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Article

Microparticles Carrying Sonic Hedgehog Are Increased in Humans with Peripheral Artery Disease

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Abstract: Sonic Hedgehog (Shh) is a prototypical angiogenic agent with a crucial role in the regulation of angiogenesis. Experimental studies have shown that Shh is upregulated in response to ischemia. Also, Shh may be found on the surface of circulating microparticles (MPs) and MPs bearing Shh (Shh+ MPs) have shown the ability to contribute to reparative neovascularization after ischemic injury in mice. In this study, the plasma number of Shh+ MPs in patients with peripheral artery disease (PAD) and control subjects without PAD. We found significantly higher number of Shh+ MPs in plasma of subjects with PAD, compared to controls, while the global number of MPs – produced either by endothelial cells, platelets, leukocytes, and erythrocytes – was not different between PAD patients and controls. Interestingly, the concentration of Shh protein unbound to MPs – which was measured in MP-depleted plasma – was not different between subjects with PAD and controls, indicating that, in the setting of PAD, the call for Shh recapitulation does not lead to secretion of protein into the blood but to binding of the protein to the membrane of MPs. These findings provide novel insights on the mechanisms through which the Shh signaling is reactivated during ischemia in humans, with potentially important fundamental and clinical implications.

Keywords: Sonic hedgehog; Microparticles; Peripheral artery disease.

1. Introduction

Sonic hedgehog (Shh) is a morphogen belonging to the hedgehog (Hh) family of proteins and is crucial during embryonic development [1]. In post-natal life, the reactivation of the Shh pathway has been observed in various organs and tissues after injury and during regeneration, as well as in tumors [2]. In multiple experimental models, it has been demonstrated that ischemia induces the recapitulation of the Shh pathway, triggering a variety of responses in endothelial cells (ECs), endothelial-progenitor cells (EPCs), smooth muscle cells (SMCs), and fibroblasts, and promoting angiogenesis and vasculogenesis [3-7].

Microparticles (MPs) are small plasma membrane fragments shed by cells after blebbing due to cell activation and/or apoptosis. They play an important role in cell to cell communication because of their ability to act at distant site as well as locally, and to propagate the functional antigens of their parent cell [8]. Angiogenesis is among the processes that may be regulated by MPs [9].

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There is experimental evidence that MPs harboring Shh (Shh+ MPs) may modulate the nitric oxide (NO) pathway, regulate the production of pro-angiogenic factors and the up-regulation of proteins involved in cell adhesion, and orchestrate several processes related to cell proliferation, differentiation and angiogenesis [10]. However, such findings have been generated only in pre-clinical settings.

The experiments presented in this paper have tested the hypothesis that, in humans with peripheral artery disease (PAD) – a prototypical ischemic cardiovascular pathology with morbidity and mortality rates higher than coronary artery disease (CAD) [11] – there is increased production of Shh, similar to what has been previously demonstrated by our groups in various animal models of ischemia [3, 12-14]. Our findings demonstrate increased levels of Shh bound to circulating MPs in the plasma of subjects with PAD, compared to controls, while Shh concentration in MP-depleted plasma is similar in subjects with and without PAD.

2. Results

The main demographic and clinical characteristics of the studied population are summarized in **Table 1**. Briefly, PAD patients and controls did not differ in terms of age (71.4±9.4 vs 70.3±8.2 p=n.s.) and presence of diabetes (44.0% vs 34.0%, p=n.s.), dyslipidemia (72.0% vs 60.0% p=n.s.), and hypertension (80.0% vs 80.0% p= n.s.). PAD patients were more frequently men (74.0% vs 54.0%, $p<0.05$) and smokers (34.0% vs 14.0%, $p<0.05$). History of myocardial infarction (MI) and stroke was reported more frequently by PAD patients than controls (42.0% vs 8.0%, $p<0.01$ and 12.0% vs 0.0%, $p<0.05$, respectively). Regarding pharmacological therapy, 84.0% of PAD patients were on single anti-platelet treatment (SAPT) (either aspirin, clopidogrel, cilostazol, or ticlopidine) and 10.0% were on double antiplatelet therapy (DAPT). On the other hand, 48.0% of controls were on SAPT and none was in DAPT. The use of statins, beta-blockers, and ACE inhibitors/ARB was not statistically different between patients and controls (72.0% vs 56.0%, 54.0% vs 52.0%, and 46.0% vs 38.0%, respectively, p=n.s.). Regarding disease severity, 15 PAD patients were in Leriche-Fontaine stage IIa (30.0%), 15 were in stage IIb (30.0%), 10 in stage III (20.0%), and 10 in stage IV (20.0%).

