

# Distinct Pattern of Inflammatory Enzyme Activities in Human Ovarian Cancer and Benign Myoma

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**Abstract:** *Objective:* Inflammatory cells and their products are significant components of malignancies. This study was performed to determine the activity of inflammatory enzymes myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG) in ascitic fluid, sera or peritoneal lavage fluid from patients with epithelial ovarian cancer (EOC).

*Methods:* Eighteen patients age ranging from 25 to 79 years (54.6±2.9 years) with epithelial ovarian cancer submitted to surgical treatment (EOC group) and 17 patients with uterine myoma (Myoma group) submitted to abdominal hysterectomy (control group) were prospectively studied. MPO and NAG activities were evaluated colorimetrically in sera, ascites or peritoneal lavage fluid obtained from the patients at the time of laparotomy.

*Results:* In a total of 18 EOC, there were stage I in 1 case (5.5%), II in 3 (16.7%), III in 11 (61.1%) and IV in 3 cases (16.7%). MPO activity in sera of EOC was higher than in the ascitic fluid from the same patients. Conversely, MPO activity was similar in sera from both EOC and myoma-bearing patients. Comparison between NAG activities in sera from both groups showed much higher values in the EOC patients. Furthermore, inflammatory enzyme activities were overall associated with the stage of the disease.

*Conclusions:* Our results show that inflammation has been positively correlated with cancer and that the pattern of a systemic inflammatory response induced by EOC differs quantitatively from that of a typical benign pelvic condition. The most important limitation lies in the fact that the number of patients and controls was relatively small. Further studies with a larger number of patients and longer follow-up are necessary to assess the accuracy of the diagnostic and prognostic impact of these results.

**Keywords:** NAG, (N-acetylglucosaminidase), MPO(Myeloperoxidase), Neutrophils, Macrophages, Enzymes, inflammatory response, Ovarian cancer, inflammation, myoma, pelvic surgery.

## INTRODUCTION

Ovarian cancer is the seventh most frequent cancer in women worldwide, and is the leading cause of death from gynecologic malignancies in most of the Western world [1]. Despite improvements in surgical management and advances in cytotoxic therapy, the overall 5-year survival rate for women with advanced disease is only 13% [2, 3]. Because ovarian cancer is often asymptomatic in its early stages, the great majority of patients have widespread disease at the time of diagnosis.

Over the past fifteen years the understanding of the inflammatory microenvironment of malignant tissues has implicated inflammatory processes as cofactors in carcinogenesis and inflammatory cells as “promoters” of tumor development [4-7]. Neutrophils and macrophages are often present in tumors and considered to affect their development. While macrophages are prominent in the stromal

compartment of practically all types of neoplasias [8] neutrophils are mainly found in tumor blood vessels. These highly versatile cells respond to the presence of *stimuli* in different parts of tumors with the release of a distinct repertoire of growth factors, cytokines, chemokines and enzymes that regulate tumor development and metastasis [8, 9]. In human breast cancers, a positive correlation between poor prognosis and the density of tumor-associated macrophages has been found [10]. Genetic studies in mice showed that decreased number of macrophages in tumor mass was associated with large reduction in rates of metastasis [10]. Among the products released by inflammatory cells, myeloperoxidase (MPO), an enzyme restricted to azurophil granules of neutrophils, has been extensively used as a marker for measuring polymorphonuclear leukocytes accumulation in tissue samples [11]. MPO catalyzes a reaction that produces hypochlorous acid, which, although toxic to bacteria, can lead to activation of some procarcinogens and damage to DNA [11-13]. Another enzyme, N-acetyl-beta-glucosaminidase (NAG), present in lysosomes, has been employed to detect macrophage accumulation/activation in a variety of animal and human tissues, including tumors [14-16]. It has been suggested that these enzymes and

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products of their activities can be released to the outside of the cells raising the potential for damage to an extracellular target. In fact, there have been attempts to use local or systemic levels of MPO or NAG activities as predictors of tumor progression in patients with breast cancer [17, 18], gastric adenocarcinoma [15] and gynecological cancers [19]. In these studies, positive association between sera or tumor levels of inflammatory enzymes activities and tumor growth has been reported. We have not found in the literature any report in which the activities of both enzymes have been determined simultaneously in ascites fluid and serum from patients bearing ovarian tumor or uterine myoma.

