

# Tryptase mast cell density, protease-activated receptor-2 microvascular density, and classical microvascular density evaluation in gastric cancer patients undergoing surgery: possible translational relevance

Michele Ammendola, Rosario Sacco, Giuseppina Vescio, Valeria Zuccalà, Maria Luposella, Rosa Patruno, Nicola Zizzo, Claudia Gadaleta, Iliaria Marech, Roberta Ruggieri, Ibrahim Furkan Kocak, Taner Ozgurtas, Cosmo Damiano Gadaleta, Giuseppe Sammarco and Girolamo Ranieri

## Abstract

**Background:** Mast cells (MCs) can stimulate angiogenesis, releasing several proangiogenic cytokines stored in their cytoplasm. In particular, MCs can release tryptase, a potent *in vivo* and *in vitro* proangiogenic factor via protease-activated receptor-2 (PAR-2) activation and mitogen-activated protein kinase (MAPK) phosphorylation. Nevertheless, no data are available concerning the relationship among tryptase MC density (TMCD), endothelial cells (ECs) positive to PAR-2 microvascular density (PAR-2-MVD) and classical MVD (C-MVD) in gastric cancer (GC) angiogenesis.

**Methods:** In this study, we analyzed the correlation of TMCD, PAR-2-MVD, C-MVD with each other and with the main clinicopathological features in GC patients who underwent surgery. A series of 77 GC patients with stage T<sub>2-3</sub>N<sub>2-3</sub>M<sub>0</sub> (classified by the American Joint Committee on Cancer for Gastric Cancer, 7th edition) were selected and then underwent surgery.

**Results:** Tumour tissue samples were evaluated by mean of immunohistochemistry and image analysis methods in terms of numbers of TMCD, PAR-2-MVD and C-MVD. A significant correlation between the TMCD, PAR-2-MVD and C-MVD groups with each other was found by Pearson *t*-test analysis (*r* ranged from 0.64 to 0.76; *p* value ranged from 0.02 to 0.03). There was no other significant correlation between the above parameters and clinicopathological features.

**Conclusions:** Our *in vivo* preliminary data suggest that TMCD and PAR-2-MVD may play a role in GC angiogenesis and they could be further evaluated as a target of antiangiogenic therapy.

**Keywords:** angiogenesis, gastric cancer, mast cells, PAR-2, tryptase

## Introduction

Mast cells (MCs) play a role in tumour angiogenesis and their involvement has been demonstrated in several animal and human malignancies [Gulubova and Vlaykova, 2009; Marech *et al.* 2014a]. MCs can secrete classical proangiogenic factors, including vascular endothelial

growth factor (VEGF), fibroblast growth factor-2, thymidine phosphorylase and interestingly, a non-classical proangiogenic factor, namely tryptase, stored in their secretory granules [Marech *et al.* 2014b; Ammendola *et al.* 2013a, 2014]. With special reference to tryptase, it induces *in vitro* microvascular endothelial cells' (ECs) proliferation in

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Correspondence to:  
**Michele Ammendola, MD**  
Department of Medical and  
Surgical Sciences, Clinical  
Surgery Unit, University  
of Catanzaro 'Magna  
Graecia' Medical School,  
Viale Europa – Germaneto,  
88100, Catanzaro, Italy  
[michele.ammendola@  
libero.it](mailto:michele.ammendola@libero.it)

**Rosario Sacco, MD,**  
**Professor**  
**Giuseppe Sammarco, MD,**  
**Professor**  
**Giuseppina Vescio, MD**  
Department of Medical and  
Surgical Sciences, Clinical  
Surgery Unit, University of  
Catanzaro 'Magna Graecia'  
Medical School, Catanzaro,  
Italy

**Valeria Zuccalà, MD**  
Health Science  
Department, Pathology  
Unit, University of  
Catanzaro 'Magna Graecia'  
Medical School, Catanzaro,  
Italy

**Maria Luposella, MD**  
Cardiovascular Disease  
Unit, 'San Giovanni di Dio'  
Hospital, Crotona, Italy

**Claudia Gadaleta, MD**  
**Rosa Patruno, MD**  
**Nicola Zizzo, MD,**  
**Professor**  
Chair of Pathology,  
University 'Aldo Moro'  
Veterinary Medical School,  
Bari, Italy

**Ibrahim Furkan Kocak, MD**  
**Taner Ozgurtas, MD,**  
**Professor**  
Department of  
Biochemistry, Gulhane  
Military Medical Academy  
Etlik, Ankara, Turkey