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Variables		PAD	Controls	p
Years of age (mean ± SD)		71.4 ± 9.4	70.3 ± 8.2	n.s.
Males, n (%)		37 (74.0)	27 (54.0)	<0.05
Smokers, n (%)		17 (34.0)	7 (14.0)	<0.01
Diabetes, n (%)		22 (44.0)	17 (34.0)	n.s.
Dyslipidemia, n (%)		36 (72.0)	30 (60.0)	n.s.
Hypertension, n (%)		40 (80.0)	40 (80.0)	n.s.
Previous MI, n (%)		21 (42.0)	4 (8.0)	<0.01
Previous Stroke, n (%)		6 (12.0)	0 (0.0)	<0.01
Therapy	SAPT, n (%)	33 (66.0)	24 (48.0)	0.04
	DAPT, n (%)	9 (18.0)	0 (0.0)	<0.01
	Statins, n (%)	36 (72.0)	28 (56.0)	n.s.
	ACE/ARB inhibitors, n (%)	27 (54.0)	26 (52.0)	n.s.
	Beta-blockers, n (%)	23 (46.0)	19 (38.0)	n.s.

Table 1. Demographic and clinical characteristics of the studied population.

When looking at the global number of MPs of endothelial origin (EMPs), no significant difference was found between PAD patients and controls (138.2±49.2 vs 130.6±62.2, p=n.s.) (**Figure 1a**). Similarly, no differences were found in terms of global number of platelet-derived MPs (PMPs) (143.9±77.1 vs 126.1±61.6, p=n.s.) (**Figure 1b**), leucocyte-derived MPs (LMPs) (126.9±116.6 vs 130.5±79.0, p=n.s.) (**Figure 1c)**, and erythrocyte-derived MPs (ErMPs) (315.5±238.7 vs 271.6±159.5, p=n.s.) (**Figure 1d**).

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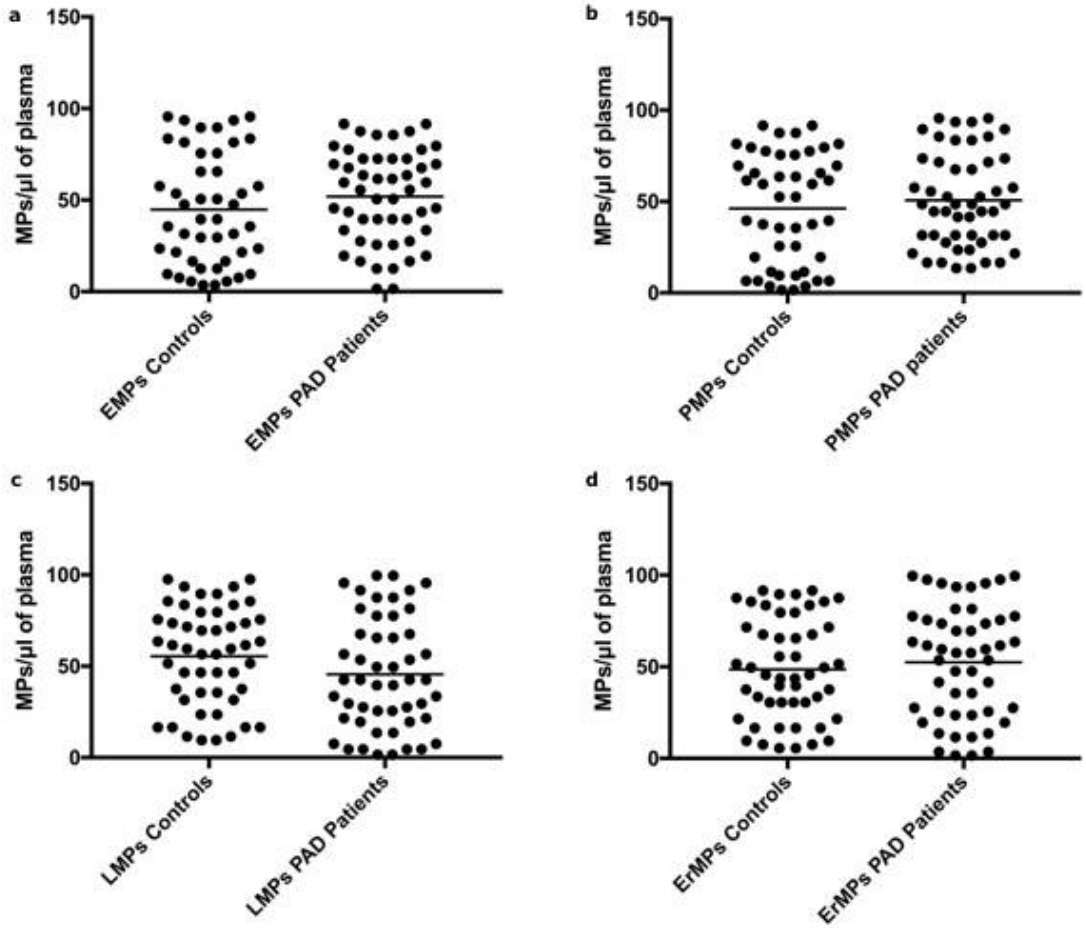

