00000272458 | hsa-miR-432  | 1207.070114                |
| ENSG00000199161 | hsa-miR-126  | 1171.006656                |
| ENSG00000207721 | hsa-miR-186  | 1166.72288                 |
| ENSG00000207947 | hsa-miR-152  | 1111.222163                |
| ENSG00000198987 | hsa-miR-16   | 1072.827202                |
| ENSG00000216099 | hsa-miR-889  | 1046.656644                |
| ENSG00000198974 | hsa-miR-30e  | 1001.082556                |
| ENSG00000199043 | hsa-miR-335  | 982.9147665                |
| ENSG00000199024.1 | hsa-miR-103a | 974.3511292                |
| ENSG00000207752 | hsa-miR-199a | 919.4531345                |
| ENSG00000207870 | hsa-miR-221  | 895.0699612                 |
| ENSG00000207864 | hsa-miR-27b  | 821.4878295                |
| ENSG00000199032 | hsa-miR-425  | 772.8305776                 |
| ENSG00000207638 | hsa-miR-99a  | 740.1333674                 |

(Continues)
| ENSEMBL gene     | miRBase ID  | Avg count (four replicates) |
|------------------|-------------|-----------------------------|
| ENSG00000207934  | hsa-miR-654 | 621.1900075                 |
| ENSG00000208008  | hsa-miR-125a| 597.3963088                 |
| ENSG00000207725  | hsa-miR-222 | 538.5877917                 |
| ENSG00000198975  | hsa-let-7a  | 515.1025809                 |
| ENSG00000207781  | hsa-miR-625 | 505.7290621                 |
| ENSG00000265253  | hsa-miR-4446| 451.4169995                 |
| ENSG00000212040  | hsa-miR-543 | 409.0533201                 |
| ENSG00000207757  | hsa-miR-93  | 403.8187224                 |
| ENSG00000272036  | hsa-miR-139 | 399.12804                   |
| ENSG00000207962  | hsa-miR-30c | 390.0986257                 |
| ENSG00000199051  | hsa-miR-361 | 389.718269                  |
| ENSG00000207970  | hsa-miR-660 | 340.2597669                 |
| ENSG00000207758  | hsa-miR-532 | 318.869568                  |
| ENSG00000208008.1| hsa-miR-125b| 315.48886                   |
| ENSG00000283170  | hsa-miR-382 | 314.8899174                 |
| ENSG00000207737.1| hsa-miR-181b| 314.5318373                 |
| ENSG00000208027  | hsa-miR-485 | 289.3005292                 |
| ENSG00000211580  | hsa-miR-769 | 283.8908085                 |
| ENSG00000199025  | hsa-miR-369 | 273.869608                  |
| ENSG00000199069  | hsa-miR-323a| 248.201048                  |
| ENSG00000198972  | hsa-let-7e  | 228.5801569                 |
| ENSG00000207569  | hsa-miR-433 | 218.3957338                 |
| ENSG00000207989  | hsa-miR-493 | 216.51903                   |
| ENSG00000202569  | hsa-miR-146b| 214.64207                   |
| ENSG00000198997  | hsa-miR-107 | 203.1544061                 |
| ENSG00000194717  | hsa-miR-494 | 200.0772964                 |
| ENSG00000199023  | hsa-miR-339 | 193.5326846                 |
| ENSG00000199082  | hsa-miR-342 | 190.9249656                 |
| ENSG00000221406.1| hsa-miR-320b| 180.4782364                 |
| ENSG00000221445  | hsa-miR-1301| 166.0596828                 |
| ENSG00000207633  | hsa-miR-505 | 149.7697394                 |
| ENSG00000207563  | hsa-miR-23b | 149.228785                  |
| ENSG00000207973  | hsa-miR-589 | 143.1520471                 |
| ENSG00000208013  | hsa-miR-652 | 133.0130015                 |
| ENSG00000211578  | hsa-miR-766 | 129.7966503                 |
| ENSG00000207965  | hsa-miR-629 | 125.3803452                 |
| ENSG00000207779  | hsa-miR-15b | 125.0632511                 |
| ENSG00000199047  | hsa-miR-378a| 121.8129584                 |
| ENSG00000199020  | hsa-miR-381 | 116.4263774                 |
