Increased vitreous shedding of microparticles in proliferative diabetic retinopathy stimulates endothelial proliferation

running title: Microparticles and diabetic retinopathy

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**Objectives:** Diabetic retinopathy (DR) is associated with progressive retinal capillary activation and proliferation, leading to vision impairment and blindness. Microparticles (MPs) are submicron membrane vesicles with biological activities, released following cell activation or apoptosis. We tested the hypothesis that pro-angiogenic MPs accumulate in vitreous fluid in DR.

**Research Design and Methods:** Levels and cellular origin of vitreous and plasma MPs from control (n=26) and diabetic (n=104) patients were analyzed by flow cytometry and their pro-angiogenic activity assessed by *in vitro* thymidine incorporation and neovessel formation in subcutaneous Matrigel plugs in mice.

**Results:** MPs of endothelial, platelet, photoreceptor and microglial origin were identified in vitreous samples. Levels of photoreceptor and microglial MPs were undetectable in plasmas, but were comparable in diabetic and control vitreous samples. Vitreous platelet and endothelial MPs levels were increased in diabetic patients, and decreased following panretinal laser photocoagulation or intravitreal anti-VEGF injection in proliferative DR. The ratio of vitreous to plasma MPs levels was calculated to estimate local formation versus potential plasma leakage. In proliferative DR, the endothelial MPs ratio -but not that for platelet- was greater than one, indicating local formation of endothelial MPs from retinal vessels and permeation of platelet MPs from plasma. Isolated vitreous MPs stimulate by 1.6 fold endothelial proliferation and increased new vessel formation in mice.

**Conclusions:** The present study demonstrates that vitreous fluid contains shed membrane microparticles of endothelial, platelet and retinal origin. Vitreous MPs levels are increased in patients with diabetic retinopathy, where they could contribute to disease progression.
Microparticles and diabetic retinopathy

Despite advances in medical care, diabetic retinopathy continues to be a leading cause of vision impairment and blindness in working-age adults (1). The pathogenesis of diabetic retinopathy is complex and has involved multiple pathways including, accumulation of polyol compounds and advanced glycation end products, increased oxidation stress and activation of protein kinase C (PKC) pathway, production of growth factors, and inflammation (1).

Although there is growing evidence for an early involvement of the neural elements of the retina (2), vision loss in diabetic retinopathy is associated with progressive alterations of the retinal vasculature, leading to the breakdown of the blood retinal barrier and pathological angiogenesis of new vessels in the vitreous cavity (1; 3). The risk of vision loss results then from macular edema, and bleeding of these new vessels (vitreous hemorrhage) or their contraction (retinal detachment).

Microparticles (MPs) are submicron membrane vesicles shed from cell surface of both healthy and damaged cells (4). Shedding of membrane MPs is a physiological process that accompanies cell growth and activation and which is enhanced by cytokines, reactive oxygen species, activation of apoptotic pathways or increases in intracellular calcium leading to cytoskeleton reorganization. Numerous studies now indicate that MPs have biological activities and may be involved in thrombosis, cell inflammation, angiogenesis and cell-to-cell communication (5-12).

Microparticles have been identified not only in human plasma but also in other tissues with high cellular activation, inflammation or apoptosis, such as human atherosclerotic plaques or synovial fluid in rheumatoid arthritis (13; 14). Plasma microparticles from different cellular origin circulate in healthy subjects and their levels increase in patients with cardiovascular disease (15; 16). Changes in circulating levels of MPs appear to be controversial in diabetic patients (17; 18), but plasma levels of platelet-derived and monocyte-derived microparticles increase with the severity of diabetic retinopathy (19; 20).

Diabetic retinopathy is associated with increased local activation or apoptosis of retinal, neural and vascular endothelial cells in the eye, both in humans and in animal models (21-24). These finding indicate that microparticles of different cellular origin might be locally generated in the eye of diabetic patients. Alternatively, the presence of microparticles in the eye could also result from an increased vascular permeability associated with diabetic retinopathy. Thus, we sought to investigate the presence of endothelial, platelet and retinal-derived MPs both in the vitreous and in the plasma of diabetic patients undergoing vitrectomy for DR and compare to that of non-diabetic patients. We also examined the potential biological effects of vitreous MPs on endothelial proliferation and new vessel formation.

