

Angiogenic properties of human endothelial colony-forming cells in Granulomatosis with Polyangiitis

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Abstract

BACKGROUND

Endothelial progenitor cells are essential for vascular homeostasis. Considering the recurrent nature of granulomatosis with polyangiitis (GPA) the aim of the study was to evaluate the angiogenic capacity of endothelial colony-forming cells (ECFC), which have the capacity for neovasculogenesis *in vitro*, of the patients with GPA, before and after plasma stimulation.

METHODS

Thirteen GPA PR3-positive patients and 15 healthy controls were included. ECFC were isolated from periferic blood and characterized by flow cytometry (FACS). Capillary tube formation (Matrigel assay) and scratching assay were measured during 24 hours. The migration assay was also performed after overnight incubation with healthy control plasma and active GPA patient plasma. Three patients with active disease where submitted to recollection of ECFC after treatment for longitudinal evaluation.

RESULTS

The culture was successful in 62% of GPA patients and 57% of controls. In the matrigel assay the ECFC from patients and controls showed similar capillary structures formation, however ECFC from inactive GPA alone showed early loss of angiogenic capacity between 15 and 24 hours. In scratching assay, there was an impairment in the proliferative capacity of the ECFC between GPA patients and controls without significant difference (12th hour, $p = 0.05$). When incubated with control plasma, ECFC of remission GPA patients showed a significant lower migration capacity after the 4th hour ($p = 0.0001$). In longitudinal analysis, ECFC isolated after treatment showed significantly lower migration rates.

CONCLUSIONS

PR3-positive remission GPA ECFC demonstrated early loss of tube formation and less proliferative capacity compared to those of healthy controls, suggesting impairment of endothelial function.

Introduction

Granulomatosis with polyangiitis (GPA) is a recurrent severe anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) characterized by the presence of pauci-immune necrotizing vasculitis and autoantibodies.[1] Anti-neutrophil cytoplasmic antibodies play a central role in the pathogenesis of GPA anti-proteinase 3 (PR3)-positive due to neutrophil activation and consequent loss of endothelial integrity.[2, 3, 4] This primary vasculitis is characterised by unsatisfactory treatment for sustained remission leading to increased cardiovascular risk and high morbidity and mortality rates.[5, 6]

In healthy individuals, vascular repair occurs by the local migration and proliferation of mature endothelial cells, known as circulating endothelial progenitor cells (EPC),[7] which are essential for vascular homeostasis. The number of EPC has been described as a possible measure of endothelial damage in ischemic diseases and its proliferative and regenerative function has been demonstrated in animal studies and clinical trials in humans.[8] Patients with vascular diseases usually have impaired vascular repair by altering the angiogenic function of EPC.[9] EPC isolated from the peripheral blood generate endothelial colony-forming cells (ECFC) *in vitro*, which have a stable culture-wide phenotype and high proliferative capacity; therefore, these cells are the most commonly used in *in vitro* studies.[10, 11]

Although it is known that endothelial damage has a key effect on AAV, little has been studied about vascular repair. Previous studies suggest that endothelial repair dysfunction in AAV patients may be due to changes in circulating EPC. [12, 13, 14] Studies involving endothelial cell cultures in AAV observed a lower proliferative capacity in patients in comparison to healthy controls. [14, 15] However ECFC were used only by Wilde et al. [16]

To date, there are no studies on the angiogenic capacity of EPC and ECFC in AAV. A better understanding of the characteristics and functions of these cells is essential to better understand the participation of the endothelium in the aetiopathogenesis of AAV and potential future therapeutic targets.

In this study, ECFC isolated from peripheral blood and cultured *in vitro* were used as the study model. The study aimed to evaluate the functional ability of ECFC through proliferation, migration, and angiogenesis in GPA patients before and after activation.

Methods

Patients and controls

Patients diagnosed with GPA were enrolled in the study. The inclusion criteria were the age at symptom onset of over 18 years, newly diagnosed but not yet treated active patients and remission GPA patients in follow-up without immunosuppression for at least 24 months, prednisone dose ≤ 10 mg/day, normal haematimetric indices, and no infection for at least 6 months. GPA was diagnosed according to ACR criteria (1990) and International Consensus Conference on the nomenclature of systemic vasculitis (Chappel Hill 2013). Disease activity was based on the Birmingham Vasculitis Activity Score (BVAS). [17, 18] Active disease was defined by clinical manifestations of recent AAV-related disease activity requiring immunosuppressive therapy. Remission was defined by the absence of clinical disease activity with a zero BVAS for at least 24 months.[19] All patients were recruited from the Rheumatology Unit at the State University of Campinas.