| ENSG00000199017  | hsa-miR-1   | 109.082889                  |
| ENSG00000199109  | hsa-miR-411 | 103.8221213                 |
| ENSG00000264864  | hsa-miR-3613| 97.42976184                 |
| ENSG00000283604  | hsa-miR-338 | 94.82506088                 |
| ENSG00000221214  | hsa-miR-548e| 92.96228251                 |
| ENSG00000211582  | hsa-miR-758 | 91.70920684                 |
| ENSG00000207990  | hsa-miR-182 | 90.79786569                 |
| ENSEMBL gene | miRBase ID | Avg count (four replicates) |
|--------------|------------|-----------------------------|
| ENSG00000207993 | hsa-miR-134 | 88.31512911 |
| ENSG00000283871 | hsa-miR-130b | 83.90167079 |
| ENSG00000207827 | hsa-miR-30a | 81.14539472 |
| ENSG00000221760 | hsa-miR-548j | 77.90232379 |
| ENSG00000207743 | hsa-miR-495 | 76.96521629 |
| ENSG00000266192 | hsa-miR-1260b | 74.37795737 |
| ENSG00000207703 | hsa-miR-7 | 74.26392405 |
| ENSG00000199088 | hsa-miR-379 | 72.24067434 |
| ENSG00000207762.1 | hsa-miR-329 | 71.91954104 |
| ENSG00000284032 | hsa-miR-29a | 68.38616085 |
| ENSG00000274466 | hsa-miR-1273h | 66.08291135 |
| ENSG00000284231 | hsa-miR-424 | 65.47781571 |
| ENSG00000199168 | hsa-miR-374a | 60.16913084 |
| ENSG00000207994 | hsa-miR-100 | 58.7905659 |
| ENSG00000207744 | hsa-miR-10b | 55.09601822 |
| ENSG00000221493.1 | hsa-miR-320c | 54.59647569 |
| ENSG00000264931 | hsa-miR-3138 | 53.8840101 |
| ENSG00000265154 | hsa-miR-151b | 53.7004073 |
| ENSG00000283152 | hsa-miR-3120 | 52.1660638 |
| ENSG00000207742 | hsa-miR-487a | 50.73182344 |
| ENSG00000207628 | hsa-miR-651 | 50.3340178 |
| ENSG00000199172 | hsa-miR-331 | 46.95913882 |
| ENSG00000207782 | hsa-miR-150 | 46.70532224 |
| ENSG00000207755 | hsa-miR-450a | 43.8147299 |
| ENSG00000283891 | hsa-miR-628 | 41.5096656 |
| ENSG00000284195 | hsa-miR-6852 | 40.13295067 |
| ENSG00000215930 | hsa-miR-942 | 39.06812916 |
| ENSG00000221745 | hsa-miR-1197 | 38.78852425 |
| ENSG00000207942 | hsa-miR-136 | 37.0613353 |
| ENSG00000207944 | hsa-miR-574 | 36.46764017 |
| ENSG00000207568 | hsa-miR-203a | 35.70390632 |
| ENSG00000216001 | hsa-miR-450b | 35.41952103 |
| ENSG00000211157 | hsa-miR-760 | 33.74964625 |
| ENSG00000221333 | hsa-miR-548k | 32.79941145 |
| ENSG00000283785 | hsa-miR-15a | 31.76970682 |
| ENSG00000208005 | hsa-miR-503 | 29.3742859 |
| ENSG00000199090 | hsa-miR-326 | 29.13483177 |
| ENSG00000263456 | hsa-miR-5189 | 28.3987136 |
| ENSG00000221754 | hsa-miR-1260a | 25.2317752 |
| ENSG00000265820 | hsa-miR-3177 | 24.77186052 |
| ENSG00000263575 | hsa-miR-4665 | 24.67203585 |
| ENSG00000207582 | hsa-miR-30b | 23.79214734 |
| ENSG00000283929 | hsa-miR-5010 | 23.58517512 |
| ENSG00000221464 | hsa-miR-1271 | 23.45850327 |
| ENSG00000207959 | hsa-miR-656 | 23.24797065 |
| ENSG00000207698 | hsa-miR-32 | 22.99690997 |

(Continues)
As a further selective step, we set a threshold for miRNAs with over 1000 reads, thus obtaining a shortlist of 39 miRNAs (Table 1), which underwent a bioinformatic top-down analysis on the mirPath v.3 online tool (reverse search). The gene ontology (GO) terms selection was performed according to the function of interest observed for PL treatment on endothelial cells, which is angiogenesis. We interrogated the database by employing five GO, including the first two with the highest hierarchy for processes of capillary and vascular formation, in particular: VASCULOGENESIS (GO_0001570), ANGIOGENESIS (GO_0001525), ENDOTHELIAL CELL ACTIVATION (GO_0042118), VASCULAR DEVELOPMENT (GO_0001944) and ENDOTHELIAL TUBE MORPHOGENESIS (GO_0061154).

