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# Chymase-positive Mast cells correlate with tumor angiogenesis: first report in pancreatic cancer patients

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**Abstract. – OBJECTIVE: Mast cells (MCs) are known to be involved in several physiological and pathological processes in humans and animals. Recently, their potential role in tumor development and angiogenesis has been investigated, arising interesting results to be potentially applied in clinics. Mast cells' granules contain a huge quantity of protease enzymes that, through different mechanisms, induce the formation of new microvessels, feeding tumor burden. Among them, tryptase and chymase are the most abundant enzymes: tryptase is well known for its multiple activities, on the contrary, the role of chymase in pancreatic cancer angiogenesis has not been investigated yet.**

**PATIENTS AND METHODS: Our research aims to correlate to each other and to angiogenesis four different tissue parameters (MCs density positive to chymase, MCs area positive to chymase, microvascular density and endothelial area) together with the main clinical-pathological characteristics in 52 patients surgically resected for pancreatiCDuctal adenocarcinoma, employing immunohistochemistry and image analysis system.**

**RESULTS: All reported tissue parameters match to confirm the correlation between chymase enzyme and angiogenesis in pancreatic cancer.**

**CONCLUSIONS: This evidence could become a starting point for a new potential therapeutic route exploiting chymase inhibitors as a novel anti-angiogenetic strategy in pancreatic cancer patients.**

*Key Words:*
Mast cell, Chymase, Chymase inhibitors, Tumor microenvironment, Angiogenesis, Pancreatic cancer, Tryptase, Microvascular density, Endothelial area.

## Introduction

Pancreatic cancer (PC) is the fourth most common cause of cancer-related death worldwide, with a 5-year survival rate of just 8%[1]. In the early stages, surgery and adjuvant chemotherapy represent the standard treatment, but approximately 60-70% of patients undergo early relapses[2]. The FOLFIRINOX regimen is the most effective treatment for the advanced disease at the expense of a severe toxicity[3]. This is the reason why FOLFIRINOX is recommended only for fit patients with Performance Status 0-1 and aged ≤ 75 years, according to the Eastern Cooperative Oncology Group. This treatment leads to an improvement in median progression-free survival (mPFS) and median overall survival (mOS) compared to gemcitabine in monotherapy of 6.4 months *vs.* 3.3 months ($p < 0.0001$) and 11.6 months *vs.* 6 months ($p = 0.001$), respectively[3]. Patients with advanced PC and BRCA 1-2 germline mutation without disease progression from at least 4 months after first-line platinum-based chemotherapy, can receive Olaparib benefitting of a longer median PFS



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compared to placebo (median 7.4 months versus 3.8 months; $p = 0.004$)[4]. Only a few therapeutic strategies are known for this type of tumor, and further investigations to study safe alternative target treatments are required[5].

Recent preliminary data[6-9] indicate that Mast Cells (MCs) are involved in tumor growth and angiogenesis in PC. MCs came from bone marrow progenitors undergoing maturation in a T cell-dependent manner in different organs of the body[10]. They are known to be involved in allergic reactions, anaphylaxis and induced immunity through the release of their secretory granules in the tissue microenvironment after different stimuli[11-13]. MCs activation is scattered by natural ligands' binding to various receptors expressed on their surface and, among them, the most important pathways are mediated by the cross-linkage to their high-affinity IgE receptor (FcεRI) and c-Kit-Receptor (c-KitR)[11-13].

MCs' granules contain a lot of cytokines, including a low quantity of classical pro-angiogenetic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), platelet-derived endothelial cell growth factor/thymidine phosphorylase (PDEC-GF/TP)[14]. MCs also contain a large amount of protease enzymes, such as chymase and tryptase, which are defined as not classical pro-angiogenetic factors[15]. MCs' differentiation into distinct phenotypes and functions, mediated by growth factors and cytokines, occurs in the tissue microenvironment[10].