In the present study we evaluate the inflammatory response in these two major pathways: local (ascitic fluid) and systemic (serum) in patients with epithelial ovarian cancer (EOC) in comparison with serum or peritoneal lavage of patients bearing non-malignant growth (myoma). We aim at providing further evidence for the association between cancer and inflammation. We will also try to determine an association between serum levels of inflammatory enzymes activities and tumor stage. This analysis might reveal causal or parallel inflammatory events involved in epithelial ovarian cancer and thus unravel factors and mechanisms underlying tumor development.

## PATIENTS AND METHODS

This study prospectively included 18 women with ovarian cancer submitted to surgical treatment (EOC group) and 17 women with uterine myoma submitted to abdominal hysterectomy (control group) who were treated at the Department of Obstetrics and Gynecology, Federal University of Minas Gerais, between January 2005 and February 2006. All ovarian tumors examined were limited to epithelial ovarian cancer. The study protocol was approved by the Ethical Committee for Research in Human Beings guidelines of the Institution. An informed consent from all patients involved was also obtained. The study group was composed of female patients with EOC admitted for surgical treatment and the control group enrolled women bearing uterine leiomyomas needing surgical removal. All women gave their informed written consent.

Patients with ovarian cancer underwent laparotomy and debulking surgery. This was the primary treatment for all patients, since none of them had previously undergone radiotherapy and/or chemotherapy. The

tumor staging was performed according to the FIGO recommendations. At the time of laparotomy serum and ascites samples were collected from each patient. Abdominal hysterectomy was performed for uterine myoma according to the modified Richardson's technique [20] and serum samples were collected at the time of hysterectomy. The peritoneal lavage (10 mL) was collected after washing the cavity with 20 mL of sterile saline. All biological samples were centrifuged at 10,000 rpm for 10 minutes and stored at -20°C until analysis.

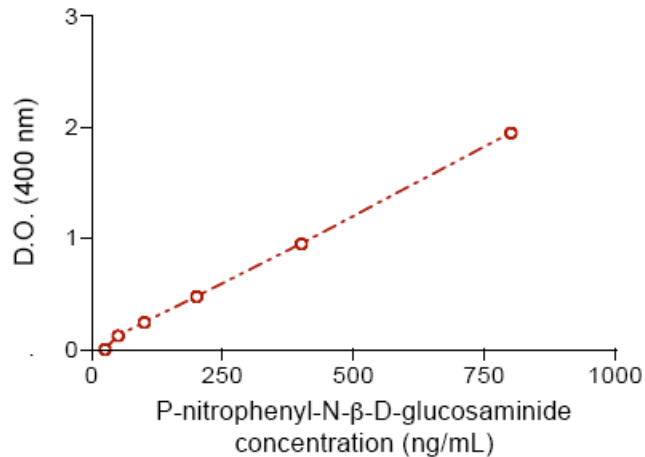
## Determination of MPO Activity

Neutrophil quantification in ascitic fluid, serum and peritoneal fluid was indirectly assessed by assaying MPO activity as initially described by Bradley *et al.* (1982) and used in my other publications in order to quantify neutrophilic inflammatory activity (16, 27, 28 e 29). An aliquot of each fluid was homogenized in 2.0 mL of phosphate buffer (pH 4.7 0.1 M NaCl, 0.02M NaPO<sub>4</sub>, 0.015M NaEDTA) and centrifuged at 12000g for 10 minutes at 4° C. The pellets were further resuspended in 2.0 mL of phosphate buffer (pH 5,4 0.05M NaPO<sub>4</sub>) containing 0.5% p/v hexadecyltrimethylammonium bromide (HTAB), followed by three cycles of freezing and thawing in liquid nitrogen. New centrifugation at 12000g for 10 minutes at 4°C was performed and the supernatant used for measuring MPO levels. A 96-well plate was filled with 25  $\mu$ L of the samples as well as 25  $\mu$ L of TMB (tetramethylbenzidine 1.6 mM) substrate diluted in DMSO. The plate was then incubated at 37° C for 5 minutes followed by the addition of 100  $\mu$ L of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub> 0.3mM) to each individual well and a second round of incubation at 37° C for 5 minutes. All assays were performed in duplicate. The reaction was stopped by the addition of 100  $\mu$ L de H<sub>2</sub>SO<sub>4</sub> 4M. MPO activity in the samples was assayed by measuring the change in absorbance (optical density; OD) at 450 nm. Results were expressed as change in OD/mL.