The controls included healthy volunteers and human umbilical vein primary endothelial cells (HUVEC).

After peripheral blood collection, isolation and expansion, ECFC were prepared on plates for angiogenesis and migration assays, both *in vitro*. The study protocol was approved after review by the local ethics

committee.

Isolation of the ECFC

ECFC were cultured according to the protocol of Lin et al. with some modifications. [20, 21] Briefly, 45 mL of peripheral blood from each participant was drawn into tubes containing sodium heparin anticoagulant (BD Vacutainer, San Jose, CA, USA). To isolate the buffy coat mononuclear cells, the samples were submitted to density gradient centrifugation for 30 min with Ficoll-Paque reagent TM Plus 1,077 (GE Healthcare, Uppsala, Sweden) after dilution in PBS phosphate buffered saline (1:1). Cell pellets were washed with PBS and re-suspended in Endothelial Basal Medium (EBM-2) (Lonza, Walkersville, MD, USA). Approximately 1×10^7 cells were cultured in 12-well plates previously treated with rat tail collagen type I (50 µg/L) (SigmaAldrich, Saint Louis, Missouri, USA) with a volume of 1.5 mL EBM-2 supplemented with the EGM-2 (Endothelial Growth Media) Single Quot kit (Lonza, Walkersville, MD, USA), which includes 10% fetal bovine serum (FBS). Plates were incubated at 37 °C and 5% CO₂, and the culture medium was refreshed daily for 7 days and every other day thereafter. Upon reaching plate confluence, the adhered cells were removed by trypsinization (Gibco Invitrogen, Grand Island, NY, USA) until the third passage at approximately 40 days of culture. After the third passage, ECFC were characterized by fluorescence-activated cell sorting (FACS), and sent to cryopreservation.

Fluorescence-activated cell sorting (FACS) analysis

Characterization of ECFC was performed using specific antibodies conjugated to fluorochromes [fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin chlorophyll protein (PerCP)] that detect specific endothelial surface markers: anti-CD31-FITC, clone MBC 78.2 (Invitrogen, Camarillo, CA, USA), anti-CD144-PE, clone TEA 1/31 (Beckman Coulter, Marseille, France), anti-CD146PE, clone 128018 (R&D Systems, Minneapolis, MN, USA), anti-VEGF R2/KDR-PE, clone 89106 (R&D Systems, Minneapolis, MN, USA), anti-CD34-FITC, My10 clone (BD, San Jose, CA, USA), anti-CD45-PerCP, clone 2D1 (BD, San Jose, CA, USA), and anti-CD133-APC, clone AC133 (Miltenyi Biotech, Auburn, CA, USA). The cytometric tubes were incubated for 30 min at 4 °C, protected from light, washed with PBS, centrifuged at 450 g for 5 min (RT), and then re-suspended in 300 µL of PBS for the acquisition of 10,000 events on a cytometer flow (FACS Calibur, Immunofluorometry Systems, Mountain View, CA, USA). Data analysis was performed using BD FACS DIVA 7.0 software (San Jose, CA, USA). Cells were considered to be ECFC when they were positive for CD31, CD144, CD146, and KDR markers, negative for CD45 and CD133, and showed decreased CD34 expression.

Matrigel assay

To access *in vitro* endothelial tube formation, ECFC and HUVEC were cultured in a 24-well plate (10×10^4 cells/well) with a basement-membrane-like substrate (Matrigel™, BD Biosciences, Carlsbad, CA) for 24 hours.[22] Images were photographed after 15 and 24 hours of incubation with an inverted microscope (Olympus IX81, Olympus, Miami, FL) coupled with a digital camera (Olympus DP72, Olympus, Miami, FL) at a 4X magnification in phase-contrast mode. Characterization of the capillary-like structures such as extremities, junctions, nodes, meshes, and segments, was performed using online ImageJ software

(National Institutes of Health, Bethesda, Maryland, USA). The experiments were performed in triplicate for each patient, control, and HUVEC.

Migration assay

To assess cell migration, ECFC were grown in a 24-well plate coated with collagen in complete EBM-2 medium supplemented until a confluent monolayer was formed. The scratch assay methodology involves introducing a thin 'wound' by scratching the bottom of the well with a sterile 200- μ L pipette tip. [23] The plate with fresh medium was photographed at zero time and every 60 min for 24 h in an Inverted Fluorescence Microscope (Zeiss LSM780-NLO, Carl-Zeiss, Jena, Germany) at a 10x magnification, with three images per well at each time point. The images obtained in this experiment were also analysed using the ImageJ software. The distance between the cells in the multiple scratched areas in each culture well was measured and quantified as the rate of wound closure. The experiments were performed in triplicate for each patient, control, and HUVEC.