MCs are commonly found in tumor microenvironment (TEM) of different human and animal cancer diseases[16]. Interestingly, one of the most relevant pro-tumoral mechanisms regards the stimulation of tumor angiogenesis through the production and release of the not-classical pro-angiogenetic factors. To this regard, the pro-angiogenetic role of MCs' tryptase has been already demonstrated by several studies[7,17-38]; in fact, mast cell density positive to tryptase (MCDPT) and mast cell area positive to tryptase (MCAPT) have been strongly correlated to tumor angiogenesis, in terms of microvascular density (MVD) and endothelial area (EA), in different human and animal tumors, including PC[7,8,26,39].

Chymase is a chymotrypsin-like enzyme that can induce tumor angiogenesis through different mechanisms[10]. First of all, it is able to convert angiotensin I to angiotensin II, a potent angiogenic factor able to stimulate production/release of VEGF[40,41]. To this regard, an angiotensin II receptor blocker could partly prevent chymase-induced angiogenesis[42]. Chymase can also convert pro-matrix metalloproteinase-9 to matrix metalloproteinase (MMP)-9, enhancing the degradation of extracellular matrix (ECM) and the subsequent VEGF release[43].

There are several data about the role of tryptase-positive MCs in tumor angiogenesis[44-53], on the contrary the pro-angiogenic role of chymase-positive MCs is still unclear. In particular, the few published studies on this topic examined only the number of chymase-positive MCs, with no analysis of the involved area.

In this exploratory study, through immunohistochemistry and image analysis system, we aimed at correlating Mast Cells density positive to Chymase (MCDPC) and Mast Cells area positive to Chymase (MCAPC) with MVD and EA in pancreatiCDuctal adenocarcinoma tissue (PDAT), in a patient population who had undergone potential radical surgery. The correlation between these parameters and the main clinical-pathological features has been also performed.

## Patients and Methods

### Study Population

We analyzed PDATs from 52 patients affected by PC with stage $T_{2\text{-}3}N_{0\text{-}1}M_0$ based on the American Joint Committee on Cancer 7th edition (AJCC-TNM) classification. All patients received potential curative resection through pancreaticoduodenectomy, distal pancreatectomy and total pancreatectomy with lymph node dissection. PDAT pathological grading was evaluated according to the World Health Organization (WHO) classification (2000 version). Table I summarizes the clinicopathological characteristics of the patient population.

**Table I.** Patient population characteristics (n = 52).

| Subgroups | No. of patients |
|---|---|
| Age | |
| • < 65 | • 33 (63.5%) |
| • > 65 | • 19 (36.5%) |
| Gender: | |
| • Female | • 27 (52%) |
| • Male | • 25 (48%) |
| Tumor site: | |
| • Head | • 22 (42.3%) |
| • Body-Tail | • 30 (57.7%) |
| TNM by AJCC Stage | |
| • $T_2N_{0\text{-}1}M_0$ | • 23 (43%) |
| • $T_3N_{0\text{-}1}M_0$ | • 29 (57%) |

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the "Mater Domini" Hospital, "Magna Graecia" University, Catanzaro (N° 242; 22 December 2016). Informed consent was obtained from all patients.

### Immunohistochemistry

Immunohistochemistry was employed to assess MCDPC, MCAPC and angiogenesis in terms of MVD and EA using a three-layer biotin-avidin-peroxidase technique. In detail, 5 μm-thick serial sections of formalin-fixed and paraffin-embedded PDAT were cut. These slides were processed with a microwave oven at 500 Watt for 10 min and then, a 3% hydrogen peroxide solution was used to inhibit the endogenous peroxidase enzyme. Subsequently, the slides were incubated with the following primary antibodies:

– Anti-CD31 (QB-END 10; Bio-Optica Milan, Milan, Lombardy, Italy) diluted 1:50 for 1h at room temperature (as a pan-endothelial marker).
– Anti-chymase (clone 2377, Abcam, Cambridge, Cambridgeshire, United Kingdom) diluted 1:100 for 1h at room temperature.