## Determination of NAG Activity

Accumulation of mononuclear cells in ascites, serum or peritoneal fluid lavage was quantified by measuring the levels of the lysosomal enzyme NAG present in high levels in activated macrophages [16, 20]. An aliquot of each fluid (1mL) was homogenized in NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega) and centrifuged (3,000 g; 10 min at 4°C). Samples of the resulting supernatant (100  $\mu$ L) were incubated for 10 min with 100  $\mu$ L of p-nitrophenyl-

N-acetyl-beta-D-glucosaminide (2,24 mM) prepared in citrate/phosphate buffer (39 mM pH = 4.5) and incubated for 10 min at 37°C. The reaction was stopped by addition of 100 $\mu$ L of 0.2M glycine buffer (pH 10.6). The reaction product was detected colorimetrically and performed at 400 nm. A standard curve with p-nitrophenol (0-500 nmol<sup>-1</sup>) was built and the results expressed in NAG activity (in nanograms) per sample milliliter (mL) (Figure 1).



**Figure 1:** N-ACETILGLICOSAMINIDASE (NAG) pattern curve – Pattern curve of substrate concentration (p-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminida) used to calculate NAG (n-acetilglucosaminidase) concentration in the samples.

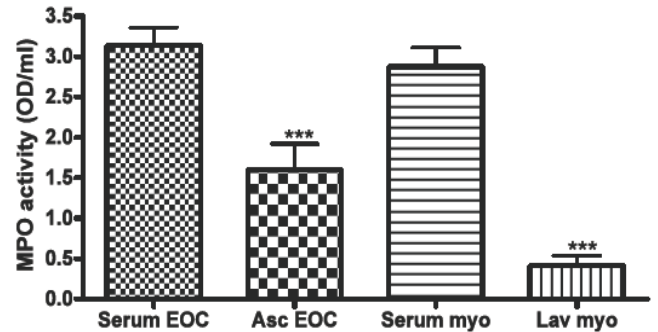
### Statistical Analysis

Results are presented as means $\pm$ s.e.m. The data were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls correction factor for multiple comparisons as a post-test. The level of significance was set at  $p < 0.05$ . This sample (18 EOC and 17 myomas) were enough to show statistical differences between groups with a confidence level of 95% a statistical power of 80% and a minimal detectable difference of one standard deviation.

### RESULTS

The age of the patients ranged from 25 to 79 years (54.6 $\pm$ 2.9 years). The stage (FIGO) was I in 1 case (5.5%), II in 3 (16.7%), III in 11 (61.1%) and IV in 3 cases (16.7%).

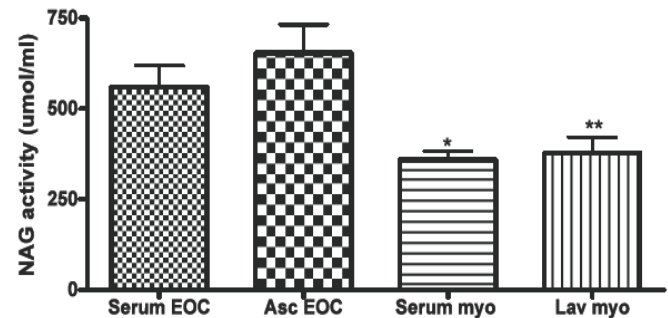
The comparison between the inflammatory enzyme activities revealed that in sera of EOC patients, MPO activity was significantly higher ( $p < 0.001$ ) than in the ascitic fluid of the same patients and comparable to the enzyme activity in sera of myoma bearing patients (Figure 2).



**Figure 2:** Levels of MPO activity in sera, ascitic fluid (asc), peritoneal lavage fluid (lav) of patients bearing EOC (n=18) or myoma (n=17).

Note: The data were analyzed using one way analysis of variance (ANOVA). \*\*\* $p < 0.001$  for comparison between EOC serum versus EOC ascites; myoma serum versus peritoneal lavage.

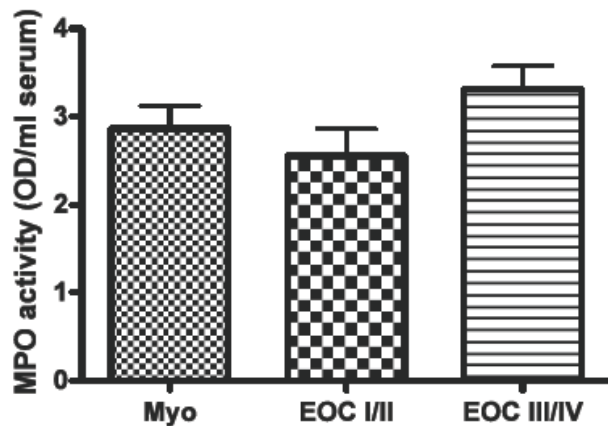
A completely different profile of activity was observed for NAG activity in both groups. Thus, inflammatory enzyme activity was similar within sera or ascitic fluid in EOC group, but significantly higher than sera or peritoneal lavage fluid in the myoma group (Figure 3).