Figure 1. Global number of EMPs (a), PMPs (b), LMPs (c), and ErMPs (d) in plasma of PAD patients and controls.

In contrast, we found a significantly higher number of Shh+ MPs in the plasma of subjects with PAD, compared to controls (70.5±19.0 vs 27.2±8.5, p<0.001) (**Figure 2a**). This increment was due to an increased number of Shh+ EMPs (22.3±21.8 vs 9.2±6.0, p<0.001) (**Figure 2b**), as well as to an increased number of Shh+ MPs derived from platelets (17.3±10.1 vs 9.9±4.6, p<0.001) (**Figure 2c**), leukocytes (15.2±7.9 vs 9.0±3.9, p<0.001) (**Figure 2d**), and erythrocytes (20.0±11.8 vs 9.8±4.6, p<0.001) (**Figure 2e**).

A summary of the results of these studies is presented in **Table 2**.

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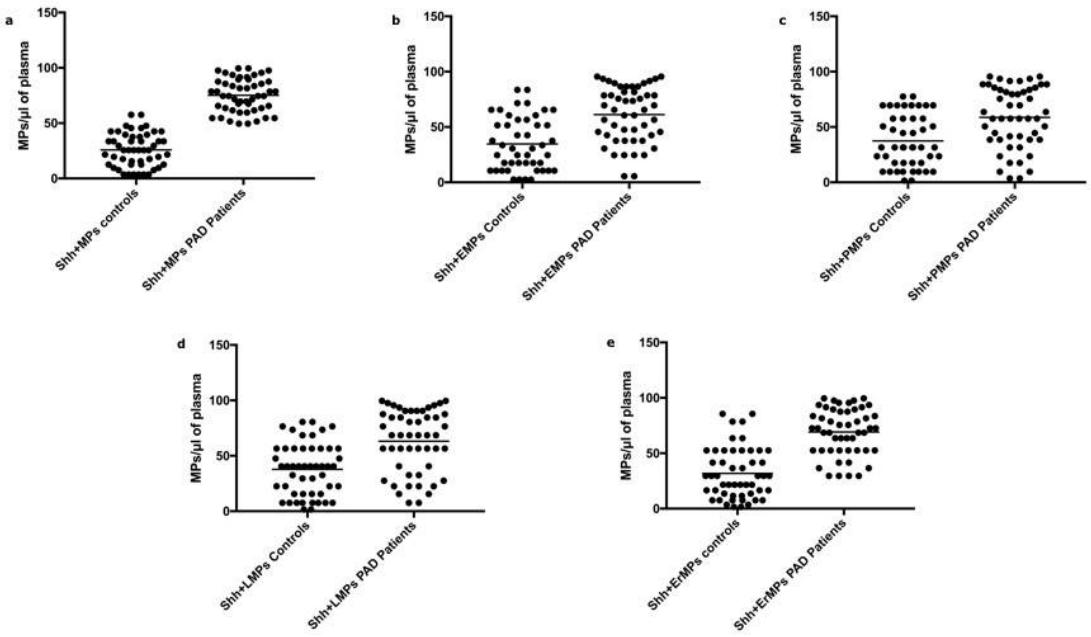

Figure 2. Global number of Shh+ MPs in PAD patients and controls (**a**); number of Shh+ EMPs (**b**), Shh+ PMPs (**c**), Shh+ LMPs (**d**), and Shh+ ErMPs (**e**) in PAD patients and controls.