| ENSEMBL gene          | miRBase ID | Avg count (four replicates) |
|-----------------------|------------|----------------------------|
| ENSG00000199053       | hsa-miR-324| 22.040929532               |
| ENSG00000207613       | hsa-miR-181c| 21.68525112                |
| ENSG00000211538       | hsa-miR-501| 21.1697488                 |
| ENSG00000207996       | hsa-miR-301a| 19.45112604                |
| ENSG00000207730       | hsa-miR-200b| 18.50465325                |
| ENSG00000199005       | hsa-miR-370| 17.84907533                |
| ENSG00000277255       | hsa-miR-7854| 17.66723576                |
| ENSG00000276365       | hsa-miR-145| 16.87146305                |
| ENSG00000199080       | hsa-miR-133b| 16.79696452                |
| ENSG00000283609       | hsa-miR-4662a| 16.70017447               |
| ENSG00000264607       | hsa-miR-3173| 16.28783901                |
| ENSG00000211514       | hsa-miR-454| 15.84741881                 |
| ENSG00000283279       | hsa-miR-376c| 15.5124361                  |
| ENSG00000263813       | hsa-miR-3679| 15.27222439                |
| ENSG00000284035       | hsa-miR-5187| 15.2522619                 |
| ENSG00000199038       | hsa-miR-210| 14.78583447                |
| ENSG00000199012       | hsa-miR-412| 13.67471599                 |
| ENSG00000264141       | hsa-miR-3928| 13.43883139                 |
| ENSG00000273932       | hsa-miR-6877| 12.95400748                |
| ENSG00000263652       | hsa-miR-548ax| 12.60596835              |
| ENSG00000253008       | hsa-miR-2355| 12.55831531                |
| ENSG00000207754       | hsa-miR-487b| 12.31700799                 |
| ENSG00000207617       | hsa-miR-3074| 12.1477769                  |
| ENSG00000284154       | hsa-miR-3605| 11.67230493                 |
| ENSG00000221463       | hsa-miR-1277| 11.5186948                  |
| ENSG00000207988       | hsa-miR-576| 11.43428817                 |
| ENSG00000207784       | hsa-miR-542| 10.87185064                 |
| ENSG00000267200       | hsa-miR-132| 10.58107814                 |
| ENSG00000221025       | hsa-miR-1250| 10.23728349                |
| ENSG00000221510       | hsa-miR-548b| 10.11483819                |
| ENSG00000283203       | hsa-miR-1246| 10.02602246                 |
| ENSG00000265565       | hsa-miR-3143| 10.02172938                 |

Note: Specifically, the selection here reported was made by including only those miRNAs with average counts >10 copies. The miRNome was performed on four replicates of PL batches. The first 39 miRNAs with a threshold of over 1000 reads are highlighted in red.

Abbreviation: PL, platelet lysate.

Table 1 (Continued)

After intersecting each GO term with the top 39 miRNA shortlist, we extrapolated potential eligible candidates for the abovementioned roles. We found that the highest overlap was with the angiogenesis gene list as displayed in the function-expression interaction network that we generated by software (Figure 5C). Afterwards, we compared the results and shortlisted the main group of 31 miRNAs and a further subset of 11 miRNAs correlating with two and all five GO categories, respectively, where three miRNAs (hsa-miR-320a, hsa-miR-25 and hsa-miR-126) were selected to quantitatively validate the seq data by real-time PCR (Table 2). Notably, miR-126 is a key regulator of angiogenesis and is known as the angio-miRNA and one of the most abundant and specific miR to endothelial cells, human platelets and
platelet-derived vesicles. Notably, the qPCR data accurately highlighted that miR-126 is the most abundant miRNA in PL, compared to miR-320a and miR-25 (Figure 5D; \( p < 0.01 \) and \( p > 0.001 \)).