MATERIAL AND METHODS
Patient eligibility: We included 130 patients who underwent vitrectomy at Lariboisiere Hospital. Baseline characteristics of diabetic (D) and control (C) patients are given in Table 1. Patients were eligible for inclusion when they had had no vitreous surgery on the same eye before and agreed to participate in this study, which was approved by our Institutional Ethics Committee Board and adhered to the declaration of Helsinki. All patients signed a written informed consent. Prior to surgery, DR was evaluated according to the simplified International DR classification (21), made on the basis of clinical data, intra-operative assessment by the surgeon, and review of fundus photographs.
Undiluted vitreous fluid samples (about 300 to 400 µl) were collected from patients’ eyes at the start of standard three-port pars plana vitrectomy for the treatment of retinal diseases. A central vitrectomy was performed after a 20” or 23” gauge sclerotomy at 4 mm from the limbus using a vitreotome (Accurus®, Alcon, Texas, USA). Vitreous samples were collected at the beginning of vitrectomy before opening the balanced salt solution infusion line to maintain intraocular pressure and homogenized by gently pipetting the suspension up and down several times. Ninety-eight samples were collected from the eyes of 93 patients with proliferative DR; in these cases, vitrectomy was performed for persistent vitreous hemorrhage and/or tractional retinal detachment and/or macular edema. Among them, 12 eyes had received an intravitreal injection of Bevacizumab one week before surgery for proliferative DR. In 11 additional diabetic patients, DR was absent, and vitrectomy was performed for epimacular membrane or retinal detachment (Table 1). The control group consisted of vitreous samples from 26 eyes of 26 non-diabetic patients with idiopathic macular hole, idiopathic epiretinal membrane, rhegmatogenous retinal detachment or with age-related macular degeneration (Table 1).

**Microparticles isolation:** Vitreous MPs were isolated from fresh vitreous drawn at beginning of the surgery. Vitreous was separated from cells and platelets after 2 centrifugations (500g; 15 min and 13,500g; 5 min). Venous blood samples (10ml) were collected on citrated tubes before the eye surgery and platelet-free-plasma (PFP) from 60 patients and was immediately prepared by successive centrifugations, according to previous studies (11). For each included patient, PFP and vitreous were frozen and stored at -80°C until subsequent use. Samples were frozen and thawed only once.

**Materials:** Monoclonal antibodies to VE-Cadherin (CD144) conjugated with PE, to human glycoprotein GPIIb (IIIa) (CD41) conjugated with PC5, as well as their corresponding isotype IgG1 were from Beckman Coulter, France. Human Annexin V solution conjugated with FITC was from Roche Diagnostics, France. Lectins from *Arachis hypogaea* Peanut Agglutinin (PNA) and from *Bandeiraea Simplicifolia* Isolectin B4 (ILB4) conjugated with FITC, were from Sigma Aldrich, France. HUVECs (passage 3-6), medium (Endothelium Cell Basal Medium) were obtained from Promocell, Heidelberg, Germany. Cells were cultured in EGM at 37°C in 95% air-5% CO2 atmosphere. Matrigel basement membrane matrix was purchased from BD Bioscience (San Jose, CA) and female C57/BL6 mice (aged of 7 weeks) from Charles River Labs, France, were used for the Matrigel plug assay.