The bound immunoreaction was visualized using a biotinylated secondary antibody, avidin-biotin-peroxidase complex (LPS, K0640, Dako, Glostrup, Hovedstaden, Denmark), and fast red or 3-Amino-9-ethyl carbazole as chromogen. Nuclear counter-staining was performed with Gill's hematoxylin No. 2 (Polysciences, Warrington, Cheshire, United Kingdom). The primary antibody was omitted in negative controls.

### Morphometrical Assay

A light microscopy with an integrated image analysis system (AXIO, Scope A1, ZEISS, Oberkochen, Baden-Württemberg, Germany) was employed. For each serial section of PDAT, the five most immunostained areas (hot spots) were assessed at low magnification ×100 (ocular lens ×10, objective lens ×10). Then, MCDPC, MCAPC, MVD, and EA were analyzed for each serial section at higher magnification x400 (ocular lens x10, objective lens ×40) (0.19 $mm^2$ area) in the five identified hot spot areas. MCDPC indicates the number of chymase-positive MCs in the selected microscopic area, while MCAPC corresponds to the area positive to chymase inside MCs, evaluated by means of a semi-automatic technique. In detail, the area with immunostained chymase was defined with the mouse of the image analysis system by the operator. Then, the software calculates the area in the complex for each considered hot spot in the microscopic field. MVD represents the count of single endothelial cells, endothelial cell clusters and microvessels, clearly separated from adjacent microvessels, independently from vessels lumen, based on the modified Weidner's method[54]. EA corresponds to the immunostained vascular area in the selected microscopic field, evaluated by means of a semi-automatic technique. To be specific, the immunostained area with anti-CD-31 was defined with the mouse of the image analysis system by the operator. Then, the software calculates the area in the complex for each considered hot spot in the microscopic field. Immunostained MCs positive to chymase and endothelial cells positive to anti-CD31 antibody were also detected in terms of immunoreactive area at ×400 magnification (0.19 $mm^2$ area). Finally, morphological details evaluations of MCs positive to chymase and endothelial cells were observed at ×1000 magnification, in oil.

### Statistical Analysis

*A*ll studied tissue parameters were quantified in terms of mean values for each single section and the whole series. All data ± 1 Standard Deviation (SD) are reported in Table II. Pearson's (r) analysis was used to investigate and determine correlations among MCDPC, MCAPC, MVD and EA, and by the Chi-square test ($\chi^2$) we investigated the correlations between all tissue parameters and the main clinic-pathological features. Statistically significant analyses had a $p<0.05$.

**Table I.** MCDPC, MCAPC, MVD, and EA in PDAT means ± standard deviations.

| | MCDPC x400 magnification (0.19 $mm^2$ area) | MCAPC x400 magnification (0.19 $mm^2$ area) | MVD x400 magnification (0.19 $mm^2$ area) | EA x400 magnification (0.19 $mm^2$ area) |
|---|---|---|---|---|
| PDAT | 13.76 ± 3.96 | 144.01 ± 44.22 $\mu m^2$ | 28.44 ± 5.86 | 202.11 ± 64.15 $\mu m^2$ |

MCDPC: mast cells density positive to chymase; MCAPC: mast cells area positive to chymase; MVD: microvascular density; EA: endothelial area; PDAT: pancreatiCDuctal adenocarcinoma tissue.

Statistical analyses were performed by the SPSS statistical software package (SPSS, Inc., Chicago, Delaware, IL, USA).