Next, we validated the role of miR-126 in the biological effects of PL and EVs on HUVECs, by comparing the sole stimuli that were the media supplemented with 10% PL or 5%, 10% and 20% EVs (with volumes and dilutions adjusted to correspond to 10% PL). Although an increasing but not statistically significant trend of miR-126 was observed among the percentages of EVs, real-time PCR testing confirmed the equivalent content of miR-126 in all media recipes (Figure 6A). The treatment with 10% EVs was the only able to significantly upregulate the levels of intracellular miR-126 in endothelial cells compared to the negative control (Figure 6B; \( p < 0.05 \)). We sought to verify whether EV-miR-126 could play a direct role in mediating angiogenesis ascribable to the transferring of this miRNA through EVs. To this aim, we transfected HUVECs with the antagoniR-126 (LNA-126) to rule out endogenous contribution. Then, we stimulated the cells with 10% EVs or PL. Results showed that HUVECs were efficiently transfected by both the LNA-126 and control, as shown by flow cytometry analysis (Figure S1a; 84.84 ± 0.4% positive cells with LNA-126 and 78.5 ± 5.15% with control). Moreover, the copy number of miR-126 in HUVEC, quantified by droplet digital PCR, was significantly decreased at the lowest levels 24 h after treatment with the LNA-126, compared to both untreated and transfection controls (Figure S1b; \( p < 0.01 \) and \( p < 0.05 \), respectively), while at 48 h the levels had increased again.

Finally, we tested the functional effects of blocking EV-miR-126 on angiogenesis and if HIF-1α or VEGF, known to upregulate miR-126 and to be abundant in PL, respectively, might be downstream targets. In physiological conditions, the Matrigel assay showed that the number of loops significantly increased in presence of LNA-126 but with treatment with 10% EV or 10% PL compared to control EBMM (Figure 6C, D, all \( p < 0.05 \) and Figure S1c,d for Matrigel with control), therefore, confirming the angiogenic effect contained in the EVs derived from PL and mediated at least partially by miR-126. The analysis of the matches to human 3′-UTRs of both HIF-1α and VEGF through the TargetScan software revealed that only VEGF is a target of miR-126-3p in the poorly conserved category (Figure 6E). Nevertheless, the validation by real-time PCR showed no modulatory effect in our system ascribable to VEGF (Figure 6E), suggesting different molecular targets controlled by miR-126.

### DISCUSSION

This study demonstrates that PL formulations are enriched with EVs, which are biologically active and efficiently sustain angiogenesis in endothelial cells. Interestingly, the EV concentration in our PL is very high and mainly composed of a small EVs subset (50–200 nm), suggesting that PL might be reinterpreted as an abundant biological source of this EVs subpopulation.

Intriguingly, in line with reports showing comparable in vitro and in vivo haemostatic properties of both platelet microparticles and PL-
derived EVs on platelets, our EVs only partially retain this feature. Our PL-derived EVs do not promote platelet aggregation, but they are able to preserve coagulation in a physiological manner, strengthening their versatile use as angiogenic/antiaggregant means for cardiovascular applications (where platelet aggregation is a critical risk factor), as a topical product for bleeding during surgery, or as a...

FIGURE 6 Evaluation of hsa-miR-126 in endothelial cells after treatment with PL-derived EVs. (A) Quantitative real-time PCR, showing a similar amount of has-miR-126 among the percentages of EVs (5%, 10% and 20%) and 10% PL when employed as stimulus. One-way ANOVA test was applied. A range of \( N = 3-16 \) experiments was performed. (B) Relative expression of has-miR-126 in HUVEC by real-time PCR after treatment with 5%, 10% and 20% EVs or 10% PL. The fold change on the basal EBM is indicated. \( * p < 0.05 \). (C) Representative optical images and (D) quantification of the matrigel assay of HUVEC after antagomir-126 (LNA_126) transfection and different treatments. Magnification ×4. White scale bar = 200 μm. \( * p < 0.05 \). (E) Targetscan analysis at the human 3′-UTR of VEGF, highlighting that it is a target of miR-126-3p in the poorly conserved category and the validation by RTPCR showing no modulatory effect of this gene in our system. A range of \( N = 3-7 \) experiments was performed. ANOVA, analysis of variance; EV, extracellular vesicle; HUVEC, human umbilical vein endothelial cell; PL, platelet lysate.
Although PL also contains a wide range of overlapping miRNAs, we found that angiogenic miRNAs (miR-320, miR-25 and miR-126) are contained in the EV cargo as well as in PL. So far, proper screening of the miRNA profile has been performed only in PRP and intact or hyperreactive platelets from healthy subjects, or in the presence of cardiovascular pathologies. Interestingly, when we profiled our PL, data have shown that the formulation reflects a similar repertoire of mature miRNAs found in human platelets and described in the literature, including the abundant miRlet-7 (a marker of platelet differentiation and maturation in megakaryocytes), or defined microRNA families (e.g., miR-25 and miR-103). By intertwining the transcriptomic expression profile with the vascular function, we have confirmed that in PL-based preparations the miRNAs quantitatively more represented are also strictly interconnected to the angiogenic function. The EVs contained in PL mirror this picture and confirm that miR-126 is the most significant miRNA in both preparations.

