# MEDICINA:

# LONGE DOS HOLOFOTES,

## PERTO DAS PESSOAS 3

Benedito Rodrigues da Silva Neto
(ORGANIZADOR)

Atena Editora
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# Medicina: longe dos holofotes, perto das pessoas 3

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# APRESENTAÇÃO

Sabemos que o trabalho do médico humanitário envolve uma grande variedade de atividades que podem girar em torno de diversas atividades. Existe um longo e vasto caminho muitas vezes pouco iluminado pelos sistemas de comunicação, mas que são uma base essencial para o desenvolvimento dessa ciência. Exemplos como de equipes médicas que atuam em situações de conflito e pós-conflito, no controle e combate às doenças epidêmicas, no atendimento emergencial às vítimas de catástrofes naturais, e garante atendimento médico às pessoas excluídas dos sistemas de saúde locais, contribuem para esse entendimento.

A proximidade com o paciente e os valores éticos necessitam ser valorizados e incentivados, pois geram possibilidades além de pressionarem grandes indústrias e governos para que medicamentos acessíveis e de qualidade cheguem às populações mais pobres do mundo.

Tendo em vista a dimensão e a importância dessa temática, a mais nova obra da Atena Editora, construída inicialmente de três volumes, direciona ao leitor um novo material de qualidade baseado na premissa que compõe o título da obra.

Situações de emergência pedem resposta rápida, com atendimento médico especializado e apoio logístico, mas falhas crônicas no sistema de saúde local, como a escassez de instalações de saúde, de profissionais qualificados e a inexistência da oferta de serviços gratuitos para populações sem recursos financeiros, também podem motivar a atuação da organização. Ou seja, uma amplitude de temas que aqui serão abordados dentro dos diversos campos de atuação dos profissionais envolvidos.

De forma integrada e colaborativa a nossa proposta, apoiada pela Atena Editora, trás ao leitor produções acadêmicas desenvolvidas no território nacional abrangendo informações e estudos científicos no campo das ciências médicas com ênfase na promoção da saúde em nosso contexto brasileiro. Desejamos que a obra “Medicina: Longe dos holofotes, perto das pessoas” proporcione ao leitor dados e conhecimento fundamentado e estruturado.

Tenham todos uma ótima leitura!

Benedito Rodrigues da Silva Neto

## SUMÁRIO

# CAPÍTULO 18

## PLATELET/LYMPHOCYTE AGGREGATES AND CD40L RECEPTORS HAVE A CRITICAL ROLE IN PROGRESSION AND METASTASIS OF GASTRIC CANCER

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**ABSTRACT: Background and objectives:** By modulating the immune system, platelets regulate several aspects of cancer-associated pathology and activated platelets could influence the mechanisms linking the CD40/CD40L system with neoplasia. Thus, the present study evaluated the activation level of circulating platelets in gastric cancer (GC), the formation of circulating platelet-leucocyte conjugates as well as the CD40L levels conjugate to leucocytes in GC patients **Methods**: Peripheral blood was taken before surgical treatment from 34 patients with gastric cancer and 30 healthy controls. Measurement of circulating platelet-leukocyte aggregates (PLAs) were performance by flow cytometry using monoclonal anti-CD41a for label platelet and CD62p and CD40L to measure of platelet activation. **Results:** We observed higher levels of platelet- T lymphocyte aggregate (P-T lymp) and platelet-B lymphocyte aggregate (P-B lymp) in the peripheral blood (PB) of GC patients with stage IV (metastatic) when compare with stages I-III, and control group ($p<0,05$). Reduced levels of CD40L+ Platelet–total lymphocyte (P-lymp) were observed at stage IV of the disease ($p<0.05$). High levels of CD62p+ platelets and CD62p+ platelets-monocyte aggregate were observed GC patients (Stages I-IV) when compare to control group ($p<0.05$). **Conclusion:** The results of this study allow us to conclude that quantitative alterations of platelet-lymphocytes aggregates (PLYS) and CD40L+ on PLYS were associated to progression and metastasis in GC.

