Distinct features of circulating microparticles and their relationship with disease activity in inflammatory bowel disease

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Abstract

\textbf{Background} There is evidence that circulating microparticles (MPs) and annexin (+) platelet-derived MPs (PDMPs) are increased in inflammatory bowel disease (IBD). The aim of our study was to characterize the abundance, origin, and annexin V binding of MPs in patients with IBD and correlate them with the disease characteristics.

\textbf{Methods} Case-control study of 46 IBD patients (23 Crohn’s disease, 23 ulcerative colitis) and 40 matched healthy controls (HC). MPs were divided according to annexin V binding, their origin was estimated based on specific cell membrane markers in plasma samples and their number was calculated via flow cytometry. Clinical and laboratory activity indices were also analyzed.

\textbf{Results} Annexin (-) PDMPs (P=0.0004), total (P=0.04) and annexin (+) monocyte-derived MPs (P=0.02) were increased and annexin (-) total MPs (P=0.0007) were decreased in IBD patients compared to HC. The annexin (+)/(-) ratio of all MP types were significantly elevated in IBD patients compared to HC (P<0.003). IBD patients with active disease displayed elevated total and annexin (+) total MPs, total, annexin (+) and (-) PDMPs compared with those in remission (P<0.05). Annexin (-) PDMPs were considerably increased in IBD patients with active compared to those with inactive disease (P=0.0013). Total and annexin (-) PDMPs were significantly correlated with most of the disease activity indices (P<0.05).

\textbf{Conclusion} The majority of circulating MPs, their counterparts and particularly annexin (-) PDMPs are increased in active IBD patients. Annexin (+)/(-) ratio proved to be the most reliable distinctive MP index between HC and IBD patients.

\textbf{Keywords} Microparticles, inflammatory bowel disease, Crohn’s disease, ulcerative colitis

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Introduction

Particular importance has been given recently to platelets (PLT) as active inflammatory components participating in the pathogenesis of chronic inflammatory diseases, such as inflammatory bowel disease (IBD) [1]. PLT deviations in IBD involve increase in their absolute number (thrombocytosis), alteration in morphological parameters (mean PLT volume decrease, PLT distribution width increase, plateletcrit increase) and disseminated activation [2]. The latter takes place in the enteric microcirculation, during inflammatory processes like IBD [3]. PLT when activated by agonists such as thrombin, calcium, collagen, complement and shear forces, increase in size, over-excrete their granular content, express elevated number of surface receptors, form PLT aggregates and over-release vesicles called microparticles (MPs).

MPs are small-sized vesicles (0.1-1 μm) released by almost any kind of eukaryotic cell during activation or apoptosis. Their
formation is a well regulated process provoked by changes in the local concentration of specific intracellular molecules (especially calcium), cytoskeleton disruption and membrane remodeling with phosphatidylserine (PS) and phosphatidylethanolamine exposure in the outer membrane layer of ancestral cells [1]. MPs acquire functions of their original cell and can influence a diverse series of physiological and pathological functions to their targets, by transferring genetic material (m-RNA, micro-RNA, DNA), membrane receptors and a series of parental molecules [1]. Thus, MPs act as conveyors of immune responses through a variety of mechanisms and as such they can be instrumental in the pathogenesis of various inflammatory disorders as IBD [1]. PLT-derived MPs (PDMPs) comprise the majority of circulating MPs, estimated at 70-90%, with a large portion of them considered originating from megakaryocytes in healthy individuals [4,5].

The objectives of our study were: (i) to compare the number of total MPs, PDMPs and monocyte-derived MPs (MDMPs) between IBD patients and healthy controls (HC); (ii) to evaluate the specific properties of certain MPs sub-populations according to their ability to bind annexin; and (iii) to investigate a possible correlation between MPs and disease characteristics in patients with IBD.