Three patients with active disease were submitted to recollection of ECFC after 6 months of treatment with cyclophosphamide for longitudinal evaluation.

Plasma influence in migration in vitro

To reproduce an environment conducive to endothelial cell activation and assess their influence on cell proliferation, ECFC were incubated overnight with plasma. For this procedure, plasma from an active GPA patient and from a healthy control was added at 10% to supplemented EBM-2 medium without foetal bovine serum. The migration assay was then performed for both conditions.

Statistical analysis

Statistical analysis was done using SAS system for Windows (Statistical Analysis System), versão 9.4. SAS Institute INC, 2002–2012, Cary, NC, USA. Variables are expressed as means with standard deviations (SDs). ANOVA was used for repeated measures over time in Matrigel assay and to compare three or more groups in plasma migration assays. The Kruskal-Wallis test was performed to compare the groups in migration assay, with the Dunn test correction for multiple comparisons. P values of 0.05 or less were considered statistically significant. The graphics were elaborated using GraphPad Prism, version 6.00 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

Results

Isolation and culture of human ECFC

Peripheral blood samples were collected from 13 GPA patients and 14 healthy controls. All GPA patients were anti-PR3 positive at the diagnosis. The culture was successful in 62% of GPA patients and 57% of controls. Nine patients presented disease activity at the time of blood collection. Culture success was observed in 55% of patients with disease activity and 75% of patients in remission.

On average, the first ECFC colonies of the GPA patients appeared on the 15th day, even as ECFC of the controls. Regarding the number of colonies, there was no significant difference at the end of the third passage between the controls and the active disease and remission patients. In one patient with active GPA, the colony count was 4 times higher than that of the others. The patients' data and ECFC culture results are summarized in Table 1.

For longitudinal evaluation, samples were collected for the isolation and culture of ECFC at diagnosis and after remission induction with cyclophosphamide in three patients. Success was achieved in pre-treatment isolation in all three cases and in only one case after immunosuppression; that case had the highest number of pre-treatment colonies.

Characterization of GPA and control ECFC

The ECFC of GPA patients showed typical endothelial cobblestone morphology. FACS analysis confirmed that the ECFC were endothelial cells expressing specific markers (CD31 (PECAM), KDR (VEGFR), CD144 (VE-Kadhrin), CD146). Moreover, as expected, the ECFC were negative for CD133 and CD45 (monocyte markers) and presented a decreased expression of CD34 markers. The control ECFC demonstrated similar results (Figure 1).

Efficiency of the ECFC in forming capillary-like structures

The Matrigel assay showed a similar number of structures formed (extremities, junctions, nodes, meshes, and segments) by the ECFC in the GPA patients compared to the control group ($p=0.18$, $p=0.57$, $p=0.49$, $p=0.76$, and $p=0.82$, respectively). When classified according to the presence or absence of disease activity, it was observed that only remission patients showed a progressive decrease in the number of most structures after 15 h (Figure 2).

Migration assay

The migration assay was performed by the scratching method and the results were analysed comparing the values of each group every 4 hours, until the closing of gap area for a total of 24 hours. There was no significant difference in the proliferative capacity of the ECFC between GPA patients and controls (12th hour $p=0.05$), even when divided according to the presence or absence of disease activity (12th hour $p=0.08$) (Figure 3).

Otherwise, comparing the average of the lowest confluence percentage that each group (GPA, controls and HUVEC) reached in 24 hours with the mean time that each group took to close the gap area, no difference was found ($p=0.20$, $p=0.91$).

Longitudinal analysis

Blood samples from three GPA patients were collected to analyse the ECFC behaviour before and after treatment. All three patients had glomerulonephritis and were PR3-ANCA positive. Successful ECFC

isolation was observed in only one case after remission induction with endovenous cyclophosphamide.

ECFC isolated after cyclophosphamide showed significantly lower migration rates than those of ECFC isolated before treatment ($p=0.0056$) (Figure 4).

Plasma influence on migration *in vitro*

To analyse the influence of plasma on the migration capacity of ECFC, the authors performed the migration assay after ECFC overnight incubation with plasma from healthy controls and an active disease patient.

Comparing the lowest percentage of gap area reached in 24 hours between the groups (active GPA, remission GPA, control and HUVEC) in both conditions of plasma, there was less migration capacity in the group of remission GPA when incubated with control plasma ($p=0.0020$).