**KEYWORDS**: Platelet, CD40L, platelets-leucocyte aggregate; gastric cancer; flow cytometry.

## 1 | INTRODUCTION

Gastric cancer (GC) is responsible for 9% of cancer deaths worldwide. Over 950,000 new cases are diagnosed each year, and about 90% of them are in advanced stage, requiring some systemic treatment [1][2]. Thus, it was valuable to explore the mechanism of gastric cancer progression such as proliferation, migration and immune escape [3]. It is now becoming clear that the tumors microenvironment, which is largely coordinate by inflammatory cells, is an indispensable participant in the oncologic process [4]

There is growing evidence demonstrating an intimate relationship between platelets and cancer, and the abundance of activated platelets in the microenvironment of a range of tumors[5][6] The interaction of platelets and tumor cells has been shown experimentally to influence metastasis as a result of both physical association and bidirectional activation [7].

Platelets modulate innate and adaptive immune responses with crucial roles in immune surveillance, inflammation and host defense. An important pre-requisite for platelet-mediated changes of immune functions involves direct engagement with different types of leukocyte [8] Under hemostatic conditions, platelets generally do not bind to leukocytes. However, when activated, platelets adhere to neutrophils and monocytes, and interactions with lymphocytes have also been identified [9]. Binding between platelets and other cell types is primarily mediated by P-selectin (also known as CD62p)[10]. P-selectin via its ligand, P-selectin glycoprotein ligand-1 (PSGL-1), has a central role in the interactions between platelets, monocytes, neutrophils and endothelial cells. P-selectin cross-links platelets and leukocytes and is a major mediator of platelet-leukocyte aggregate formation [11].

Activated platelets also express CD40L (also known as CD154). The role of CD40/CD40L in carcinogenesis is widely examined. The mechanisms linking the CD40/CD40L system and the soluble form of CD40 ligand (sCD40L) with neoplasia are nowadays a topic of intensive research [12]. Platelets expression of CD40L has been shown to affect dendritic cells as well as B and T lymphocytes, suggesting that it provides a communicative link between innate and adaptive immunity[13][11][9] Studies on CD40L have exposed that it enhances antineoplastic immune response, inhibits tumor growth, and induces apoptosis of cancer cells [14] [15]. Subsequent reports have suggested that in many tumors, CD40 activation by its ligand results in a completely contrary situation, i.e., enhancement of tumor growth and progression [16] [17].

The study of platelet function in cancer patients has many challenges. Activated platelets and aggregates seem to be crucially involved in cancer immunity and understand how it works in gastric cancer could open new possibilities of biomarkers and treatment, moreover, several pro-tumorigenic and cancer immunological relevant mechanisms can be addressed as well. The aim of the study was the evaluation of circulating platelet-leukocyte aggregates (PLAs) and platelet activation in patients with gastric cancer and compare PLAs levels regarding clinical stage.

## 2 | RESULTS

### 2.1 High PLAs levels of metastatic gastric cancer patients

The interaction between platelets and leukocytes, and endothelium can occur in various ways. PLAs have been detected in the blood of humans with a variety of diseases and are now considered as one of the most sensitive markers related to platelet activation.

This study, we observed reduced levels of T lymphocytes (T-Lymp) (Figure 1A) and increased levels of Platelet – T lymphocytes (P-T lymp) and Platelet – B lymphocytes (P-B lymp) in peripheral blood of gastric peripheral blood of gastric cancer patients with stages stage IV (metastatic) when compare to stages I-III (no metastatic) and control groups ($p<0,05^*$) (Figure 1B and D). No significant differences were observed in the levels of B lymphocytes (B-Lymp) between CG patients and controls groups (Figure 1C).