**MPs labeling and flow cytometry analysis:** Analyses were performed on a Coulter EPICS XL flow cytometer (Beckman Coulter, France) by two independent examiners, unaware of the subject status. Sixty (60) µl of human vitreous and 20 µl of PFP were incubated with either anti CD41-PC5 (10µl), anti CD144-PE (20µl) antibodies or their respective isotypic immunoglobulins. MPs expressing phosphatidylserine (PS) were labeled using FITC conjugated Annexin V solution in the presence of CaCl2 (5mM, final concentration) according to the recommendation of the supplier. MPs from retinal origins were labeled with lectins. FITC-conjugated lectins were diluted in PBS to reach the final concentration of 100µg/ml. Sixty (60) µl of human vitreous was incubated with either lectin from *Arachis hypogaea* Peanut Agglutinin (PNA)-FITC (20µg) or lectin from *Bandeiraea Simplicifolia* Isolectin B4-FITC (4µg) (25; 26). Their respective controls were pre-incubated with D-Galactose (80mM; 30 min) (Sigma Aldrich, France)(27-29). Microparticles were gated as events with a 0.1-1µm diameter identified in forward
scatter and side scatter intensity dot-plot representation, using standards synthetic beads of 1µm in diameter (Polyscience AG, Switzerland) (Figure 1). MP concentration was assessed by comparison to calibrator Flowcount beads (Beckman Coulter, 10 µm diameter) with a pre-determined concentration.

**Isolation of vitreous MPs for assessment of cell proliferation and angiogenesis:** To study properties of vitreous MPs on HUVECs proliferation and angiogenesis, MPs were isolated from vitreous samples pooled from 6 consecutive patients with proliferative diabetic retinopathy. Patients that have had previous intravitreal anti-VEGF therapy injection or complete panphotocoagulation were excluded. Microparticles were pelleted and washed three times in EGM medium (20,500g; 150 min). At the end of the last centrifugation, the supernatant was filtered with 0.2µm then 0.1µm Acrodisc® PF Syringe Filter (PALL Life Sciences, France) and used as vehicle. Levels of endothelial CD144+ MPs, the most abundant vitreous MPs sub-population in patients with PDR, were determined in each pellet using flow cytometry and all proliferation experiments were performed at the final concentration of 65 CD144+MPs/µL. Vitreous MPs isolated using this procedure did not contain detectable levels of VEGF when determined by immuno-assay (minimal detection limit of 3.3 pg/ml; n=6).

**Assessment of cell proliferation:** Endothelial cell proliferation was evaluated by ³H-thymidine (Amersham, Biosciences) incorporation in sub confluent quiescent HUVECs. At 70% of confluence, endothelial cells were rendered quiescent by replacing FCS 10% by 0,1% BSA for 24 hours. Endothelial cells were then incubated during 48 hours with either FCS 10% (used as a positive control), vitreous MPs from patients with proliferative diabetic retinopathy (corresponding to a final concentration of 65 MP/µL of microparticles of endothelial origin) or vehicle. The concentration of MPs used in these experiments was the highest concentration obtained after isolation of vitreous MPs from patients with PDR. After 48 hours, ³H-thymidine was added (1µCi/well) for 16 hours at 37°C. Endothelial cell proliferation was stopped by freezing (– 80°C), and free thymidine was then separated by filtration on 1.2 µm filters. The counting of the radioactivity remaining on the filters (to determine thymidine incorporated in endothelial cells) was then realized, after adding a Microscint® solution (40µL/well), in a Microplate luminescence counter (Perkin Elmer, Boston, USA).

**Matrigel angiogenesis assay:** All animal experiments were performed in accordance with institutional animal care guidelines as described earlier (30). Briefly, 500 µL of Matrigel (free of growth factors) mixed with vitreous microparticles (reaching a level of 5000 CD144+ MPs of endothelial origin) or vehicle, was injected subcutaneously in the back of sixteen 7-week-old C57/BL6 mice. After seven days, mice were euthanized by i.p. injection of 0.1 mL of pentobarbital (Ceva Sante Animale, Libourne, France) and Matrigel plugs were excised. Specimens were fixed with formaldehyde 4% overnight at 4°C and embedded in paraffin. Serial sections (5 µm thick) were submitted to Masson Trichrome staining. Two pathologists graded the samples in a masked fashion and counted capillary structures (each area surrounded by few contiguous cells forming a patent lumen was considered a capillary structure) present on three different sections for every Matrigel plug (X10, X20 and X40 magnification, Olympus BH-2, Leica).