The data were also explored comparing the values of each group (active GPA, remission GPA, control and HUVEC) every 4 hours until the closing of gap area. When incubated with plasma from an active disease patient, although ECFC of GPA patients showed a decreased migration capacity compared to the ECFC of controls, no statistical significance was found (12th hour $p=0.16$, 16th hour $p=0.36$). In addition, it was observed a higher proliferative capacity was in the subgroup of ECFC from active disease patients, also without statistical significance (remission patients, $p=0.31$ and controls, $p=0.74$).

Considering the influence of control plasma, ECFC of remission GPA patients evidenced a significant lower migration capacity after the 4th hour ($p=0.0001$) (Figure 5).

Discussion

This study aimed to evaluate endothelial cells of PR3-positive GPA patients through an *in vitro* ECFC angiogenesis assays. Considering the recurrent nature of the disease, investigation of these mechanisms may lead to a better understanding of endothelial injury and its perpetuation and inadequate repair. This study showed an early decrease in the ability of ECFC to form capillary-like structures and an intriguing alteration of proliferation capacity *in vitro* in remission GPA patients when compared to controls.

In ANCA-positive AAV patients, the interaction between neutrophils and endothelial cells is a crucial factor in endothelial damage, leading to imbalance. Angiogenesis is overriding for vascular homeostasis and consists of EPC migration and local proliferation of mature endothelial cells, influenced by plasma factors, adhesion molecules, and hypoxia. Patients with vascular diseases usually have impaired vascular repair by altering the angiogenic function of EPC. [24, 25]

Previous studies report that the number of circulating EPC in AAV patients may vary according to disease activity, as well as predict relapses.[6, 9, 26, 27] The present study did not evaluate the levels of EPC. Little is studied about the repair of endothelial microcirculation in AAV. In view of the strong evidence of the participation of EPC in vascular repair, its study is essential. In order to analyze the regenerative capacity

of EPC, some previous studies have evaluated its proliferation in culture. The results showed a deterioration of the growth system of the EPC. However, these results come from colony-forming units-endothelial cells (CFU-EC) assays, with no capacity for neovascuogenesis.[14, 15] In the study of Patschan *et al*, the authors also founded greater expression of PR3 associated with less proliferation. Considering the ECFC supposed EPC that produce a well-differentiated endothelial progeny that is suitable for ex vivo analysis of endothelial function it is imperative to identify possible changes in the behaviour of these cells to elucidate potential future interventios. Wilde *et al* were the first to study ECFC cultures in AAV patients and observed a decreased differentiation and proliferation capacity. The authors also describe reduced ECFC growth capacity in relapsing disease.[16]

This study is the first to evaluate the angiogenic capacity of ECFC in AAV patients. Considering the isolation and cultivation of ECFC, the success obtained was similar to that previously described as ranging from 50–80% depending on the protocols used.[28–30] The mean age of patients with GPA was higher than that of controls, both in the total group and in those with isolation efficiency. However, the authors believe that age has not had an influence, as previously reported in previous studies. [11] In this study, the ECFC differentiation and proliferation capacity was not impaired in GPA patients, in contrast to the findings of Wilde *et al*. The authors point out that a patient with active disease had a much higher number of colony, which raised the average of the GPA group. In the present study, a specific group of patients, all with PR3-ANCA, was evaluated. Patients with active disease were newly diagnosed and never treated, and those in remission were without immunosuppression for more than 24 months. These differences in the study population may explain the different results.

Though the difference was not significant, the authors noticed a mild increase in the number of *in vitro* tube formation in the Matrigel assay of the ECFC from GPA patients compared to those of controls. Interestingly, the number of capillary structures formed by ECFC from patients with inactive GPA alone decreased between 15 and 24 h, showing an early loss of angiogenic capacity with derangement in the vascular structures. Impaired angiogenic function had already been reported by Holmén C *et al* in GPA patients, but not with ECFC but with CFU-EC. [15]

In the scratch assay, when assessing the proliferative capacity, ECFC migration in GPA patients was observed to be similar when compared to control patients and HUVEC. As this was an *in vitro* study and cytokines and growth factors are known to influence EPC function, the migration experiments were reproduced with plasma from a healthy control and a patient with active GPA. Incubation with both types of plasma reduced the proliferative capacity of remission GPA ECFC compared to controls ECFC. Curiously, significant difference was found only with healthy control plasma. It was not possible to define its exact influence on the cells of patients in remission. Nevertheless, these findings support the previous reports of microvascular endothelial dysfunction [8] and may contribute to poor vascular repair.