**Patients and methods**

**Patients**

This is a prospectively designed case-control study. IBD patients (8 hospitalized and 38 outpatients) were recruited from the Gastroenterology Department of Venizelion General Hospital of Heraklion, Crete, Greece. All patients and controls included in the study were Caucasians and were carefully selected regarding medical history, because MPs measurements can be heavily influenced by a wide range of disorders [1,6]. Those with a history of malignancy, cardiovascular disease, severe renal or respiratory failure, hepatic, thyroid, rheumatologic or hematologic disorders, thromboembolic events, diabetes mellitus, dyslipidemia or established autoimmune diseases, recent pregnancy or delivery (<6 months) and blood transfusion during the past 3 months, were excluded. The control group consisted of age- and gender-matched blood donors.

IBD diagnosis was based on the European Crohn’s and Colitis Organization guidelines [7,8]. Montreal classification was adopted for disease phenotype [9]. Disease activity was evaluated at baseline using Crohn’s Disease Activity Index (CDAI) for Crohn’s disease (CD) and simple clinical colitis activity index (SCCAI) for ulcerative colitis (UC) patients [10,11].

**Definitions**

Anemia was defined using the World Health Organization (WHO) criteria: hemoglobin (Hb) <13 and <12 g/dL for men and women respectively [12]. Thrombocytosis was defined as an absolute PLT count >400 K/µL, based on the whole blood count analyzer’s standardized normal range (150-400 K/µL). Active disease was defined as a CDAI score >150 for CD and a SCCAI score >3 for UC patients [10,11].

**MP preparation**

Whole blood (10 mL) was collected in an atraumatic fashion using a 21-gauge needle. Two tubes (7 mL) were used for whole blood cells count and serological analyses. The remnant whole blood (3 mL) was put into one tube with sodium citrate anticoagulant and was processed within 1 h for MPs evaluation. MPs were obtained using a 3-step centrifugation process. Initially, the sample was centrifuged at 1.500 g for 15 min at room temperature. Plasma was transferred into a new tube leaving 200 µL of supernatant on top of the red blood cells. The latter was centrifuged for 2 min at 13.000 g to remove PLT debris. The remaining supernatant was transferred into another tube, and 2 aliquots of 250 µL for each sample were stored at -80°C until further assayed. In the final step, plasma was centrifuged at 18.000g for 20 min at 4°C, after quick thawing at 37°C, to obtain PLT-free plasma. The supernatant was discarded, while keeping 50 µL at the bottom of the tube in which the MP pellet was present. Subsequently, 150 µL Annexin V binding buffer (10 mM Hepes/140 mM NaCl/2.5 mM CaCl₂ in distilled H₂O) was added into the MP suspension at 4°C. All buffers were sterile-filtered with a 0.2 µm filter (Whatman, NJ, USA).

**MP labeling**

One hundred µL of each final sample (MPs-Annexin suspension) were transferred in two TruCount™ tubes (BD Biosciences, NJ, USA); the first served as negative control sample and the second for the MP calculation. Into the latter, 10 µL Annexin V-FTTC, 20 µL CD14-PerCP clone 47-3D6 (mouse IgG1) and 20 µL CD41α-PE clone HIP8 (mouse IgG1) were added and the MP suspension was incubated for 30 min in the dark at 4°C. The samples were then analyzed on a BD LSRII flow cytometer with the FACSDiva software (BD Biosciences, NJ, USA), after 400 µL Annexin V buffer was added in each of the TruCount™ tube. CD41 and CD14 were used for the detection of PDMPs and MDMPs, respectively. TruCount™ tubes with a known number of fluorescent beads were used for absolute quantification of MPs, using the following formula.

$$\text{Absolute count of MPs/µL} = \left( \frac{\text{events in a region containing beads per Trucount-tube}}{\text{events in a region containing absolute count cells}} \right) \times \frac{\text{beads per Trucount-tube}}{\text{test volume in µL}}$$

The number of beads per test-tube was provided by the manufacturer and the test volume was 25 µL. All reagents were purchased from Immunostep S.L. (Salamanca, Spain).
Flow cytometry

For the detection of MPs by flow cytometry, size calibration and MP-size gate was conducted with Megamix (American Diagnostic, Hauppauge, NY, USA), a mixture of microbeads of three different sizes (0.5 μm, 0.9 μm, and 3.0 μm). This MP gate excludes the electronic background noise using a threshold, which was set to 200 (Fig. 1).