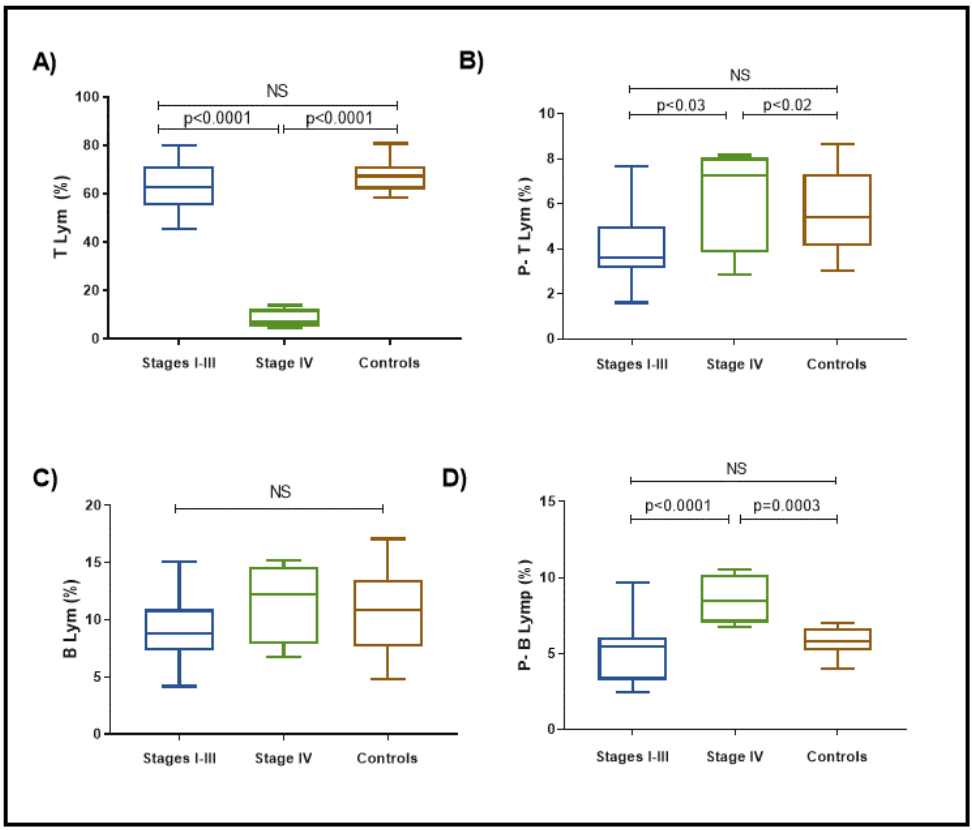


Figure 1. Percentage levels of: A) T lymphocytes (T-Lymp), B) Platelet – T lymphocytes (P-T lymp); C) B lymphocytes (B-Lymp) and D) Platelet – B lymphocytes (P-B lymp) in peripheral blood of gastric cancer patients with stages I-III (n=27), stage IV (n=7) and control group (n=30). Kruskal-Wallis test was performed. *p<0.05. NS: No significant.

## 2.2 Analyses of the levels P-Lymp, P-neutrophils and P-monocytes with CD40L expression in peripheral blood of CG patients

We observed high levels of platelet - total lymphocytes aggregates (P-Lymp) and reduced levels of CD40+ P-lymp in blood of CG patients with stage IV compared to stages I-III and controls groups (p<0,05; Figure 2A and B). No significant differences were observed in the levels of Platelets-neutrophils aggregate (P-neutrophils), CD40L+ P-neutrophils, Platelets-monocytes aggregate (P-monocytes) and CD40L+ P-monocytes between CG patients and controls groups (Figure 2C-F).