**Statistical Analysis:** Microparticles levels are expressed as median and range and analyzed by Kruskall-Wallis non-parametric analysis to compare levels between more than 2 groups, followed by Mann-Whitney analysis of two groups when appropriate. Other data were
expressed as mean ± SEM according to the normality of distribution and analyzed by ANOVA followed by appropriate Bonferroni tests or by Student tests. In addition, Chi-Square test was performed for frequency analysis of Matrigel angiogenesis assays. Statistical analysis was performed with SPSS 10.0 software for Windows (SPSS Software, USA). Differences were considered significant at $p < 0.05$.

**RESULTS**

**Characterization of circulating and vitreous MPs:** Microparticles were identified by flow cytometry analysis as events with a 0.1-1µm positively labeled by specific antibodies (Figure 1). Specific MP populations were defined (Figure 1): endothelium-derived MPs (EMP; CD144+ or VE-cadherin positive) and platelet-derived microparticles (PMP; CD41+; glycoprotein IIb positive), as previously reported elsewhere (11). Photoreceptor derived MPs (PNA MP; PNA+; peanut agglutinin positive) and microglial derived MPs (ILB4 MP; ILB4+; isolectin B4 positive) were also identified.

Plasma levels of AnnexinV+, platelet CD41+ and endothelial CD144+ MPs were not different in C and D patients (Table 2). MPs from retinal origin (PNA+ or ILB4 + MPs) were undetectable in plasma samples (Table 2). Vitreous levels of AnnexinV+, platelet CD41+ and endothelial CD144+ MPs were all markedly increased in Diabetic compared with Control patients ($p=0.035; 0.018$ and $p<0.001$, respectively) (Figure 2). MPs of endothelial origin, identified as expressing VE-cadherin (CD144), were the most abundant MPs sub-population in vitreous samples from diabetic patients. Vitreous levels of AnnexinV+, endothelial CD144+ and platelet CD41+ MPs were increased in patients with proliferative diabetic retinopathy (PDR), compared with non-proliferative DR ($p=0.007; p=0.017$ and $p=0.018$, respectively) (Table 2). In addition, vitreous levels of AnnexinV+, platelet CD41+ and endothelial CD144+ MPs were augmented in diabetic patients with PDR when compared to control subjects ($p=0.004$, 0.003 and <0.0001, respectively; (Figure 2). However, there was no difference between vitreous levels of retinal MPs (ILB4+ and PNA+) in diabetic patients and control patients. Furthermore, circulating levels of endothelial and platelet-derived MPs were not different between control and diabetic patients with or without PDR (Figure 2).

The ratio of vitreous to plasma MPs levels indicates the importance of intraocular formation versus potential plasma leakage of MPs from microvessels in vitreous fluid (Figure 3). For all markers, the ratio was increased in PDR compared to control. This ratio was markedly greater than unity for only endothelial CD144+MPs in proliferative DR ($p=0.02$), indicating either an intraocular shedding or an abnormal clearance of endothelial MPs.

**Effect of therapies on vitreous MPs levels in Proliferative Diabetic Retinopathy:** In patients with proliferative DR, complete panretinal photocoagulation (PRP) significantly decreased vitreous endothelial MPs levels ($p=0.037$), to reach values not different from those obtained in control patients. Complete laser treatment tended to decrease vitreous levels of AnnexinV+, CD41+ or PNA+ MPs, but did not reach statistical significance (Figure 4). One week prior to vitrectomy, twelve patients with PDR received an intravitreal injection of Bevacizumab (50µL; 25µg/µL), a humanized recombinant antibody binding all isoforms of VEGF-A, which causes at least short-term involution of retinal neovascularization, therefore reducing the risk of hemorrhage during surgery (Figure 4). Bevacizumab treatment significantly decreased vitreous levels of AnnexinV+ MPs and endothelial CD144+MPs ($p=0.04$, $p=0.02$, respectively) and led to a complete disappearance of
platelet-derived CD41+MPs in PDR vitreous samples (Figure 4). However, anti-VEGF treatment did not affect levels of photoreceptor MPs (PNA+). Following Bevacizumab treatment, vitreous levels of endothelial CD144+MPs were no longer different from those found in control patients. Vitreous MPs induce endothelial cell proliferation and new vessel formation: Vitreous MPs or supernatant, as previously prepared, were incubated with subconfluent quiescent HUVECs during 48 hours (n=4). Vitreous MPs increased significantly ³H-thymidine incorporation by 1.6 fold when compared to MPs vehicle (vitreous supernatant; p=0.029). Fetal calf serum (10%; used here as a positive control) increased it by 2.4 fold (p=0.003) (Figure 5A). Vitreous MPs did not contain detectable levels of VEGF when determined by immuno-assay, with a minimal detection limit of 3.3 pg/ml (n=6). In the in vivo Matrigel plug assay, vitreous MPs induced significantly endothelial cell migration (Score 1) and new vessels formation (Score 2) when compared to vehicle (vitreous supernatant; Score 0) (p=0.001) (Figure 5B).