## Results

The Immunohistochemical assay and image analysis study of MCDPC, MCAPC, MVD and EA in PDAT at ×400 magnification demonstrated the following data: MCDPC = 13.76±3.96 SD; MCAPC = 144.01±44.22 SD μm$^2$; MVD = 28.44±5.86 SD; EA= 202.11±64.15 SD μm$^2$ (Table II; Figures 1A, 1B). Several details of the tissue parameters were also evaluated at ×600 (ocular lens ×10, objective lens ×60) or ×1000 magnification (ocular lens ×10, objective lens ×100) (Figures 1C, 1D, 2A, 2B, 2C, 2D). Pearson's analysis highlighted a significant correlation among all parameters (Figure 3). The correlations between MCAPC and EA (r = 0.78, $p$ = 0.01), MCDPC and EA (r = 0.69, $p$ = 0.02), MCAPC and MVD (r =

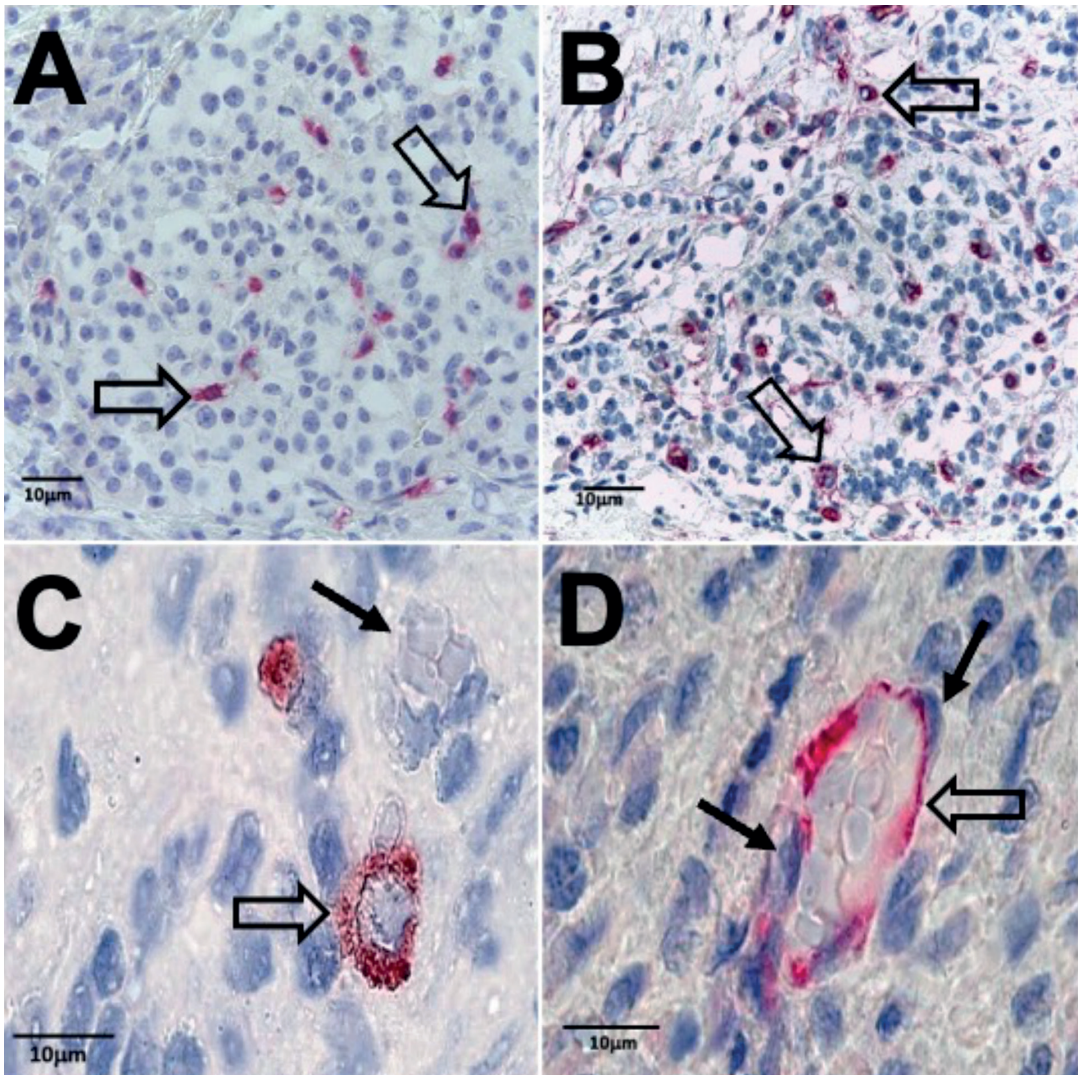


**Figure 1.** Tissue sections. **A,** Magnification ×400, 0.19 mm$^2$ area, immunostaining with the primary anti-chymase antibody. High MCDPC, arrows indicate single red-stained MCs, please note blue nuclei. **B,** Magnification ×400, 0.19 mm$^2$ area, immunostaining with the primary anti-CD31 antibody. High MVD, arrows indicate single red-stained microvessels, please note blue nuclei. **C,** The strongest magnification at light microscopy, ×1000 in oil, immunostaining with primary anti-chymase antibody. The big arrow indicates a MC with a cytoplasm fullfilled with red-stained positive chymase granules. The central blue nucleus is also clearly evident. The small arrow indicates a microvessel. Please note several ialin translucid dysmorphic red cells in its lumen. **D,** The strongest magnification at light microscopy, ×1000 in oil, immunostaining with the primary anti-CD31 antibody. The big arrow indicates a red-stained vessel with several ialin translucid dysmorphic red cells in its lumen as an internal positive control. Please note the nuclei of the endothelial cells (*small arrows*).