**Roberta Ruggieri, MD**  
**Iliaria Marech, MD**  
**Cosmo Damiano Gadaleta,**  
**MD**  
**Girolamo Ranieri, MD**  
Diagnostic and  
Interventional Radiology  
Unit with Integrated  
Section of Translational  
Medical Oncology,  
National Cancer Research  
Centre, 'Giovanni Paolo II',  
Bari, Italy



the matrigel assay and displayed *in vivo* the capillary growth on the chick embryo chorioallantoic membrane, conversely, suppressed by tryptase inhibitors [Ammendola *et al.* 2013b; Marech *et al.* 2016b; Ribatti *et al.* 2011]. This proangiogenic stimulus induced by tryptase is mainly mediated *via* protease-activated receptor-2 (PAR-2) that belongs to the G protein-coupled receptor family [Blair *et al.* 1997; Stack and Johnson, 1994; Fajardo and Pejler, 2003; Itoh *et al.* 2005]. Four forms of PARs have been reported (PAR-1 through PAR-4). In particular, PAR-2 can be activated by proteases such as trypsin and tryptase. These proteases cleave the N terminus to generate a tethered ligand, which interacts and activates the receptor [Rickard *et al.* 2005; Matej *et al.* 2007; Morris *et al.* 2006; Ammendola *et al.* 2013c, 2014; Donato *et al.* 2014; Ranieri *et al.* 2009]. Signaling *via* PAR-2, expressed on ECs, elicits activation of the major members of the mitogen-activated protein kinase (MAPK) phosphorylation family and induces EC proliferation. PAR-2 activation also leads to the production of other proangiogenic factors, such as VEGF, interleukin-8 (IL-8), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) [Ammendola *et al.* 2015; Ribatti and Ranieri, 2015; Malfettone *et al.* 2013; Soreide *et al.* 2006; Darmoul *et al.* 2001; Uusitalo-Jarvinen *et al.* 2007; Liu and Mueller, 2006].

In literature, no data have been published on the relationship among tryptase MC density (TMCD), ECs positive to PAR-2 forming microvascular density (PAR-2-MVD) and classical MVD (C-MVD) in gastric cancer (GC) angiogenesis [Yano *et al.* 1999; Sedda *et al.* 2014; Ribatti *et al.* 2010; Wang *et al.* 2013a; Zhang *et al.* 2012].

In this preliminary study, we analyzed the numbers of TMCD, PAR-2-MVD and C-MVD to analyse whether they correlated with each other in primary tumour tissue from GC patients undergoing surgery. The correlation among the above analysed parameters and the main clinicopathological features has been also performed.

## Materials and methods

### Study population

A series of 77 GC patients with stage T<sub>2-3</sub>N<sub>2-3</sub>M<sub>0</sub> (classified by the American Joint Committee on

Cancer for Gastric Cancer, 7th edition) diagnosed with preoperative gastric endoscopy were selected and underwent to curative resection. Surgical approaches used were open total and subtotal gastrectomy, with D2 lymph node dissection. Patients were staged according to the American Joint Committee on Cancer 7th edition (AJCC-TNM) classification [Washington, 2010; Tamura *et al.* 2011; Verlato *et al.* 2014; Mrena *et al.* 2015]. All patients did not have distant metastases on computed tomography of the thorax, abdomen and pelvis. All samples evaluated in this study were of adenocarcinomas' histological type. The clinicopathological features of the patients are summarized in Table 1. Full ethical approval and signed consent from individual patients were obtained. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of 'Mater Domini' Hospital, 'Magna Graecia' University, Catanzaro.

### Immunohistochemistry

For the evaluation of TMCD, PAR-2-MVD and C-MVD, a three-layer biotin-avidin-peroxidase system was utilized [Ranieri *et al.* 2007]. Briefly, 6 µm-thick serial sections of formalin-fixed and paraffin-embedded tumour sample were cut. Sections were then microwaved at 500W for 10 minutes, after which, endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. Tumour sections were incubated with the following primary antibodies: antitryptase (clone AA1; Dako, Glostrup, Denmark) diluted 1:100 for 1 hour at room temperature specific for MC identification; anti-PAR-2 (C-17; sc-8205, Santa Cruz Biotechnology, Texas, USA), diluted 1:50 for 1 hour at room temperature and anti-CD34 antibody (QB-END 10; Bio-Optica Milan, Italy) diluted 1:50 for 1 hour at room temperature as a pan-endothelial marker, respectively. The bound antibody was visualized using a biotinylated secondary antibody, avidin-biotin-peroxidase complex and liquid permanent red (LPS, K0640, Dako, Glostrup, Denmark). Nuclear counterstaining was performed with Gill's haematoxylin no.2 (Polysciences, Warrington, PA, USA). The primary antibody was omitted in negative controls.