DISCUSSION

This study reveals for the first time the presence of submicron membrane vesicles shed from platelet, endothelial and retinal cells in human vitreous samples of patients undergoing vitrectomy and demonstrates the specific vitreous accumulation of endothelial microparticles in patients with proliferative diabetic retinopathy. In addition, we show that vitreous microparticles isolated from patients with diabetic retinopathy stimulate endothelial cell proliferation and formation of new vessels, suggesting that they could contribute to retinal angiogenesis. Presence of shed-membrane microparticles has been reported in several human body fluids, such as plasma or synovial fluid (13; 15; 16). Plasma levels of MPs of different cellular origins are increased in patients with high atherothrombotic risk or inflammatory diseases (15; 16; 31). In this study, we observed no significant differences in circulating MP levels between diabetic patients and the control group. However, our diabetic group included mostly patients with type 2 diabetes, a disease for which conflicting results on changes in circulating MPs have been reported (17-20). Another potential confounding factor is that significant numbers of patients from the control group have cardiovascular risk factors such as hypertension or dislipidemia, which are known to be associated with increased circulating levels of MPs (32; 33).

Proliferative diabetic retinopathy is associated with ocular increases in oxidative stress, protein glycation, growth factors, inflammatory cytokines and cell apoptosis, which could all stimulate the shedding of membrane microparticles from retinal or vascular cells (1; 4; 15; 16; 21; 22; 24; 34; 35). We observed significant increases in the overall pool of vitreous AnnexinV+ microparticles in proliferative diabetic retinopathy when compared to control patients or to patients with non-proliferative diabetic retinopathy. In addition, we demonstrate the presence of MPs positively labelled with either *Arachis hypogaea* Peanut Agglutinin or *Bandeiraea Simplicifolia* Isolectin B4 in vitreous samples but not in plasma, indicating their photoreceptor or microglial origin, respectively (25-29). If anything, levels of MPs derived from photoreceptors or microglia tend to increase in patients with proliferative diabetic retinopathy, but the difference did not reach statistical significance. These MPs originate from cells localized in deeper retinal layers and may be released in vitreous fluid following the tear of the retinal internal limiting membrane. Microfracture of the internal limiting membrane is a likely hypothesis in our control patients, who were
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included for epimacular membrane or macular hole, and even more so in patients with proliferative diabetic retinopathy, where proliferating microvessels need to perforate the internal limiting membrane (36; 37).

The present study also demonstrates increased vitreous levels of MPs originating from vascular or circulating cells such as platelets. Diabetic retinopathy is characterized by an augmented vascular permeability and microparticles present in the blood could permeate through the leaky vascular wall into the vitreous fluid. By the same token, vitreous MPs expressing endothelial VE-cadherin may originate from the circulating pool of MPs, but they may be also locally generated from the retinal new vessel wall. To appreciate the relative importance of local formation versus potential leakage of MP from microvessels into the vitreous fluid, we determined the ratio of vitreous to plasma MPs levels. This ratio was greater than unity only for endothelial CD144+ MPs in diabetic patients with proliferative DR, suggesting that significant numbers of endothelial MPs found in the vitreous fluid could be generated from local microvascular endothelial cells in proliferative diabetic retinopathy or that clearance of endothelial MPs was abnormal. On the contrary, the ratio for platelet CD41+ MPs was lower than unity, favouring the interpretation that platelet MPs present in proliferative DR vitreous fluid likely originate from the plasma.