Forward scatter (FSC-A) and side scatter (SSC-A) were set to logarithmic scale. The absolute count of MPs was measured setting the stop condition for TruCount™ beads at 10,000 events with the maximum acquisition rate of 3,000 events/sec. To separate true events from background noise, we defined MPs as particles smaller than 1.0 μm in diameter that expressed surface antigens (CD14 or CD41) compared to the negative control. CD14^+CD41^- were defined as MDMPs and CD14^-CD41^+ as PDMPs. Based on the Annexin V staining, MPs were dichotomized into annexin (+) or (-).

Statistical analysis

Results were presented as absolute numbers (percentage) or median value (interquartile range, IQR) or mean values ± standard deviation. Kolmogorov and Smirnov test was used to evaluate distribution normality. Comparisons of the values between independent groups were traced with the use of Mann-Whitney U test or Student’s t-test. Pearson’s correlation with Spearman’s rho (r) was used for assessing the relationship between the continuous parameters measured. A P value <0.05 was considered statistically significant. Statistical analysis involved use of GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA).

Ethical considerations

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki (7th revision, 2013), was approved by the Ethics Committee of our hospital and signed informed consent was obtained from all patients at baseline.

Results

Patient characteristics

A total of 46 IBD patients (23 CD and 23 UC) and 40 HC participated in our study. Patient baseline characteristics are presented in Table 1. Nine of 46 IBD patients (19.6%) presented with anemia and 15 (32.6%) displayed thrombocytosis at baseline.

MP absolute number comparison between groups

Annexin (-) total MPs were significantly lower in IBD patients compared to HC (P=0.0007). On the contrary, annexin (-) PDMPs, total MDMPs and annexin (+) MDMPs were higher in IBD compared to HC (P<0.05) (Table 2). The ratio of annexin (+)/(-) in total MPs, PDMPs and MDMPs of IBD patients were higher than those of HC (P<0.05) (Table 2).

Subsequently, we investigated the association of the MPs with disease activity. IBD patients with non-active disease demonstrated lower levels of total MPs, annexin (-) total MPs and annexin (-) PDMPs compared to HC (P<0.05). Active IBD patients showed higher levels of annexin (+) total MPs, total and annexin (+) PDMPs and total and annexin (+) MDMPs.
compared to HC (P<0.05). Finally, the comparison of active vs. non-active IBD patients revealed significantly higher total MPs, annexin (+) total MPs, total PDMPs, annexin (+) PDMPs and annexin (-) PDMPs in those with active disease (P<0.05) (Table 3). The most profound difference between active and non-active patients was observed in annexin (-) PDMPs (P=0.0013).

The ratios of annexin (+)/(-) in total MPs, PDMPs and MDMPs of non-active IBD patients were significantly higher than those of HC (P<0.05). In addition, the ratio of annexin (+)/(-) in total MPs and MDMPs of active IBD patients were higher than those of HC (P=0.009 and P=0.004, respectively). Nevertheless, there was no significant difference in the annexin (+)/(-) MP subtypes ratio between the active and non-active IBD patients (Table 3). Representative dot plots of a control and a patient for quantification of MPs are presented in Fig. 2.