### Morphometrical assay

Light microscopy integrated with an image analysis system (Quantimet-500 Leica, Wetzlar,

**Table 1.** Clinicopathological features of patients ( $n = 77$ ).

Age	
≤65	31 (40%)
≥65	46 (60%)
Gender	
Male	43 (56%)
Female	34 (44%)
Tumour site	
Cardia	11 (14%)
Lesser curvature	7 (9%)
Greater curvature	9 (12%)
Body and fundus	24 (31%)
Pyloric area	26 (34%)
TNM by AJCC stage and type by Lauren classification	
T <sub>2-3</sub> N <sub>2</sub> M <sub>0</sub>	47 (61%)
T <sub>2-3</sub> N <sub>3</sub> M <sub>0</sub>	30 (39%)
Intestinal type	45 (58%)
Diffuse type	32 (42%)
Histologic grade	
G1-G2	56 (73%)
G3	21 (27%)

TNM, classification of cancer staging; AJCC, American Joint Committee on Cancer.

Germany) was utilized [Ranieri *et al.* 2007]. In tumour sections, immunostained areas (hot spots) were selected at  $\times 100$  magnification and TMCD (Figure 1A), PAR-2-MVD (Figure 1B) and C-MVD (Figure 1C) were assessed at  $\times 400$  magnification (0.19 mm<sup>2</sup> area).

### Statistical analysis

Mean values  $\pm 1$  standard deviation (SD) of all the tissue-evaluated parameters are reported in Table 2. Correlations between TMCD, PAR-2-MVD, and C-MVD were calculated using Pearson's ( $r$ ) analysis. Correlations among the all analyzed parameters and the main clinicopathological features listed in Table 1 were performed by Chi-square test ( $\chi^2$ ). A  $p < 0.05$  was considered significant. All statistical analyses were performed with the SPSS statistical software package (SPSS, Inc., Chicago, IL).

### Results

Immunohistochemical staining by using the antibodies antitryptase, anti-PAR-2 and anti-CD34,

demonstrates that tryptase-positive MCs are well recognizable as red-stained ovoid cells with thin prolongations and generally, they are located in perivascular position (Figure 1A). A close topographic association between TMCD and PAR-2-MVD and between TMCD and C-MVD was often observed. The mean value  $\pm$  SD of TMCD, PAR-2-MVD and C-MVD was  $10.87 \pm 4.21$ ,  $24.32 \pm 8.67$  and  $27.22 \pm 9.12$  respectively, and these results are summarized in Table 2.

There was a significant correlation between TMCD and C-MVD ( $r = 0.71$ ,  $p = 0.03$ ), between PAR-2-MVD and C-MVD ( $r = 0.76$ ,  $p = 0.02$ ), and between TMCD and PAR-2-MVD ( $r = 0.64$ ,  $p = 0.02$ ) (Figure 2). There was no correlation between TMCD, PAR-2-MVD and C-MVD and the main clinicopathological features found.