Persistent vascular damage may favour a continuous immune activation with more relapses. [6, 27]

Longitudinal assessment of a patient before and after 6 months of treatment with corticosteroid and cyclophosphamide showed a lower migration rate. After treatment, ECFC had a lower proliferative

capacity. Interference of immunosuppression is a possible but questionable factor in light of the success in isolation and the large number of colonies obtained in this case. This finding confirms the previously described results from remission patients' cells.

The main limitations of this study include the difficulty of including a larger number of patients because of its monocentric design and the rarity and severity of the disease, which often requires immunosuppression. Another important limitation is the difficulty in successfully growing ECFC. *In vitro* assays are known to provide an accessible and reliable model for investigating the role of ECFC in angiogenesis, though choosing the ideal methodology remains challenging and may limit the interpretation of these findings.

This study took an initial step toward an improved understanding of the course of these cells in angiogenesis. The possibility of intrinsic endothelial cell changes in GPA patients that compromise their angiogenic capacity has been revealed. By evaluating capillary formation and scratch assays together, the ECFC of remission GPA patients showed decreased angiogenesis compared to controls. Accordingly, the authors hypothesize whether the commitment of vascular repair function in patients with anti-PR3-positive GPA may be related to its recurrent nature. Future studies are needed to elucidate which combinations of factors may improve the vascular homeostasis mechanisms in GPA.

Conclusion

In summary, PR3-positive remission GPA ECFC demonstrated impairment of angiogenic function based on tube capillary formation and proliferative capacity compared to those of healthy controls, suggesting altered endothelial function. These findings highlight the need for further studies about endothelial function in patients with GPA, which may lead to new therapeutic approaches.

List Of Abbreviations

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV)

Circulating endothelial progenitor cells (EPC)

Endothelial colony-forming cells (ECFC)

Fetal bovine serum (FBS)

Fluorescence-activated cell sorting (FACS)

From colony-forming units-endothelial cells (CFU-EC)

Granulomatosis with polyangiitis (GPA)

Human umbilical vein primary endothelial cells (HUVEC)

Declarations

ETHICS APPROVAL AND CONSET TO PARTICIPATE

The research was evaluated and approved by the local research ethics committee (CEPUNICAMP). CAAE number: 38488914.4.0000.5404

CONSENT FOR PUBLICATION

Not aplicable. The images used are entirely unidentifiable and there are no details on individuals reported within the manuscript.

AVAILABILITY OF DATA AND MATERIAL

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

COMPETING INTERESTS

The authors declare that they have no competing interests.

FUNDING

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AUTHORS CONTRIBUTION

All authors contributed equally to the study.

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Table

[Please see the supplementary files section to view the table.]

Figures

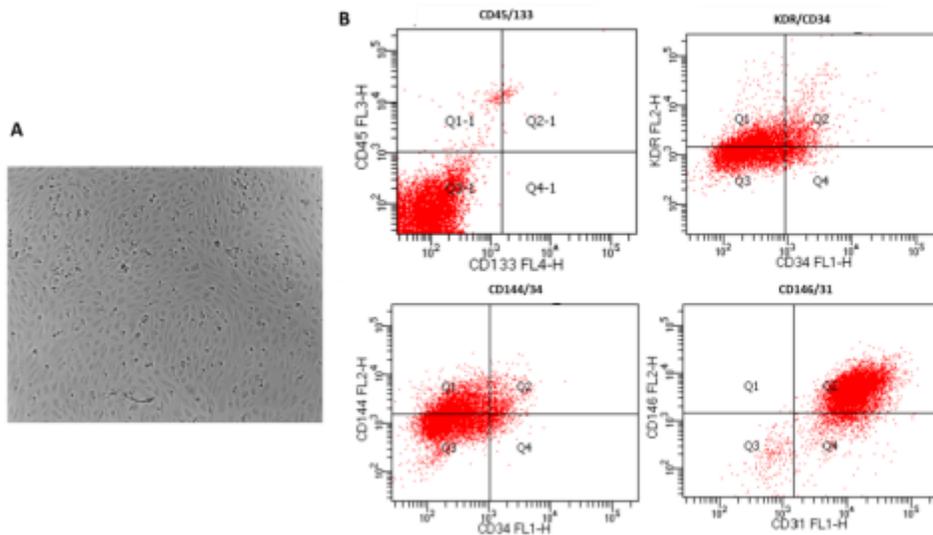


Figure 1

(A) The characteristic cobblestone morphology of confluent ECFC on a collagen plate from a GPA patient. (B) FACS analysis: the histograms demonstrate that the ECFC were positive for endothelial cell markers (CD31 (PECAM), KDR (VEGFR), CD144, CD146), negative for monocyte markers (CD133 and CD45), and showed decreased CD34 markers. Histograms are based on a GPA patient's ECFC culture and are representative of the GPA patients and controls.