	PAD	Controls	p
Shh+ MPs/µl (mean ±SD)	70.5±19.0	27.2±8.5	<0.001
EMPs/µl (mean ±SD)	138.2±49.2	130.6±62.2	n.s.
- Shh+ EMPs/µl (mean ±SD)	22.3±21.8	9.2±6.0	<0.001
PMPs/µl (mean ±SD)	143.9±77.1	126.1±61.6	n.s.
- Shh+ PMPs/µl (mean ±SD)	17.3±10.1	9.9±4.6	<0.001
LMPs/µl (mean ±SD)	126.9±116.6	130.5±79.0	n.s.
- Shh+ LMPs/µl (mean ±SD)	15.2±7.9	9.0±3.9	<0.001
ErMPs/µl (mean ±SD)	315.5±238.7	271.6±159.5	n.s.
- Shh+ ErMPs/µl (mean ±SD)	20.0±11.8	9.8±4.6	<0.001

Table 2. Number and type of circulating MPs in PAD patients and controls.

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We found detectable levels of Shh protein in the MP-depleted plasma of all the subjects included in the study. However, there was no difference between subjects with PAD and controls (47.5±8.5 pg/ml vs 53.5±25.8 pg/ml, p=n.s.) (**Figure 3**).

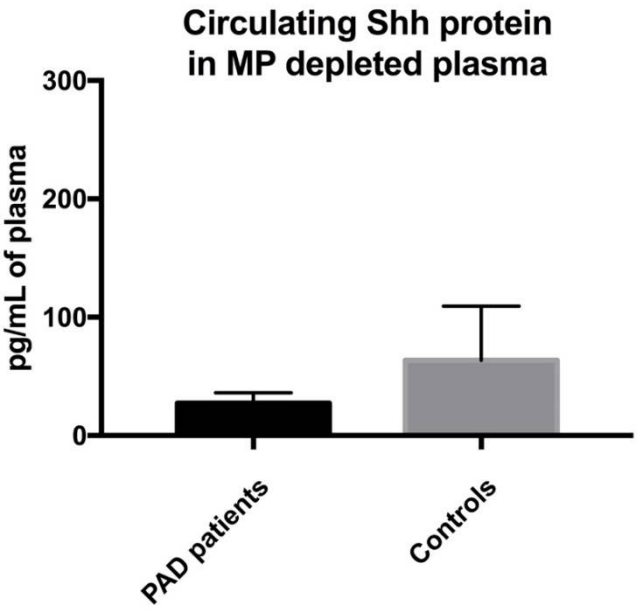

Figure 3. Levels of Shh protein in MP-depleted plasma of PAD patients and controls.

3. Discussion

We have previously demonstrated, in a number of experimental animal models, that ischemia has the ability to reactivate the Shh signaling pathway. For instance, upon induction of hindlimb ischemia in mice, there is strong upregulation of Shh within the ischemic area [12]. Recapitulation of the Shh pathway also occurs upon induction of myocardial ischemia, as well as in experimental models of diabetic vascular disease, in rats and rabbits [13, 14]. In ischemic conditions, the reactivation of the Shh pathway is functionally important, since Shh is a potent angiogenic agent able to upregulate various families of angiogenic growth factors and is considered an important pathway for tissue repair and regeneration [3, 15-17]. Recently, it has also been demonstrated that, in the ischemic skeletal muscle of mice, endogenous Shh has also the ability to limit inflammation [18].

This study was aimed to evaluate whether upregulation of Shh occurs also in humans in the setting of ischemia. We decided to study patients with PAD, because this is a prototypical ischemic pathology and is characterized by the fact that important muscular masses are exposed to severe degrees of ischemia over a prolonged period of time. We found that the number of MPs bearing Shh is significantly increased in subjects with PAD compared to controls. Importantly, we did not find an increment in the global number of circulating MPs of endothelial, platelet, leukocyte, or erythrocyte origin, but only in those MPs that carry Shh, thus suggesting a specific role for this type of MPs in this pathological condition.