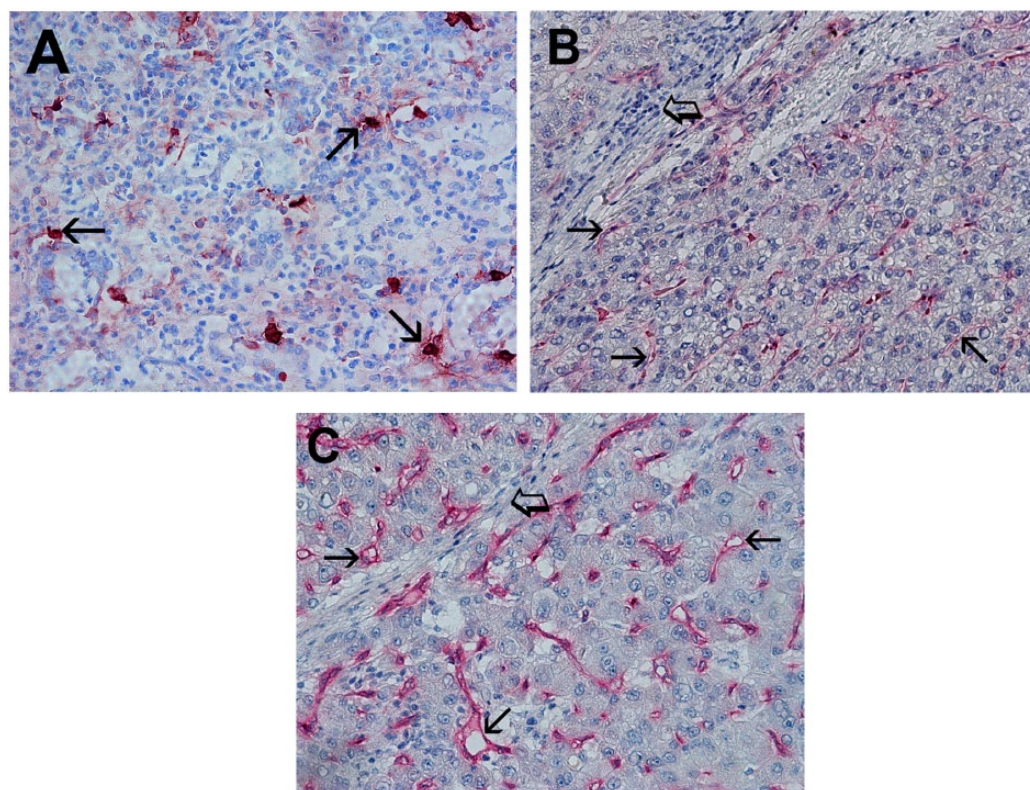
### Discussion

Currently, a lot of data supported the central role of angiogenesis in GC development and progression but few data regarding the role of MCs in GC angiogenesis have been published [Geng *et al.* 2014; Wang *et al.* 2013a; Zhao *et al.* 2012]. In particular, in a study performed by Mukherjee and colleagues, the authors studied MC density in tissue from patients with gastric ulcers and in tissue from GC patients [Mukherjee *et al.* 2009]. In the above study, the histochemical method of toluidine blue was employed to identify and count MC density. Data from this study indicated that MC density in benign gastric ulcers and in cancers was much higher than control and correlated with angiogenesis.

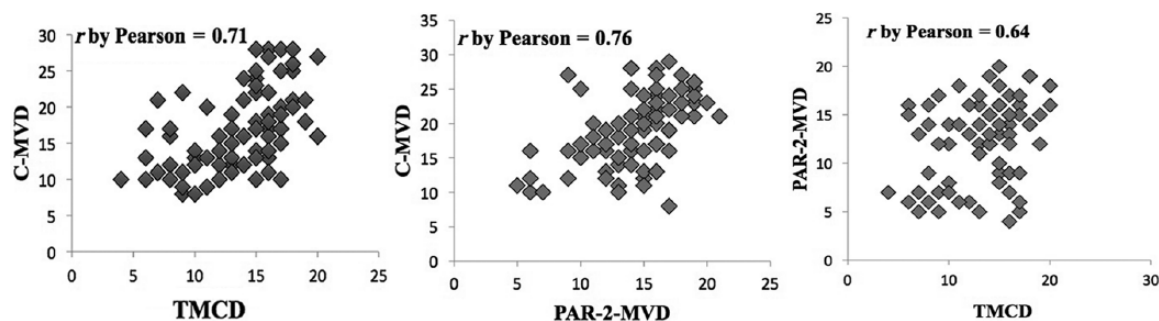
Ribatti and colleagues studied tumour samples from GC patients by mean of immunohistochemistry employing antitryptase and antichymase antibodies to stain MCs. In this study, a correlation between MVD and tryptase and chymase-positive MCs with histopathological type was found [Ribatti *et al.* 2010].

Interestingly, MCs have been shown as important players in tumour angiogenesis because of the release of proangiogenic factors stored in their secretory granules [Bhattacharyya *et al.* 1998; Marech *et al.* 2016a].

In the tumour microenvironment, MCs can be activated in different ways such as: c-Kit receptor activation and phosphorylation by stem cell factor,



**Figure 1.** (A) Primary gastric cancer [GC] tissue section. Many scattered red immunostained mast cells positive to the antitryptase antibody. Each arrow indicates single mast cells,  $\times 400$  magnification ( $0.19 \text{ mm}^2$ ). (B) Primary GC tissue section. Each small arrow indicates single red immunostained microvessel positive to the antiprotease-activated receptor-2 antibody. Big arrows indicate stromal collagen and lymphocytes infiltrate,  $\times 400$  magnification ( $0.19 \text{ mm}^2$ ). (C) Primary GC tissue section. Each small arrow indicate single red immunostained microvessel positive to the anti-CD34 antibody. Big arrow indicates stromal collagen with scattered lymphocytes,  $\times 400$  magnification ( $0.19 \text{ mm}^2$ ).