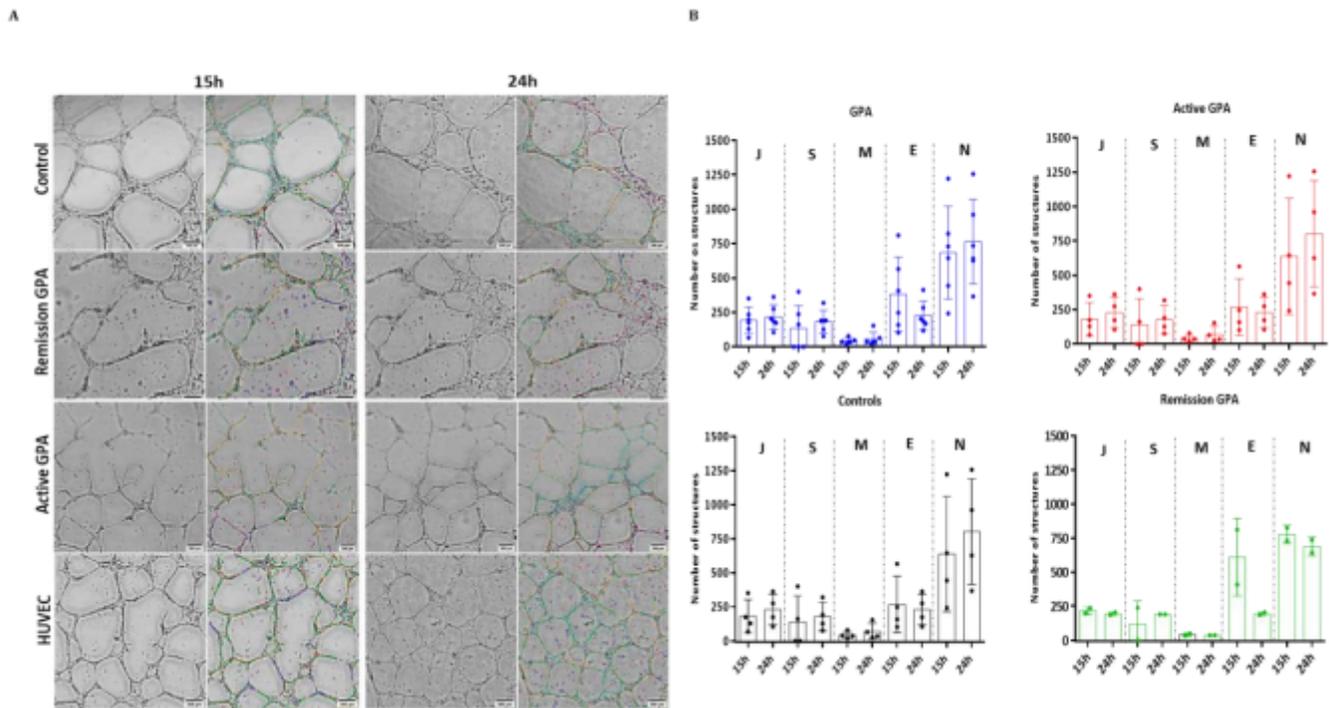


Figure 2

Matrigel assay: (A) ECFC from active and remission GPA patients formed capillary-like structures (J= junctions, S= segments, M= meshes, E= extremities, and N= nodes) comparable to those observed in healthy control ECFC. ECFC from GPA patients, controls, and HUVEC were able to form capillary-like structures; (B) Angiogenesis analysis showed a higher angiogenesis capacity in GPA ECFC, without statistical significance. A progressive increase in the structures formed after 24 h was evidenced in the active GPA patients, controls, and HUVEC, but not in the remission GPA patients. The data are representative of the GPA patients and controls.