Previous experimental studies have shown that Shh carried by MPs has the ability to induce NO release from ECs, trigger changes in the expression and phosphorylation of enzymes related to the NO pathway, and decrease production of reactive oxygen species [1]. It has also been reported that Shh+ MPs are capable to induce the formation of capillary-like structures in vitro and increase the expression of proangiogenic factors in cultured ECs [19]. More recently, it has been shown that Shh+ MPs are able to modulate neovascularization in a murine model of hind-limb ischemia, correct angiotensin II-induced hypertension and endothelial dysfunction in mice, and reduce infarct size in a rat model of cardiac ischemia-reperfusion injury [10, 20, 21]. In this scenario, our findings are consistent with the hypothesis that Shh+ MPs might be an underestimated player in the pathophysiology of ischemic disease and support the concept that MPs harboring Shh may have a

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role in contrasting endothelial dysfunction and contribute to the regulation of angiogenesis after ischemic injury.

Also, we determined the concentration of Shh protein in MP-depleted plasma of subjects with PAD and controls, finding no differences between the two groups. This is an interesting finding which might suggest that, in the setting of ischemia, the call for Shh recapitulation would not lead to the secretion of protein into the blood, where it would be inactivated by inhibitory proteins, but to the binding of the protein to the membrane of MPs, in order to warrant its activity and the delivery of the message in a protected environment. This would be consistent with previous reports in the literature, that have clearly demonstrated that Shh carried by MPs is functionally active and has its main target in ECs. In particular, using in vitro assays, it has been shown that Shh+ MPs are able to stimulate ECs to release NO, produce proangiogenic factors, and form capillary-like structures [19]. Shh+ MPs have shown their functional potentials also in vivo, when injected into rodents to stimulate angiogenesis [10], correct endothelial dysfunction [20], and reduce myocardial infarct size [21]. Thus, anchoring Shh on the surface of MPs might be a strategy used by producing cells to protect Shh from plasmatic inhibitory proteins, provide the lipid and protein environment necessary to maintain Shh activity, and deliver the active protein to target cells.

This study has some potential limitations. First, it has been conducted on a relatively number of patients and thus deserves confirmation in larger samples. Also, we have not assessed the functional properties of Shh+ MPs. In particular, it would be interesting to use in vitro and in vivo models of angiogenesis to determine whether MPs isolated from the plasma of subjects with PAD have different angiogenic capacities compared to MPs isolated from the plasma of subjects without PAD. Finally, it should be mentioned that previous data by other groups have found differences between PAD patients and controls in terms of number of circulating MPs of endothelial [22] or platelet [23] origin, in contrast with our findings. However, while in such previous studies control patients were subjects without cardiovascular diseases, or even healthy volunteers, our controls were subjects with carotid plaques.

In conclusion, this study shows that Shh bound to MPs is increased in subjects with PAD, while plasmatic Shh is not. Our findings provide novel insights on the mechanisms through which the Shh signaling is reactivated in the course of ischemia, with potentially important fundamental and clinical implications.

4. Materials and Methods

4.1. Patients

PAD subjects (n=50) and controls (n=50) were enrolled among patients consecutively admitted to the Vascular Medicine Outpatient Clinic of the A. Gemelli University Hospital of Rome, Italy and the Angiology Unit of the San Giacomo Hospital of Castelfranco Veneto, Italy, between 01 August 2015 and 30 September 2016. Inclusion criteria for patients were age>18 years, Caucasian race, and presence of clinically relevant PAD. Diagnosis of PAD was made in accordance with the criteria established by the Ad Hoc Committee on Reporting Standards of the Society for Vascular Surgery and the International Society for Cardiovascular Surgery [24]. Severity of the disease was determined using the Leriche-Fontaine classification (stage IIa, IIb, III and IV). At the time of inclusion, each subject underwent clinical examination, ankle-brachial index (ABI) measurement and lower limb Doppler ultrasound. Inclusion criteria for controls were age>18 years, Caucasian race and no clinical and ultrasound evidence of PAD. Controls were mainly identified among individuals undergoing clinical and ultrasonographic follow-up for asymptomatic carotid plaque at the above mentioned vascular centers. In all controls, PAD was excluded by appropriate clinical interview, physical examination, ankle-brachial index (ABI) measurement, and lower limb Doppler ultrasound. For both groups, exclusion criteria were cancer (current or previous), chronic inflammatory diseases, infectious diseases, autoimmune diseases, thrombotic diseases of the venous system, and thrombophilia. The study was conducted in accordance with the ethical standards of the Helsinki declaration and its later amendments and with the ethical standards and approval of the Università