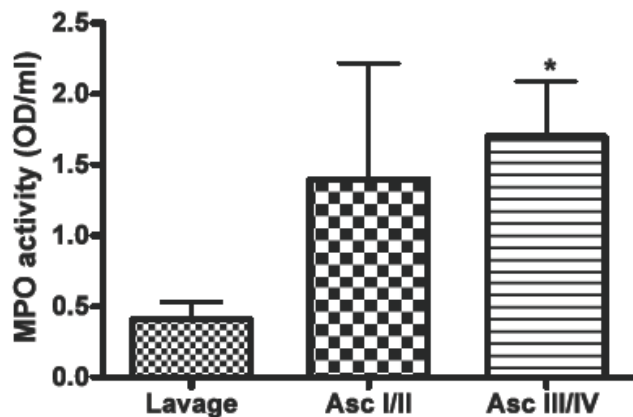
**Figure 3:** Levels of NAG activity in sera, ascitic fluid (asc), peritoneal lavage fluid (lav) of patients bearing EOC (n=18) or myoma (n=17).

Note: EOC – Epithelial ovarian cancer. The data were analyzed using one way analysis of variance (ANOVA). \* $p < 0.05$  and  $p < 0.01$  for comparison between EOC serum versus EOC ascites; myoma serum versus peritoneal lavage, respectively.

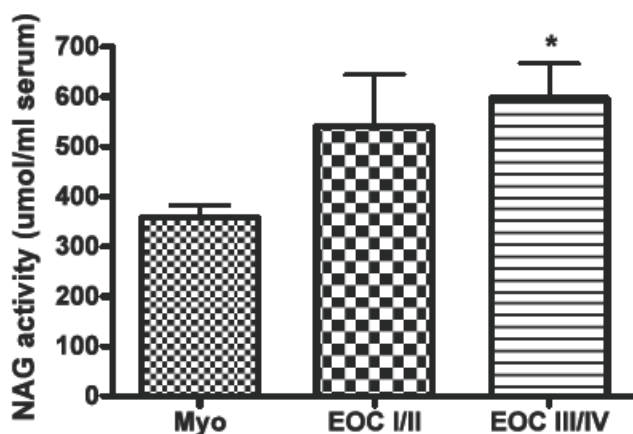
These data were further analyzed to give indication of possible association between disease stage and levels of enzyme activities. Although MPO activity in sera of both groups (myoma or EOC) showed similar levels (Figure 4), this activity was significantly higher in ascitic fluid of EOC patients stage III/IV compared with peritoneal lavage fluid of myoma-bearing patients (Figure 5). NAG activity was higher in serum (596 $\pm$ 70  $\mu$ mol/mL; stage III/IV) and ascites (684 $\pm$ 90  $\mu$ mol/mL) of tumor-bearing patients in different stages compared with serum (359 $\pm$ 24  $\mu$ mol/mL) or peritoneal lavage fluid (380 $\pm$ 42  $\mu$ mol) of the myoma group (Figures 6 and 7).



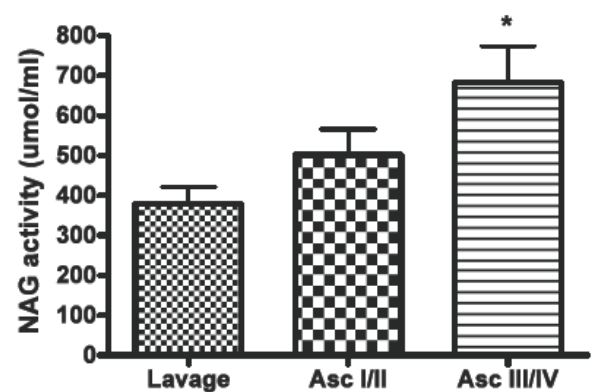
**Figure 4:** MPO activity in sera of patients bearing EOC according to disease stage (n=18) or myoma (n=17). Note: EOC – Epithelial ovarian cancer. The data were analyzed using one way analysis of variance (ANOVA).



**Figure 5:** MPO activity in ascitic fluid of patients bearing EOC according to disease stage (n=18) or myoma (n=17). Note: EOC – Epithelial ovarian cancer. The data were analyzed using one way analysis of variance (ANOVA). \* $p < 0.05$  lavage fluid versus ascitic fluid EOC stage III/IV.