### Table 1 Demographic and clinical characteristics of inflammatory bowel disease patients included in the study

|                          | Healthy controls (n=40) | Total IBD (n=46) | IBD active (n=19) | IBD non-active (n=27) |
|--------------------------|------------------------|------------------|------------------|----------------------|
| Age (years) (mean±SD)    | 39.5±10.3              | 43.3±12.7        | 43.4±13.9       | 41.8±11.9           |
| Females (N, %)           | 17 (42.5)              | 21 (45.7)        | 9 (47.4)        | 12 (44.4)           |
| CD/UC (%)                | -                      | 23/23 (50/50)    | 12/7 (63.2/36.8) | 11/16 (40.7/59.3)   |
| Disease duration at baseline (years) (mean±SD) | - | 8.5±7.6 | 9.1±8.3 | 8.2±7.1 |
| Smokers (N, %)           | -                      | 21 (45.7)        | 12 (63.2)       | 9 (33.3)            |
| Disease location (N, %)  | -                      | 0/1/22 (0/4.4/95.6) | 0/0/7 (0/0/100) | 0/1/15 (0/6.2/93.8) |
| L1, Ileitis              | 8 (34.8)               | 2 (16.7)         | 6 (54.5)        |                      |
| L2, Colitis              | 0 (0)                  | 0 (0)            | 0 (0)           |                      |
| L3, Ileo colitis         | 15 (65.2)              | 10 (83.3)        | 5 (45.5)        |                      |
| L4, Upper GI             | 5 (21.7)               | 1 (8.3)          | 4 (36.4)        |                      |
| Disease behavior (N, %)  | -                      | 13 (56.5)        | 6 (50)          | 7 (63.6)            |
| B1, Inflammatory         | -                      | 6 (26.1)         | 4 (33.3)        | 2 (18.2)            |
| B2, Strictureing         | -                      | 1 (4.4)          | 0 (0)           | 1 (9.1)             |
| B3, Penetrating          | -                      | 3 (13)           | 2 (16.7)        | 1 (9.1)             |
| P, Perianal              | -                      | 15±131           | 250±74          | 47±88               |
| Disease activity (mean±SD) | -                  | 153±131         | 250±74          | 47±88               |
| CDCAI                    | 2±3                    | 4±2              | 0±0             |                      |
| SCCAI                    | 2.3±4.2                | 4.4±5.6          | 0.8±1.6         |                      |
| ESR (mm/1h)              | 37.1±23.9              | 48.7±23.6        | 29.4±21.2       |                      |
| Extraintestinal manifestations (N, %) | - | 19 (41.3) | 9 (47.4) | 10 (37) |
| Arthritic                | -                      | 17 (36.9)        | 9 (47.4)        | 8 (29.6)            |
| Skin                     | -                      | 5 (10.9)         | 3 (15.8)        | 2 (7.4)             |
| Ocular                   | -                      | 2 (4.4)          | 1 (5.3)         | 1 (3.7)             |
| Major IBD-related surgery (N, %) | - | 4 (8.7) | 1 (5.3) | 3 (11.1) |
| Treatment (N, %)         | -                      | 24 (52.2)        | 7 (36.8)        | 17 (58.6)           |
| 5-ASA                    | -                      | 3 (6.5)          | 2 (10.5)        | 1 (3.7)             |
| Systemic CS              | -                      | 10 (21.7)        | 5 (26.3)        | 5 (18.5)            |
| Azathioprine             | -                      | 2 (4.5)          | 1 (5.3)         | 1 (3.7)             |
| Methotrexate             | -                      | 14 (3)           | 7 (36.8)        | 7 (25.9)            |

Values are mean±SD for continuous data or numbers (percentages) for discrete data

5-ASA, 5-aminosalicylic acid; anti-TNF-a, anti-tumor necrosis factor-a; CD, Crohn's disease; CDCAI, Crohn's disease activity index; CRP, C-reactive protein; CS, corticosteroids; DL, deciliter; ESR, erythrocyte sedimentation rate; IBD, inflammatory bowel disease; mg, milligram; mm, millimeter; SCCAI, simple clinical colitis activity index; UC, ulcerative colitis

**Correlation of circulating MPs with disease characteristics**

Gender, smoking, IBD disease type (UC or CD) and phenotype but also the presence of extraintestinal manifestations, history of IBD-related surgeries, appendectomy or tonsillectomy and use of different IBD medications were not associated with any type of MPs. Body mass index correlated with total MDMPs and annexin (+) MDMPs (r=-0.305, P=0.033, and r=-0.383, P=0.009). The presence of anemia, including iron deficiency anemia, or thrombocytosis were also not correlated with MPs. However, ferritin levels were correlated with annexin (+) (r=0.0332, P=0.05) and annexin (-) PDMPs (r=-0.335, P=0.049) and folate levels with total PDMPs (r=-0.508, P=0.022) and annexin (-) PDMPs (r=-0.508,
None of the red blood cells (hematocrit, Hb, red blood cells distribution width, mean corpuscular volume) and PLT (mean PLT volume, plateletcrit, PLT distribution width) measured parameters were correlated with MPs. Only white blood cell count displayed a positive correlation with the total PDMPs ($r=0.330$, $P=0.025$).