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Cattolica del Sacro Cuore/Fondazione Policlinico Universitario A. Gemelli IRCCS (protocol number 1827/15). Informed consent was obtained from all patients included in the study.

4.2. MP analysis

MPs isolation and analysis was performed accordingly with current methodological guidelines [25]. Blood was collected into sodium citrate vacutainer tubes from a peripheral vein using a 21-gauge needle and processed within 1 hour from the from the withdrawal. Samples were centrifuged at 450g for 20 min at room temperature to collect platelet-rich plasma (PRP) and then at 1500 g for 20 min to generate platelet-free plasma (PFP). Analysis were conducted using a FC500 Flow Cytometer (Beckman Coulter) equipped with 2 laser lines (488 and 633 nm). The cytometer was preliminary calibrated by using the fluorescent Megamix beads (Biocytex) covering the MP (0.5 and 0.9 μm) and platelet size ranges (0.9 and 3 μm). The upper and the outer limits of MP gate were established just above the size of the 0.9 μm beads in a forward (FS) and side scatter (SS), the lower limit was the noise threshold of the instrument, and an absolute minimum threshold of 1 was set for the SS in order to reduce the background noise. MPs were identified as particles between 0.1 and 1 μm in size according to their light scattering. A total of 150.000 events were acquired for each sample. Selected samples were serially diluted in order to avoid coincidence and reduce artifacts. EMPs, PMPs, LMPs, and ErMPs were defined as vesicles positive for either CD144, CD42b, CD45, or CD235, respectively. Shh+ MPs were identified as vesicles positive for Shh. Shh+ EMPs were defined as vesicles double positive for CD144 and Shh (**Figure 4a**). Shh+ PMPs were defined as vesicles double positive for CD42b and Shh (**Figure 4b**). Shh+ LMPs were defined as vesicles double positive for CD45 and Shh (**Figure 4c**). Shh+ ErMPs were defined as vesicles double positive for CD235 and Shh (**Figure 4d**). For Shh labelling, 150 μl of PFP were incubated for 30 min in the dark at room temperature with 3 μl (1:50) of PE-labeled anti-Shh antiboby (R&D Systems). For CD144 labelling, 3 μl (1:50) of FITC-labeled anti-CD144 antibody (BD Pharmigen) were used. For CD42b labelling, 3 μl of FITC-labeled anti-CD42b antibody (Beckman Coulter) were used. For CD45 labelling, 3 μl of FITC-labeled anti-CD45 antibody (Beckman Coulter) were used. For CD235 labelling, 3 μl of FITC-labeled anti-CD235 antibody (Beckman Coulter) were used. An equal volume of Flow Count fluorospheres (Beckman Coulter) was added to the samples in order to determine MP concentration. Values are reported as number of MPs in 1μl of PFP (number/μl).