**Figure 6:** NAG activity in sera of patients bearing EOC according to disease stage (n=18) or myoma (n=17). Note: EOC – Epithelial ovarian cancer. The data were analyzed using one way analysis of variance (ANOVA). \* $p < 0.05$  myoma sera versus EOC sera stage III/IV.



**Figure 7:** NAG activity in ascitic fluid of patients bearing EOC according to disease stage (n=18) or myoma (n=17). Note: EOC – Epithelial ovarian cancer. The data were analyzed using one way analysis of variance (ANOVA). \* $p < 0.05$  lavage fluid versus ascitic fluid stage III/IV.

## DISCUSSION

Inflammation has been positively correlated with cancer for many years, and in the past few years, the connection between inflammation and cancer has been explored in greater detail as various components of the inflammatory reaction have been better defined [4-7]. In this regard, the pelvic peritoneum in ovarian cancer patients has been shown to exhibit a general pattern of chronic inflammation, represented primarily by differentiated monocytes/macrophages, and distinct from the one seen in benign ovarian or other benign pelvic tumors [22]. The inflammatory environment of the tumor-host interface may contribute to tissue reorganization, tumor cell invasion, angiogenesis, capillary leakage, and the production of ascites [22, 23]. We have studied local and systemic inflammatory response in patients with ovarian cancer by measuring comparatively MPO and NAG activities in sera and ascitic fluid of EOC or myoma-bearing patients. The overall inflammatory process as assessed by the measurement of the inflammatory enzymes, locally or systemically, was increased (about two-fold), in EOC patients as compared with the myoma group (NAG). Thus, confirming and expanding the positive association between inflammatory processes and development and progression of tumors of earlier studies.

Increased myeloperoxidase or other inflammatory markers in plasma/sera of subjects with a variety of cancers, including gynecological tumors has been reported [22, 19, 23]. In our study, MPO activity level in sera of EOC patients was similar to that of myoma-bearing patients. MPO activity however was higher in ascitic fluid of cancer patients compared with peritoneal

lavage fluid of myoma patients and associated with ovarian cancer stage. The results of the measurement of NAG activity are also compatible with the notion of co-dependence between inflammation and tumor progression. The levels of enzyme activities were about two-fold in both sera and ascitic fluid of EOC patients compared with the activities of sera or peritoneal lavage fluid of the myoma group. Furthermore, the enzyme activity in sera or ascitic fluid of stage I/II patients was clearly lower than the activity level of stage III/IV patients. The number of stage I/II patients was not enough to show any statistical significance and it may be difficult to increase the number of patients in stage I/II since ovarian cancer is often asymptomatic in its early stages, the great majority of patients have widespread disease at the time of diagnosis. Again our data are in agreement with elevated NAG activities detected in biological fluids of cancer patients bearing thyroid, gastric or breast tumors [15, 18]. Our study, however, is unique in that the activities of both enzymes have been determined quantitatively and simultaneously in sera, ascites fluid or peritoneal lavage fluid from patients bearing two distinct pathological conditions (epithelial ovarian tumor or uterine myoma). Collectively, our results show that the pattern of a generalized inflammatory response induced by EOC differs quantitatively from the pattern of a typical benign pelvic condition. It could be argued that the comparison of peritoneal lavage fluid to ascites is inherently flawed for any marker of acute response because lavage is introduced. Peritoneal lavage cytology however is an established procedure used in ovarian cancer staging and diagnosis. Determination of enzyme activity was performed using the same methodology in all samples which reduces variations in the results encountered.

The role of inflammatory cells and their products, in respect to cancer is, on the one hand, to perform cytotoxic activity and on the other, to promote invasion and metastasis. Both inflammatory enzymes, MPO and NAG have been shown to possess proteolytic activity allowing for invasion and metastasis [14, 25], however, whether the suppression of these enzymes is likely to decrease/ameliorate the systemic inflammatory response and to increase survival rates of cancer patients remains to be investigated.

The most important limitation lies in the fact that the number of patients and controls was relatively small. Further clinical studies with larger series, association with other markers, and follow-up of patients may establish the real importance of inflammatory markers

in ovarian cancer as peritoneal inflammation may play a causative role in ovarian tumor development and metastasis [26]. In this regard, if ever proved effective, these markers might become a useful strategy to overcome the current limitations in ovarian cancer diagnosis as well as assess the accuracy of the diagnostic and prognostic impact of these results.

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