We investigated the effects of two treatments of proliferative diabetic retinopathy on vitreous levels of MPs. Complete laser photocoagulation causes regression of preretinal neovascularization by destroying the outer layers of the retina and improving the oxygen diffusion from the choroid to the inner retina (38-41), whereas intravitreal injection of the anti-VEGF antibody Bevacizumab leads to a complete resolution of angiographic leakage of neovascularization and to a rapid involution of retinal neovascularization (42-45). Both treatments decreased levels of vitreous endothelial CD144+ MPs to values no longer different from those found in control patients. These observations further reinforce our findings that diabetic proliferative retinopathy is associated with a specific increase in local shedding of endothelial microparticles originating from new vessels. Furthermore, Bevacizumab decreased vitreous platelet CD41+ MPs to undetectable levels, a finding consistent with the decrease vascular leakage reported for the anti-VEGF ocular therapy (42; 46).

The present study shows that vitreous MPs stimulate in vitro endothelial proliferation and in vivo new-vessel formation in a Matrigel plug model. This finding is in agreement with previous studies showing that MPs of different cellular origins (platelet, leucocyte or endothelial) are pro-angiogenic (8-10; 34; 47). However, the present data contrast with studies reporting the anti-angiogenic effect of MPs of endothelial or lymphocyte origin, which is associated with the stimulation of oxidative stress (48; 49). The reasons for this discrepancy are unknown but could result from the different nature or composition of MPs. Because of the paucity of MPs in vitreous samples and their low recovery after isolation, we were unable to identify the cell origin of vitreous MPs that affect endothelial cells. Although the pro-angiogenic effect of platelet-derived MPs is mediated in part by growth factors (8), the mechanism of the proliferative effect of vitreous MPs unlikely involves VEGF, as levels of this growth factor were below detection in isolated vitreous MPs samples. Because of the low recovery of the isolation procedure, the present data do not permit to examine the pro-angiogenic effect of vitreous MPs from non-proliferative diabetic retinopathy nor from control patients. For similar reasons, we could not evaluate the biological effects of increasing concentrations of vitreous MPs.
In conclusion, we identified the presence in vitreous fluid of membrane microparticles shed from retinal, vascular and circulating cells and their significant increase in patients with proliferative diabetic retinopathy where they could contribute to disease progression.

ACKNOWLEDGMENTS
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REFERENCES
Figure Legends:

Figure 1: Representative traces of flow cytometry analysis of AnnexinV+, platelet, endothelial photoreceptor, and microglial MPs in human vitreous samples. 
A-E: Size-selected events (0.1 to 1µm) are plotted as a function of their fluorescence for specific AnnexinV-FITC binding (FL1) (A), lectin PNA-FITC labeling (B), antihuman CD144-PE labeling (C), antihuman CD41-PC5 labeling (D) or lectin ILB4-FITC labeling (E). Grey shaded areas represent unspecific labeling determined either in the absence of calcium (A), in the presence of fluorescent isotypic antibodies (C,D) or in the presence of D-Galactose (B,E).

F: Ultrastructural analyses of vitreous microparticles. Transmission electron microscopic picture of vitreous section showing clustering vitreous vesicles with negative staining: following samples embedding in Epon 812, thin sections were layed on a carbon coated grid, stained with 7.6% uranyl acetate and 0.4% lead citrate and then examined by electron microscopy (Philips, Eindhoven, The Netherlands, Tecnai, Fei, USA). Scale bar: 0.1 µm.

Figure 2: Vitreous levels of AnnexinV+ (AnnV+), platelet (CD41+), endothelial (CD144+), photoreceptor (PNA+) and microglial (ILB4+) microparticles in diabetic (grey bar) and control patients (open bar). Data are expressed as numbers of MPs per µl and given as median (horizontal bar), 25th and 75th percentile (boxes), and 10th and 90th percentile (error bar). The asterisk indicates a significant difference when compared to control patients.