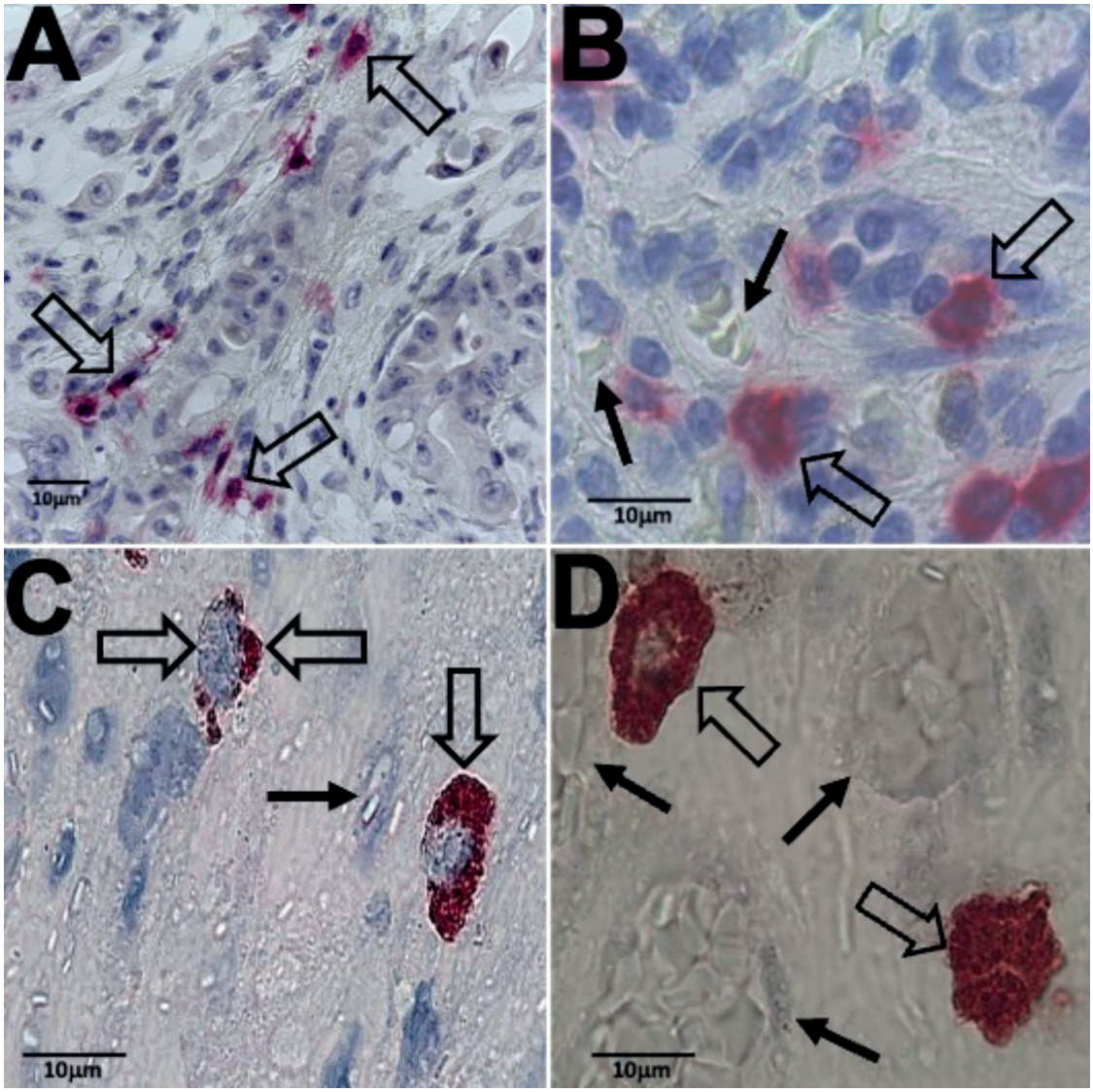


**Figure 2.** Tissue sections immunostained with the primary anti-chymase antibody. **A,** Magnification ×600, fast red chromogen. Arrows indicate single red-stained MCs, please note dark blue nuclei. **B,** Magnification ×1000 in oil, fast red chromogen. Big arrows indicate single red-stained MCs, please note dark blue nuclei. From a morphological point of view, several microvessels are evident (*small arrows*) with several ialin translucid dysmorphic red cells in their lumens. **C,** The strongest magnification at light microscopy, ×1000 in oil, 3-Amino-9-ethyl carbazole chromogen. The big arrow indicates a MC with a cytoplasm fullfilled by red-brown stained positive chymase granules, the central blue nucleus is also clearly evident. The double big arrow indicates a partially chymase degranulated MC. From a morphological point of view, two microvessels are evident (*small arrows*), please note the red blood cells in their lumens. **D,** The strongest magnification at light microscopy, ×1000 in oil, 0.06 $mm^2$ area, 3-Amino-9-ethyl carbazole chromogen, with no Gill's Hematoxylin nuclei contro-coloration. Please note MCs with a cytoplasm fullfilled by red-brown stained positive chymase granules. From a morphological point of view, three vessels are evident (*small arrows*), please note the several red blood cells in their lumens.

0.79, $p = 0.01$), MCDPC and MVD (r = 0.73, $p = 0.02$), MVD and EA (r = 0.81, $p = 0.01$), MCAPC and MCDPC (r = 0.86, $p = 0.009$). No further correlation among MCDPC, MCAPC, MVD, EA, and the main clinical-pathological characteristics was found.

## Discussion

All *in vitro* and *in vivo* studies so far published confirm that an increased MCs infiltration is associated with tumor angiogenesis in numerous animal and human malignancies,