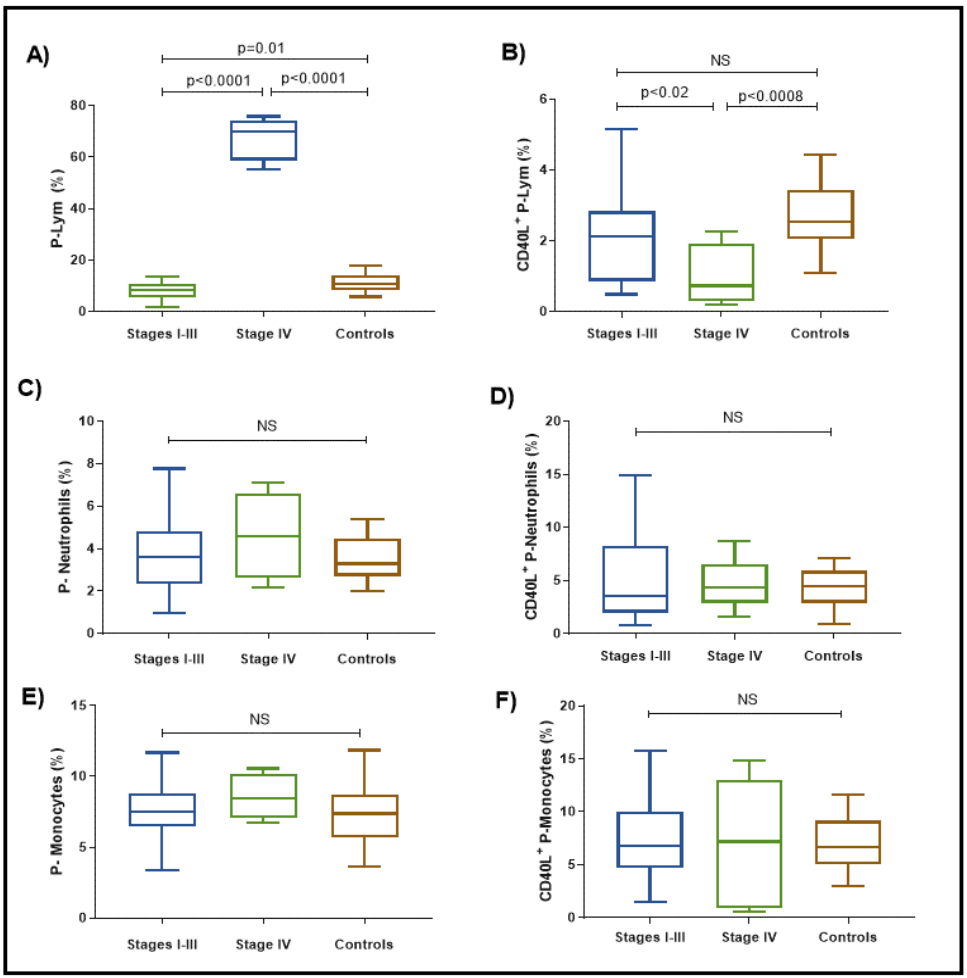


Figure 2. Percentage levels of: A) Platelet - Total lymphocytes aggregates (P-Lymp) B) CD40+ P-lymp; C) Platelets-neutrophils aggregate (P-neutrophils); D) CD40L+ P-neutrophils; E) Platelets-monocytes aggregate (P-monocytes); and E) CD40L+ P-monocytes in the peripheral blood of gastric cancer patients with stages I-III (n=27), stage IV (n=7) and control group (n=30). Kruskal-Wallis test was performed. *p<0.05. NS: No significant.

### 2.3 Analyses of the levels CD62p+ CD41a+, CD62p+ P-neutrophils and CD62p+ P-monocytes in blood of GC patients

We observed high levels of activated platelets (CD62p+ CD41a+) and activated platelets-monocytes aggregate (CD62p+ P-monocytes) in GC patients with stages I-III and stage IV when compared to controls ($p<0,05$) (Figure 3A and 3C). No significant difference between in the levels of CD62p+ P-neutrophils between CG patients and controls groups (Figure 3B).