Regarding the correlations between activity indices and MP measurements, erythrocyte sedimentation rate (ESR) was positively correlated with total MPs, annexin (+) and (-) total MPs, total PDMPs, annexin (-) PDMPs and annexin (-) MDMPs (Table 4). C-reactive protein (CRP) correlated with total MPs, annexin (-) PDMPs and total PDMPs (Table 4).

### Discussion

In this study we found that the majority of MPs and of their counterparts were increased in IBD patients and especially in those with active disease compared to HC. The annexin (-) PDMPs showed the strongest association with disease activity as well as with laboratory inflammatory biomarkers. Moreover, annexin (+)/(-) ratio proved to be the most reliable distinctive MPs index between HC and IBD patients.

Published data suggests that IBD is associated with increased plasma annexin (+) MPs. Andoh et al reported that annexin...
(+ PDMPs, measured by ELISA, were elevated in active IBD compared to HC and that they were significantly associated with markers of disease activity [13]. Moreover, Pamuk et al did not detect any difference in annexin (+) PDMPs between IBD patients and HC using flow cytometry [14]. Chamouard et al described an elevation of annexin (+) MPs in CD but not in UC compared to HC utilizing an immunocapture assay [15]. Furthermore, they showed a significant decrease in circulating MPs after infliximab induction therapy. Palkovits et al, reported elevated annexin (+) PDMPs, leukocyte annexin (+) MPs and tissue factor (+)-annexin (+) MPs in an effort to explain the high procoagulant activity in IBD patients [16].

Finally, Leonetti et al reported that CD patients with active disease displayed higher total circulating MPs, annexin (+) MPs, PDMPs, endothelial-MPs, leukocyte-MPs, activated leukocyte MPs and PDMPs compared with HC [17].

Additionally, in our study we observed increased annexin (+) total MPs, total and annexin (+) PDMPs and total and annexin (+) MDMPs in patients with active IBD compared to HC. Also, total MPs and annexin (-) total MPs were decreased and annexin (-) PDMPs were increased in IBD patients (total cohort) compared to HC. Finally, total MDMPs and annexin (+) MDMPs were elevated in both total and active IBD patients compared to HC. Certain discrepancies in results among
studies are possibly due to limitations related to different study designs and protocols used for MPs isolation. The lack of a standardized method for MPs calculation makes these deviations probably unavoidable.

The initial view was that all MPs expose PS on their surface, which, in turn, allows them to bind to annexin V. Only a limited number of studies used merely size and surface antigen markers to define MPs [18-21]. This is the explanation why there were only limited annexin (-) MP and PDMP measurements in the previously mentioned IBD studies. The allegation that MPs form only when PS exposes was soon questioned by studies [22] that demonstrated the presence of several annexin (-) MPs originating from different cell types in certain diseases [23,24]. Connor et al confirmed the existence of annexin (-) PDMPs and demonstrated that the vast majority of PDMPs (80%) fail to bind to annexin V in human PLT poor plasma [25]. These annexin (-) PDMPs are produced from PLT with decreased activation according to markers expressed on their surface. Thus, the annexin-PS bond represents a true reflection of MP procoagulant activity [26]. The latter view is supported by studies confirming that the most robust procoagulant MPs are those exposing PS and tissue factor, which have a crucial role in coagulation cascade. PDMP procoagulant capacity is estimated to be 50-100 times greater than PLT [27] and their absence is causing a rare bleeding disorder called Scott syndrome. The increased number of annexin (+) total MPs and PDMPs in our active IBD patients compared to non-active ones or HC could justify to some extent the procoagulant predisposition of IBD, which is strongly connected to disease activity [28].