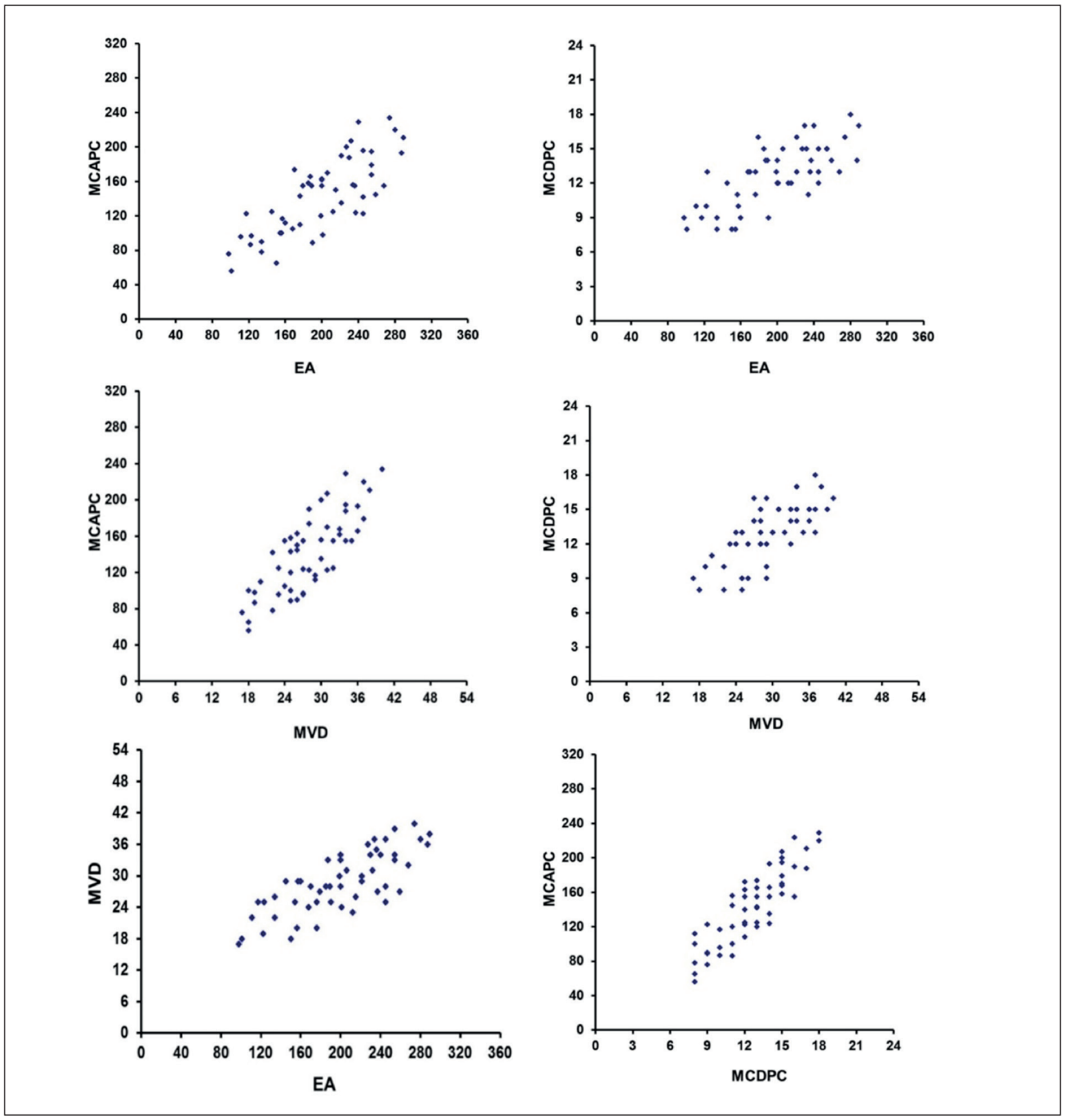


**Figure 3.** Correlations by Pearson analysis between MCAPC and EA (r = 0.78, $p = 0.01$), MCDPC and EA (r = 0.69, $p = 0.02$), MCAPC and MVD (r = 0.79, $p = 0.01$), MCDPC and MVD (r = 0.73, $p = 0.02$), MVD and EA (r = 0.81, $p = 0.01$), MCAPC and MCDPC (r = 0.86, $p = 0.009$).

including PC[45-47,49,53,55-68]. Moreover, several experimental data[69,70] showed that MCs tryptase plays an important pro-angiogenic role in a lot of human cancers. To this regard, in a previous study[7,8,26], we have already demonstrated that MCDPT and MCAPT are strongly associated with tumor angiogenesis in PC, both in terms of MVD and EA.