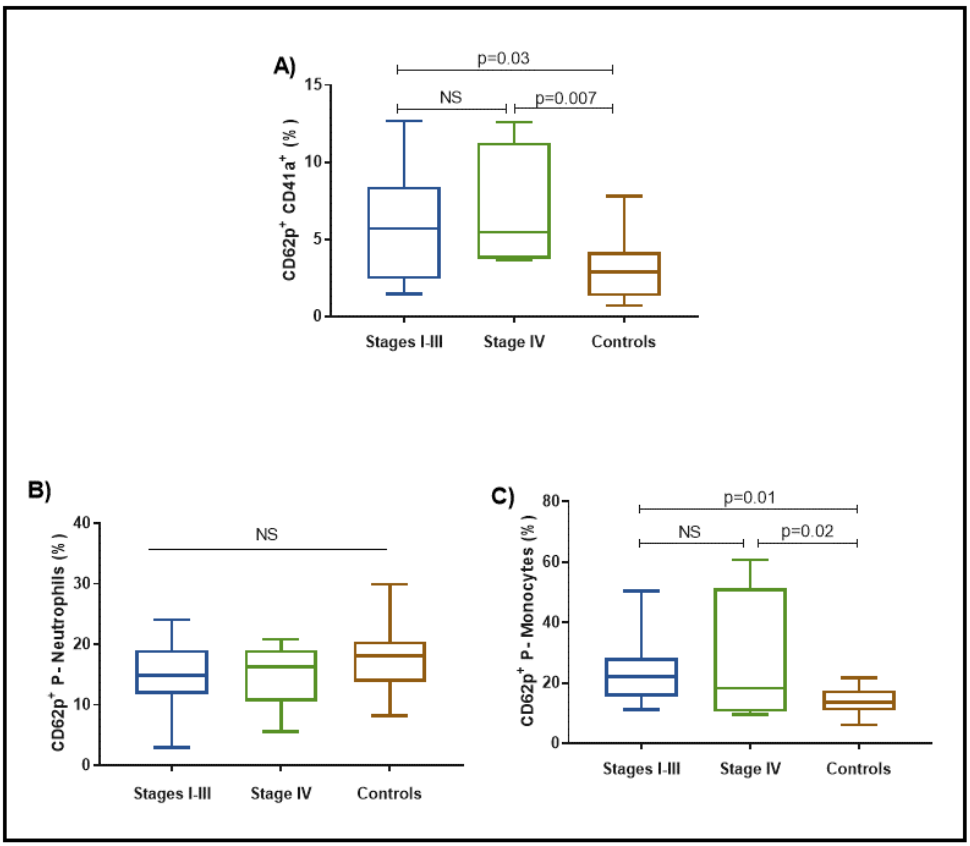


Figure 3. Percentage levels of: A) activated platelets (CD62p+ CD41a+); B) activated platelets-neutrophils aggregate (CD62p+ P-neutrophils) C) activated platelets-monocytes aggregate (CD62p+ P-monocytes) in peripheral blood of gastric cancer patients with stages I-III (n=27), stage IV (n=7) and control group (n=30). Kruskal-Wallis test was performed. *$p<0.05$. NS: No significant.

## 3 I DISCUSSION

Platelets, besides their specialized role in hemostasis and atherothrombosis, actively modulate innate and adaptive immune responses with crucial roles in immune surveillance, inflammation and host defense. An important pre-requisite for platelet-mediated changes of

immune functions involves direct engagement with different types of leukocytes [8]. Platelet activation and concomitant degranulation enables platelets to engage with leukocytes via soluble factors and physical interaction facilitated by a variety of receptors [18]. Most importantly receptors involved in direct interactions include platelet P-selectin (CD62p) and CD40 ligand (CD40L), as well as PSGL-1, CD40 and Mac-1 (integrin αMβ2, CD11b/CD18) on leukocytes [19].

To the best of our knowledge, our study is the first to investigate the associations between parameters of platelet activation (CD62p+ and CD40L) as well as the associations of these biomarkers with PLAS formation and GC. We observed a close correlation between the plasma levels of P-T lymp and P-B lymp and disease, especially metastatic GC. Activated platelets play multiple roles in the progression of tumor metastasis, including facilitation of tumor cell epithelial-mesenchymal transition (EMT), degradation of surrounding extracellular matrix (ECM), increasing vascular permeability, and aiding in the establishment of malignancies in distant tissues [20]. Besides that, we find out the same levels of B-lymphocytes among the CG and control group and a reduction in T lymphocyte levels in stage IV patients of the disease. Platelet-released platelet factor 4 (PF4) that inhibits T cell proliferation and regulates the functional activities of T cells via binding to glycosaminoglycan and that could justify the results [21-26].