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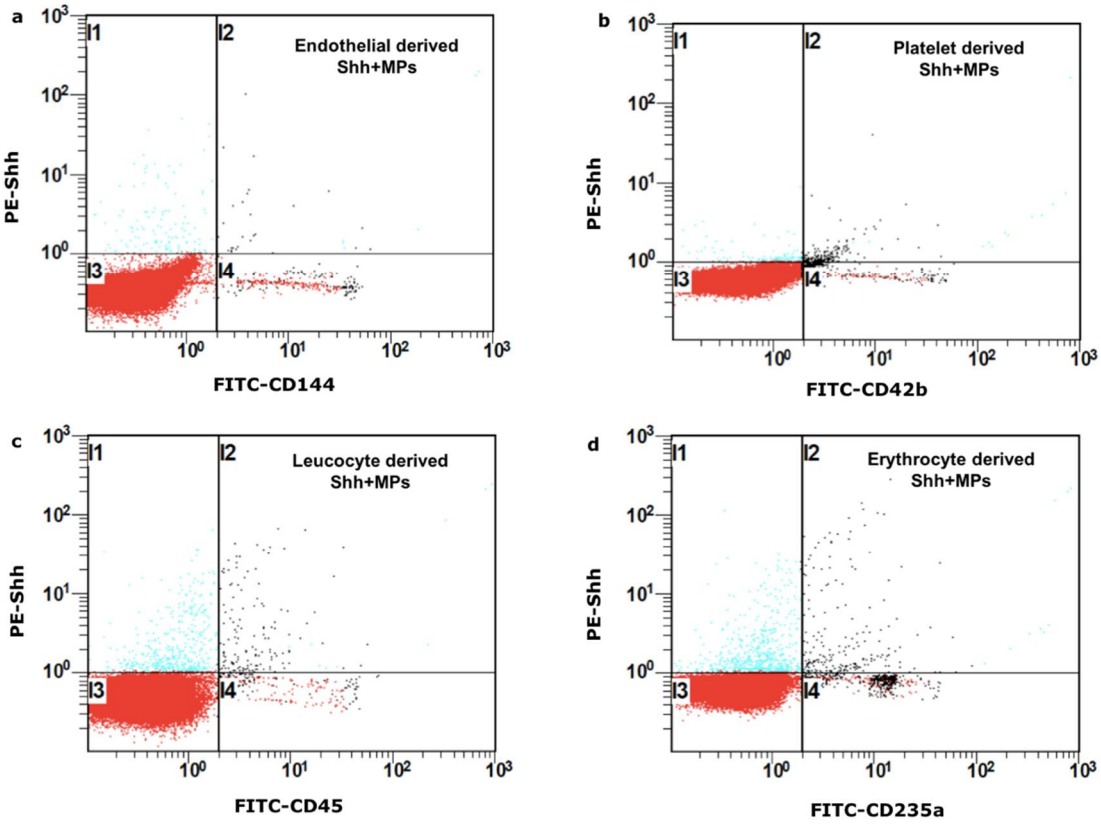

Figure 4. Size-selected events are plotted according to their fluorescence for specific CD144, CD42b, CD45, CD235a, and Shh binding. Events included in the I4 section were considered Shh+ EMPs (**a**), Shh+ PMPs (**b**), Shh+ LMPs (**c**), and Shh+ ErMPs (**d**).

4.3. Plasma collection, MP depletion of plasma, and determination of Shh protein concentration

Blood was collected from PAD patients and controls into sodium citrate vacutainer tubes. Plasma was obtained by centrifugation of blood at 1,570 x g at 4°C for 20 minutes. To generate MP-depleted plasma, we used the method described by Crowford and coll. [22]. In brief, the top 90% of plasma was collected and first centrifuged again at 1,570 x g at 4° C for 20 minutes and then depleted of MPs through further high speed centrifugation. The supernatant was kept as MP-depleted plasma. Shh levels were determined by using a dedicated ELISA test (R&D Systems).

4.4 Statistical analysis

Results are presented as mean ± SD. Since data were not always normally distributed, the non-parametric Mann-Whitney U test was used for comparison of two data sets. Correlation between the number of circulating MPs and clinical variables was evaluated by Pearson's correlation test. Differences were considered statistically significant for $p<0.05$. Calculations were performed with Prism 7 (GraphPad Software) and IBM SPSS Statistics for windows, Version 20.0, released 2011 (IBM Corp).

Author Contributions: Igor Giarretta, Ilaria Gatto and Roberto Pola conceived and designed the study. Igor Giarretta, Ilaria Gatto and Margherita Marcantoni performed the experiments. Igor Giarretta, Giulia Lupi, Ada Truma, Dario Pitocco, Diego Tonello, Angelo Porfidia, Adriana Visonà, Eleonora Gaetani and Paolo Tondi enrolled patients. Ilaria Gatto, Igor Giarretta and Roberto Pola were responsible for the data management. Igor Giarretta performed the data analysis. Igor Giarretta wrote the first draft of the manuscript. Roberto Pola edited the manuscript and wrote the revised version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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