The angio-miRNA miR-126 is one of the most abundant miRNA expressed in platelets. Sharing with miR-320 (that we also found as highly represented) the unique expression also in endothelial cells, miR-126 is able to downregulate adhesion molecules (e.g., VCAM-1) upon the influence of specific cytokines (i.e., VEGF), therefore contributing to endothelial migration, proliferation, activation and vascular inflammation. Exosomes enriched in miR-126 are strictly correlated with protection from ischemic events and atherosclerosis progression. Changes in circulating levels of miR-126 have been described in patients with acute ischemic stroke, coronary artery disease or type 2 diabetes. Moreover, vascular development and integrity are sustained by miR-126 in zebrafish and mice, whereas in vivo silencing of miR-126 impaired angiogenesis upon ischemic insult. Thus, miR-126 appears as a potential biomarker and therapeutic target for angiogenesis. Nonetheless, the transfer of miRNAs in the form of EVs under physiological and pathological conditions from platelets to endothelial cells (and vice versa), and the modality by which biological functions are sustained, are still under intensive investigation.

Our data confirm that miR-126 of platelet origin plays a key role in the angiogenic homeostasis of endothelial cells. Accordingly, results highlight that HUVECs increase intracellular levels of miR-126 upon stimulation with EVs of PL origin, by adding exogenous miR126 by EV transfer when the endogenous miR-126 is silenced. Thus, our results demonstrate that a fraction of the angiogenic effect induced by the whole PL preparation is directly ascribable to the EV cargo, specifically to platelet-derived miR-126.

This study has some limitations. Although we found that VEGF is a target of miR-126, we could not observe any modulatory effect in our system, suggesting that alternative mechanisms are needed to be verified. Only a few of them have been already described. For instance, the DNA methyltransferase, playing a role in hypoxia tolerance, has been found as a target of miR-126 contained in exosomes. Further mechanisms can coexist, including the reduction of cell apoptosis, the overactivation of autophagy through Beclin, the novel delivery system by apoptotic bodies through CXCL12 or the inhibition of the negative regulators of the VEGF-axis. More importantly, the angiogenic properties of both EVs and PL cannot be explained uniquely by the miR-126. The miRNome here described suggests the presence of additional miRNAs with similar functions.
For instance, the exosomal derived-miR25 has been found to promote angiogenesis, vascular permeabilization, metastatic niches in cancer and involvement in cardiovascular disorders.116,117

To date, the individual contribution of EVs within PL has not been fully elucidated in terms of regenerative angiogenesis. Certainly, the methodology to manufacture PL severely impacts the quantity and quality of EVs within the formulations and in particular that employed to concentrate, lyse or activate platelets118 in the formulation. Accordingly, PL preparations with excessive heterogeneity of EV content might result in parallel different downstream signalling and pathways activated with a wide range of unpredictable biological effects, also depending on cells potentially targeted by clinical PL preparations.

In conclusion, PL-based formulations are a source of both biologically available miRNAs and EVs defining the hallmark of platelet origin. The EVs reflect the ‘angiogenic physiology’ of PL, confirming that a cell-free therapy approach may be a novel effective strategic tool in clinical applications.

Future investigations will be required to unveil the role of downstream targets of different miRNAs potentially preserved in EVs of platelet origin, and how additional processes not limited to angiogenesis are modulated, including immunomodulatory functions and paracrine effects.

**AUTHOR CONTRIBUTIONS**

Antonella Bordin performed the main experiments. Maila Chirivi, Marika Milan and Roberto Rizzi performed 3D constructs. Francesca Pagano performed qPCR of small RNA seq. Marco Iuliano and Eleonora Scaccia isolated the EVs. Orazio Fortunato and Giorgio Mangino performed NTA and cytofluorimetry. Xhulio Dhori developed the droplet digital PCR. Elisabetta De Marinis performed the droplet digital PCR. Alessandra D’Amico and Fabio Pulcinelli performed all experiments on aggregation and oxidative states. Selenia Miglietta performed the TEM. Vittorio Picchio the matrix for the angiogenic network of the small RNA seq. Elisabetta De Falco and Isotta Chimenti reviewed and edited the manuscript. Elena De Falco conceived the study and wrote the paper.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

**DATA AVAILABILITY STATEMENT**

Main data generated or analysed during this study are included in this article, and detailed data are available from the corresponding authors on reasonable request.

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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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