Despite the increase in levels of P- lymp found in CG patients with metastases (stage IV), reduced levels of CD40L + P-lymp were observed at this stage of the disease, whereas patients without distant metastases had higher levels of CD40L + P-lymp. Our results demonstrate that CD40L immune responses at stage IV GC are involved in the progression and metastasis. Platelets are an abundant and systemic source of CD40L. When platelets were reported to express functional CD40L, it became intriguing to hypothesize that platelets could somehow participate in or influence adaptive immune responses [27]. Platelets can directly stimulate proliferation and antibody production of B cells in vitro via CD40L-mediated cell–cell contact. CD40L is identical in terms of structure and physiological function to membrane bound CD40L expressed in activated T-lymphocytes and other cells [28]. It can notably generate signals for the recruitment and extravasation of leukocytes. It induces, through the engagement of CD40, the secretion of chemokines and the expression of adhesion receptors in endothelial cells. It provides a powerful link between platelets and the immune system: CD40L expressed on activated platelets induces dendritic cell maturation, B-cell isotype switching, and augments CD8+ T-cell responses in both in vitro and in vivo models [29][27][30][31]. The CD40 – CD40L interaction is very important for the host defense against cancer.

In this paper, we did not observe quantitative changes in P-monocytes, P-neutrophils, CD40L + P-monocytes and CD40L + P-neutrophils in CG patients when compared to controls. On the other hand, there was an increase in activated platelet levels (CD41a + CD62p +) and CD62p + P-monocytes in CG patients (figure 3), with or without distant metastasis.

The platelet-monocyte aggregates have longer persistence in peripheral blood and were shown to be more sensitive markers of in vivo platelet activation than other platelet surface markers. Platelets can induce production of pro-inflammatory factors from other cells in the tumor microenvironment and in this way indirectly promote recruitment of leukocytes to primary and metastatic tumors. For example, platelets have been shown to induce CCL2 expression in tumor cells, and CCL2 can recruit monocytes that promote metastatic seeding at distant sites [32-34].

The role of platelets in the progression of malignant tumors has gained attention, moreover, the complex interactions among tumor cells, leukocytes, and platelets induced a specific expression profile in microvascular endothelial cells, characterized by the up-regulation of several molecules previously associated with metastasis including CCL5 [35]. The (RANTES)/CCR5 pathway is important for mediating physiological processes required for tumor invasion and progression [36]. Platelets, together with leukocytes and tumor cells, induced CCL5 secretion from endothelial cells in an experimental model of colorectal cancer, which enhanced metastatic seeding due to recruitment of monocytes [37]. Metastasis is the major cause of cancer-associated mortality and therapeutic options are limited since the underlying mechanisms remain incompletely understood. Our studies provide new insights into the molecular pathways that mediate progression of GC.

One of the most interesting results found in this study was the reduced level of T-lymphocytes associated with metastatic stage of GC (figure 1). The decline in T-lymphocytes observed in gastric cancer patients may reflect both reduced proliferation and clonal expansion, or exhaustion of T cells with increased apoptosis these cells. Some immune checkpoints may be involved as well. Rolfes et al 2018 showed an increase of programmed death-ligand 1 (PD-L1) expression in circulating blood platelets in head and neck squamous cell carcinoma (HNSCC)(33). PDL1 binds to the immune checkpoint protein programmed cell death protein 1 (PD-1) expressed on antigen-specific CD8(+) T cells, induce the exhaustion of T cells and increased apoptosis these cells. These authors also extended to other cancer types and found increased PD-L1 expression on platelets from patients with lung cancer [38]. What mechanism(s) might underlie the decline in T-lymphocytes upon the development of GC? Thus, GC can lead to the long-term impairment of lymphoid homeostasis, a condition that may need to be addressed for immunotherapy.