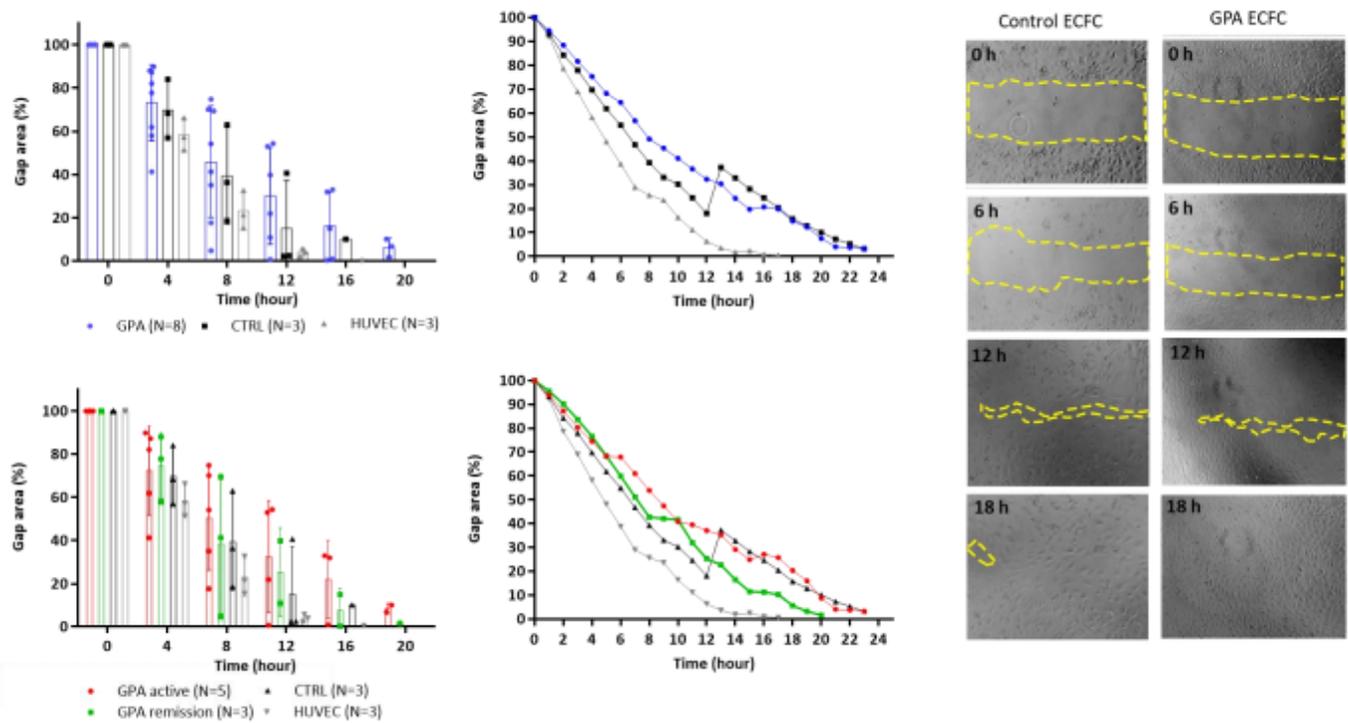


Figure 3

Migration assay: (A) ECFC proliferation in controls and GPA patients after overnight incubation with EBM-2 (37 °C). The gap area is marked in yellow. The ECFC from patients, controls, and HUVEC were able to migrate; (B) ECFC migration photographed hourly for 24 h after the scratch. The dates were also evaluated in terms of disease activity. ECFC from remission GPA patients had similar proliferative behaviour compared to that of controls. The photographs and data are representative of the GPA patients and controls.