Figure 3: Ratio of vitreous to plasma levels of AnnexinV+ (AnnV+; open bar), platelet (CD41+; black bar) and endothelial (CD144+; grey bar) microparticles in control patients and in diabetic patients with either proliferative DR or non-proliferative diabetic retinopathy (NP-DR). The asterisk indicates a ratio significantly greater than unity.

Figure 4: Vitreous levels of AnnexinV+ (AnnV+; panel A), platelet (CD41+; panel B), endothelial (CD144+; panel C) and retinal (PNA+; panel D) microparticles in controls (open bars) and diabetic patients with proliferative diabetic retinopathy (PDR; gray bars) who underwent either no or incomplete laser panphotocoagulation (PRP), complete PRP or intravitreal injection of Bevacizumab prior to vitrectomy. The asterisk indicates a significant difference when comparing with patients with proliferative DR with no or incomplete PRP.

Figure 5: A/ HUVECs proliferation assay (n=4). ³H-thymidine incorporation with the vehicle (vitreous supernatant) was used as the baseline (100%). Levels of ³H-thymidine incorporation are represented with Endothelium Cell Basal Medium (DMEM), FCS (2%) and vitreous microparticles (MPs). Vitreous MPs (estimated as 65 CD144+MPs/µl for the endothelial subpopulation) increased ³H-thymidine incorporation by 1.6 fold when compared to vehicle (vitreous supernatant without CD144+MPs) (p=0.029). Fetal calf serum (10%) increased it by 2.4 fold (p=0.003). B/ Matrigel Scoring and angiogenesis assay. Serial sections of Matrigel were quantified by two independent pathologists. The presence of capillary structures with massive cells invasion was noticed (2). In some plugs, angiogenesis response was limited to cell invasion (1). Low concentration of vitreous MPs (10 EMP/µl; 5 000 EMPs added to 500 µL of Matrigel) induced endothelial cell migration (Score 1, n=6) and new vessels formation (Score 2, n=3) compared to vitreous supernatant (Score 0, n=12) (p=0.001).
**Table 1**: Baseline characteristics of the patients, ophthalmology features, and performed therapies. The asterisk indicates a significant difference between diabetic and control patients. Data given in brackets are expressed as % of the number of patients included in each group. (PDR: proliferative diabetic retinopathy).

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<th>DIABETIC</th>
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Table 2:
Plasma levels of MPs in control patients (C) and in patients with or without proliferative diabetic retinopathy. Data are expressed as number of microparticles per µl. Microparticles were identified of platelet (CD41+MPs) and endothelial origin (CD144+MPs) or from retinal origin (PNA+MPs, ILB4+MPs). AnnV+MPs: microparticles expressing phosphatidylserine and positively labeled with Annexin V. Microparticles levels expressed as median and range were analyzed by Kruskall-Wallis non-parametric analysis, followed by Mann-Whitney analysis of two groups when appropriate.

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<td>AnnV+MP</td>
<td>1223 (7-5686)</td>
<td>1436 (130-2871)</td>
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<tr>
<td>CD41+MP</td>
<td>765 (25-5018)</td>
<td>718 (68-1763)</td>
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<td>CD144+MP</td>
<td>98 (14-901)</td>
<td>190 (31-609)</td>
</tr>
<tr>
<td>PNA+MP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ILB4+MP</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 1

[Images of flow cytometry graphs and electron micrographs]
Figure 2

![Figure 2](image)

Figure 3

![Figure 3](image)
Figure 4

A

B

C

D

Figure 5A

Thymidine incorporation (% vehicle)
Figure 5B

- **Graph:**
  - Y-axis: Matrigel Score
  - X-axis: Vehicle, MPs
  - Data points indicate a significant difference (p=0.001)
  - Visual representations for Score 0, Score 1, Score 2

- **Legend:**
  - Mats = Mats
  - Vehicle

**Caption:**

*Matrigel Score vs. Treatment (Vehicle vs. MPs) showing a significant difference (p=0.001).*