Although the results are promising, the number of patients enrolled was relatively small. Prospective studies on larger cohorts of patients are needed to confirm the results achieved and further studies need to elucidate and confirm more precisely the roles and mechanisms for CD40L during the gastric cancer development. Since CD40L and CD40 molecules are strong immune stimulators, they are considered useful in anticancer therapy [39]. However, understanding the balance between the benefits and risks are crucial to the best approached.

## 4 | MATERIALS AND METHODS

### 4.1 Study and subjects

This is a cross-sectional study performed in CG patients undergoing treatment for potentially curable in Hospital Cancer Pernambuco between 2017 to 2018. Laboratory analysis was in the Translational Research Laboratory of the Instituto de Medicina Integral Prof. Fernando Figueira. (IMIP) The inclusion criteria in this study was histologically confirmed adenocarcinoma of the stomach and all patients were newly diagnosed cases and none of patients had received chemotherapy, radiotherapy or immunotherapy before sampling. We collected information on clinical characteristics of the patients prospectively. Cancer Staging was performed according to the 7th edition of the American Joint Committee on Cancer TNM criteria [40]. The study protocol was reviewed and approved by the Hospital de Cancer de Pernambuco Ethics Research Committees (CAAE: 66228917.8.0000.5205).

Peripheral blood was taken before surgical treatment from 34 patients with gastric cancer (10 women and 24 men, aged 31-89 years), In all the gastric cancer patients adenocarcinoma in I (14,8%), II (32,35%), III (32,35%) and IV (20,5%) stages according to UICC/TNM classification was found. Histopathological gastric cancer classification according to Lauren revealed intestinal type cancer in 28% of the patients, diffuse cancer in 56 %, and no determinate in 16% of the cases. Lymph node involvement were detected in 63% of the cases, and distant metastases in 20,5% of the patients. In 72% of the patients was determined as G3, and in 28% - G1/G2 (Table 1). None of the patients performed radiotherapy or chemotherapy before peripheral blood collection. Individuals with autoimmune disease, infectious diseases and primary cancer were excluded. The basic patient demographic characteristics are summarized in table 1.

Control group were 30 healthy persons (10 women and 20 men), aged 30 – 85 years (median 53 years). These volunteer donors had not been on any medication including aspirin or non-steroid anti-inflammatory drugs during the previous two weeks. Controls subjects who were referred had no previous history of malignancy or auto-immune disease.

| PATIENTS CHARACTERISTICS | N=34 (%) |
|---|---|
| **AGE** | |
| Median (range) | 51 (31 – 89) |
| **SEX** | |
| Male | 24 (70,5) |
| Female | 10 (29,5) |
| **LYMPH NODE INVOLVEMENT** | |
| Negative | 10 (37) |
| Positive | 17 (63) |

| | |
|---|---|
| **DISTANT METASTASIS** | |
| No | 27 (79,5) |
| Yes | 7 (20,5) |
| **TNM STAGE*** | |
| I | 5 (14,8) |
| II | 11 (32,35) |
| III | 11 (32,35) |
| IV | 7 (68) |
| **HISTOLOGIC GRADE** | |
| Differentiated (G1/G2) | 10 (28) |
| Undifferentiated (G3) | 24 (72) |
| **LAUREN CLASSIFICATION** | |
| Intestinal | 9 (28) |
| Diffuse | 19 (56) |
| No determinated | 5 (16) |

Values are presented as number (%) or median (range). *Classification according to the American Joint Committee on Cancer 7th edition.

Table 1. Basic characteristics of CG patients (n=34).

### 4.2 Sample Preparation

Venous Blood samples were collected in EDTA tubes and maintained at 4 oC until being processed within 60 minutes of collection. Immunophenotyping of peripheral blood was performed by flow cytometry on a BD FACSVerse™ (BD Biosciences). For sample preparation, we performed the protocols described by Harding, et al (2007), Bournazos et al (2008) and Macey et al 2002 [41-43].