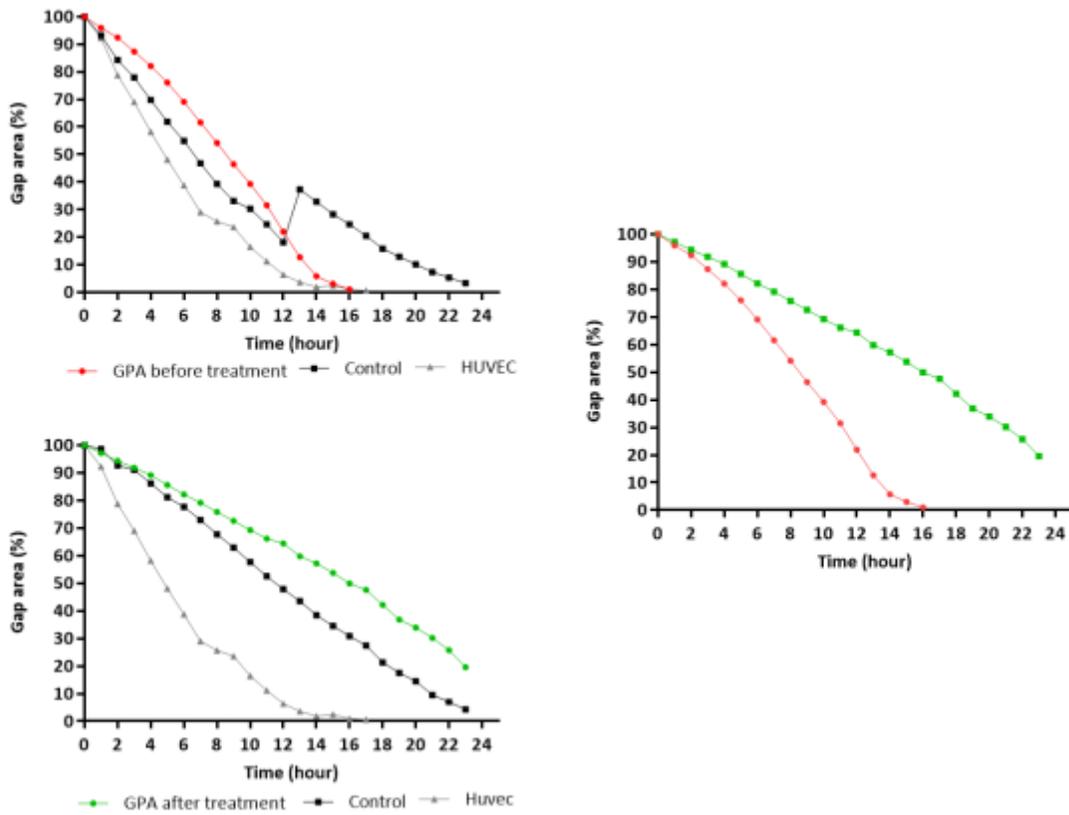


Figure 4

Longitudinal analysis: ECFC migration assay from a GPA patient before and after treatment showing clearly reduced proliferative capacity after remission.

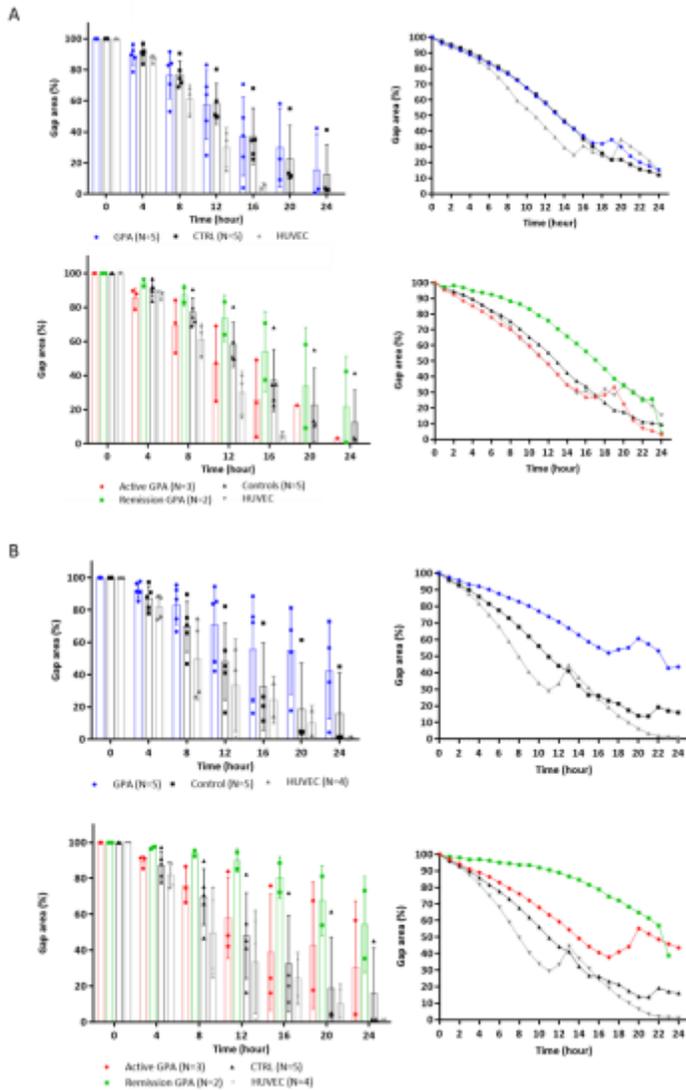


Figure 5

Migration assay after plasma stimulus: (A) ECFC from GPA patients after overnight incubation with 10% active plasma. ECFC from patients, controls, and HUVEC results were able to migrate and the results were similar between GPA patients and controls. (B) ECFC from GPA patients after overnight incubation with 10% control plasma. ECFC from patients, controls, and HUVEC were able to migrate. Considering disease activity, patients in remission presented a significant lower migration capacity compared to controls. The data are representative of the GPA patients and controls.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.docx](#)