We also measured, for the first time, annexin (-) MPs as well as their PLT and monocyte counterparts in IBD patients and correlated them with disease activity and other parameters. We found that the majority of total MPs in our study populations were annexin (-), which nevertheless is not the case for total PDMPs, suggesting that most of the MPs originate from a non-PLT cell type or that some of them are of unknown nature as suggested by Nielsen et al [29]. Moreover, annexin (-) total MPs were significantly lower in IBD patients, mainly in those with non-active disease compared to HC. This could be attributed to a consumption of annexin (-) MPs in IBD patients in target tissues, like the intestinal capillaries, due to the formation of intra capillary thrombi. Similar findings were observed in preeclamptic women with reduced number of circulating blood MPs and accumulation of PDMPs in fibrin deposits in the placenta [30]. In addition, the non-PLT originating annexin (-) MPs (endothelial or red blood cell population) in IBD patients, could aggregate in circulating leukocyte-leukocyte or leukocyte-PLT complexes which are increased, leading to underestimation of their amount in peripheral blood [14]. Finally, factors yet unknown could result in inhibition of annexin (-) MP production in IBD patients.

On the other hand, annexin (-) PDMPs were augmented in our patients compared to HC. This suggests that annexin (-) PDMPs are linked to disease activity, confirmed by the significant correlation of annexin (-) PDMPs with disease activity indices (CRP, CDAI, ESR). PS exposure on the outer membrane is an apoptotic index, important for macrophage recognition and cleavage of the apoptotic material [31]. Thus, annexin (-) MDMPs and PDMPs may serve as a long-lasting reservoir of MPs perpetuating inflammatory response in IBD.

Regarding the MPs that express PS on their surface, the annexin (+) PDMPs of HC were only 63.7% of the annexin (+) total MP. At the same time, the proportion of annexin (+) PDMPs in IBD patients was close to 75% of annexin (+) total MPs, a rate comparable with the result from other studies [4]. Furthermore, annexin (+) total MPs and annexin (+) PDMPs were elevated in active patients compared to non-active and HC, presenting an association with disease activity. CD is known to be mediated by an uncontrolled activation of mucosal T-lymphocytes and infliximab induction therapy is linked to significant increase in T-lymphocytes-mediated apoptosis in gut mucosa [32]. If this apoptotic mechanism applies also to other cell series, it could be a reasonable explanation for the increased annexin (+) PDMPs in active IBD patients compared to those in quiescent state and HC. Therapy intensification with the use of anti-tumor necrosis factor-α agents and other immunomodulators to control inflammation could justify an increased apoptotic process including annexin (+) PDMP formation.

There are certain limitations in our study mainly in the used methodology. We selected flow cytometry, considered the gold standard for MP measurement. However, it remains laborious and intensive, with inter-laboratory variations regarding study protocols and instrument types, limiting the reproducibility of the results. High centrifugation speeds, buffers containing calcium, freezing, storage and thawing of plasma samples, all can be potential confounding variables, but unavoidable steps in protocols for MP evaluation. Another limitation could be the relatively small number of IBD patients recruited, especially for conducting subgroup analyses, although it represents one of the largest published on this field. The exclusion criteria were strict, ensuring a significant lack of influence from non-IBD factors regarding MP measurements.

### Summary Box

**What is already known:**

- Platelets (PLT) are active inflammatory components participating in the pathogenesis of chronic inflammatory diseases including inflammatory bowel disease (IBD)
- Total and certain cellular origin circulating annexin (+) microparticles (MPs) may be increased in IBD patients

**What the new findings are:**

- The majority of circulating MPs and their counterparts were increased in active IBD patients
- Annexin (-) PLT-derived MPs showed the strongest association with disease activity as well as with laboratory inflammatory biomarkers
- Annexin (+)/(-) ratio proved to be the most reliable distinctive MP index between IBD patients and healthy controls
In conclusion, our results demonstrated that the majority of MPs and of their counterparts are elevated in active IBD patients compared to those with non-active disease and/or HC. PDMPs, especially the annexin (-) ones, were significantly associated with disease activity. In addition, annexin (-) PDMPs were positively correlated with inflammatory biomarkers. Thus, PDMPs and their annexin (+) and (-) subtypes could have a key role in affecting specific mechanisms of the inflammatory cascade seen in IBD and contributing to its pathogenesis and clinical manifestations. Nevertheless, there is still a lot of clinical and laboratory work that needs to be done to clarify the exact role of MPs and the ways that these particles can become beneficial in everyday clinical practice in IBD.

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