### 4.3 Measurement of circulating platelet-leukocyte aggregates (PLAs) by flow Cytometry

Two milliliters of red blood cell lysis buffer (FACS Lysing Buffer; Becton Dickinson, Mountain View, CA, USA) was added to a 50-mL aliquot of peripheral blood, incubated for 20 min at room temperature, and protected from light. Next, 2 mL concentrated 1× phosphate-buffered saline (PBS) was added to wash the cells, and samples were centrifuged at 300 × g for 5 min at 20°C. Was added five microliters of monoclonal anti-CD3 for label T Cells, anti-CD20 for label B cells [44] anti-CD41a (GpIIb/IIIa) for label platelets, anti-CD62p (GMP 140) and anti-CD40L for label platelets activation, anti-CD16 for label neutrophils [45] and anti-CD14 for label monocytes [46] (BD, Pharmigen, San Diego, CA, USA) and incubated at 20 oC for 20 min in the dark, as described by Granja T, et al. 2015) [47][48]. Washing of cells was performed with 2 mL Phosphate Buffered Saline (PBS) (1×), and cells were then centrifuged at 300 × g for 5 min at 20°C. The flushing procedure was repeated twice. After washing, the supernatants were discarded, and the cells were analyzed with 250µL

saline on a flow cytometer (FACSVERSE; Becton Dickinson, Sunnyvale, CA, USA). For flow cytometry, 20,000 cellular events were acquired, and the results were analyzed by FACSuite program (Becton Dickinson).

The CD41a antibodies were used for platelet labeling and CD62p to measure percentages of platelet activation. Platelet - Total lymphocytes aggregates (P-Lymp and CD40+ P-lymp) was performed using anti-CD41a and anti-CD40L on total lymphocytes gate. Platelet – T lymphocytes (P-T lymp) was performed using the monoclonal anti-CD3/anti-CD41a; Platelet – B lymphocytes (P-B lymp) was performed using the monoclonal anti-CD20/anti-CD41a; platelet-monocyte aggregates (P-monocytes and CD40+ P-monocytes) were analyzed by labeling with monoclonal antibodies anti-CD41a, anti-CD14 and anti-CD40L in monocytes gate. Platelet-neutrophil aggregates (P-Neutrophils and CD40L+ P-neutrophils) with monoclonal antibodies anti-CD41a, anti-CD16 and anti-CD40L in granulocytes gate. For T and B cell immunophenotyping, were used monoclonal antibodies anti-CD3 (T Lymp) and anti-CD20 antibodies (B-Lymp), respectively. Analyzes were expressed as percentages (%).

### 4.4 Statistical analysis

Statistical analysis was performed using GraphPad® Prism 7 (GraphPad Software Inc., USA). All variables were analyzed in terms of normality using Shapiro-Wilk normality tests. Kruskall-wallis U-test were used to analyze quantitative variables. Quantitative data are expressed as median and interquartile interval (IQR 25-75). Categorical variables are expressed as number (N) and as percentage (%). A value of $p<0.05$ was considered significant.

## 5 I CONCLUSIONS

In conclusion, understanding the platelet activation pathways and potential biomarkers could promise new diagnostic and therapeutic possibilities in monitoring the disease activities and responses to treatment. Because platelet-tumor cell interactions induce platelet activation and aggregation, it is reasonable to interfere with this process as a therapeutic intervention. Therefore, platelets are a promising therapeutic target for the attenuation of metastatic events in GC. We have dissected some mechanisms (CD40L) governing the interplay between T lymphocytes cells and the immunosuppressive microenvironment. Most significantly, we have found distinct way of immune regulation in gastric cancer that can be exogenously manipulated to cancer immunotherapy.

## AUTHOR CONTRIBUTIONS

C.A.C.L. and M.R.M. conceived and designed the experiments; R.L.S. and L.C.T. performed the experiments; C.A.C.L., M.R.M, J.P.A.S. and L.C.T. analyzed the data; R.L.S

and L.C.T. contributed reagents/materials/analysis tools; C.A.C.L. and M.R.M. wrote the paper. L.C.T. responsible for funding acquisition and supervised the study.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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