**Figure 2.** Correlation between tryptase mast cells' density (TMCD) and classical microvascular density [C-MVD] ( $r = 0.71$ ,  $p = 0.03$ ), protease-activated receptor-2 microvascular density (PAR-2-MVD) and C-MVD ( $r = 0.76$ ,  $p = 0.02$ ), TMCD and PAR-2-MVD ( $r = 0.64$ ,  $p = 0.02$ ). TMCD, tryptase mast cell density; PAR-2-MVD, protease-activated receptor-2 microvascular density; C-MVD, classical microvascular density.

IgE mechanism mediated by T-lymphocytes–MC interaction and other microenvironmental stimuli [Ammendola *et al.* 2016a; Patrino *et al.* 2014].

After intensive or piecemeal activation, degranulation of secretory granules occurs, depending on the MC-activation mechanism, and MC-derived

**Table 2.** Tryptase mast cell density, protease-activated receptor-2 microvascular density and classical microvascular density means  $\pm$  standard deviation as a function of gastric cancer tumour tissue, respectively.

Tissue	TMCD 400 $\times$ [0.19 mm <sup>2</sup> ]	PAR-2-MVD 400 $\times$ [0.19 mm <sup>2</sup> ]	C-MVD 400 $\times$ [0.19 mm <sup>2</sup> ]
Primary tumour	10.87 $\pm$ 4.21	24.32 $\pm$ 8.67	27.22 $\pm$ 9.12

TMCD, tryptase mast cell density; PAR-2-MVD, protease-activated receptor-2 microvascular density; C-MVD, classical microvascular density.

proangiogenic factors are released in the tumour microenvironment, stimulating angiogenesis [Ribatti and Ranieri, 2015; Wasiuk *et al.* 2009]. Among them, tryptase has been characterized as a powerful nonclassical angiogenic factor [Marech *et al.* 2016b; Norrby, 2002; Visciano *et al.* 2015; Marone *et al.* 2015].

Preclinical data showed that tryptase is an agonist of PAR-2 in vascular ECs that stimulates their proliferation. Signaling *via* PAR-2 on ECs elicits activation of the major members of the MAPK phosphorylation family and contributes to proliferation of ECs and angiogenesis. Experimental data also suggest that PAR-2 activation leads to the production of other proangiogenic factors, such as VEGF, IL-8, IL-6, GM-CSF and M-CSF [Malfettone *et al.* 2013; Soreide *et al.* 2006; Darmoul *et al.* 2001; Uusitalo-Jarvinen *et al.* 2007; Liu and Mueller, 2006; Yano *et al.* 1999; Sedda *et al.* 2014; Ribatti *et al.* 2010; Wang *et al.* 2013b; Zhang *et al.* 2012; Washington, 2010; Tamura *et al.* 2011; Verlatto *et al.* 2014; Mrena *et al.* 2015; Loffredo *et al.* 2014; Zhang *et al.* 2013; Rasmussen *et al.* 2012; Chang *et al.* 2013; Ammendola *et al.* 2016b]. With special regard to GC, it is important to underscore that the role of TMCD in angiogenesis has been little investigated and no data have been published regarding MVD in terms of PAR-2 endothelial expressing cells. Our results demonstrated an association between TMCD, PAR-2-MVD and C-MVD, supporting the central role of tryptase as a main proangiogenic factor in primary GC tissue. On the other hand, these first data need to be interpreted with caution, due to following limitations: the medium size of analyzed samples series; the lack of a previous standardized method to evaluate PAR-2-MVD and the possible inter-observer variability in the evaluation of PAR-2-MVD. Although our results are preliminary and they need to be confirmed to further awaited studies, it is intriguing to speculate that the inhibition of MC degranulation by means of c-Kit receptor

tyrosine kinase inhibitors (e.g. masitinib) or the inhibition of tryptase by means of gabexate mesilate or nafamostat mesilate, which could be novel antiangiogenic strategies worthy of clinical investigation [Erba *et al.* 2001; Mori *et al.* 2003; Humbert *et al.* 2010; Marech *et al.* 2014c; Deplanque *et al.* 2015; Ammendola *et al.* 2016c].

### Author contributions

Ammendola M and Ranieri G conceived the research. Vescio G, Kocak IF and Ozgurtas T performed the critical review of the literature. Ammendola M Sammarco G Sacco R performed surgical procedures. Zuccalà V, Patruno R, C Gadaleta, Zizzo N contributed to immunohistochemistry and tissue's study. Marech I, Ruggieri R, Luposella M and Gadaleta CD elaborated data analysis. All authors wrote the manuscript. Ranieri G reviewed the manuscript.

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### Conflict of interest statement

The authors declare that there is no conflict of interest.

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