Deepening in the topic, MCs can also synthesize, store and release Chymase, another protease belonging to the serine proteases family[10]. Chymase-positive MCs are usually placed around blood vessels in various tissues and organs[53], so that their localization has been supposed to have a role in the vascularization process. Our immuno-morphological results show that this

topographiCDistribution is also confirmed in PC (Figures 2B, C, D), even if chymase-positive MCs role in tumor angiogenesis has not been fully established yet.

*In vitro* studies[40] support the role of chymase as pro-angiogenic factor, in the conversion of angiotensin I to angiotensin II, a potent angiogenic factor stimulating VEGF expression. Chymase also converts pro-matrix metalloproteinase-9 to matrix metalloproteinase (MMP)-9, enhancing the degradation of ECM and the subsequent release of the VEGF stored in it[43].

VEGF-A expression is largely induced by high levels of hypoxia inducible factor-1α (HIF-1α), too. Both HIF-1α and VEGF-A stimulate PC invasion through angiogenesis and promote tumor cell proliferation and metastasis[71,72]. The VEGF pathway was investigated in mouse models of human PC, demonstrating that the tumor burden was reduced until 80% when an antisense VEGF-A were injected in human PC cells[73]. At the same time, the injection of diphtheria toxin in the vasculature led to inhibition of VEGF-A protein synthesis with a significant reduction in tumor burden. Similarly, the MVD and tumor volume were considerably reduced by injecting a soluble form of the VEGFR-1 decoy receptor[74].

Several clinical trials have already evaluated safety and efficacy of VEGF inhibitors in PC. To be specific, Kindler et al[75], in a phase III trial, tested Bevacizumab *vs.* gemcitabine plus placebo in advanced PC patients with no statistical difference in terms of efficacy parameters. Another phase III trial evaluated gemcitabine plus erlotinib with or without bevacizumab in 301 advanced PC patients. However, the triplet schedule did not significantly improve OS despite the significant PFS extension[76]. A few studies[53,77,78] examined the relationship between chymase and angiogenesis in some types of tumor models. Ribatti et al[53] investigated the angiogenic action of human recombinant tryptase and chymase in *vivo* chorioallantoic membrane assay. They demonstrated that both proteases showed similar angiogenic activity, comparable to the angiogenic response induced by VEGF. In particular, several new microvessels were evident at the histological examination of the sponge trabeculae.

Moreover, a study[77] on a mice skin tumor model demonstrated that expressions of MCs' chymase and tryptase were increased during tumor progression and correlated with angiogenesis.

In an experimental hamster sponge implant model, Muramatsu et al[78] demonstrated the angiogenic role of chymase by transfecting human prochymase cDNA and by injecting purified chymase into implanted sponges. In fact, they revealed as chymase is able to convert Angiotensin I in II, stimulating bFGF-related angiogenesis. At the same time, they demonstrated that the use of chymase inhibitors, such as chymostatin, reduced MC-mediated angiogenesis.

In a mouse model of human papillomavirus 16-related epithelial cancer, the authors detected both an increased number of MCs and angiogenesis, as well as the high activity of chymase, during disease progression towards invasive squamous cancer[79].

Experimental data showed that the incubation of mouse hyperplastic ear skin with mouse chymase and subsequent embedding in a medium enriched with bovine capillary endothelial cells led to increased proliferation and migration of the endothelial cells[79]. This activity seemed to be dependent to the ability to cleave pro-MMP-9 in active MMP-9 and the subsequent release of sequestered pro-angiogenic factors in the ECM[79], as discovered by Coussens et al[80]. These pre-clinical data strongly support the pro-angiogenetic role of chymase.

As for clinical research, very few results on the pro-angiogenetic role of MCs' chymase in human tumors have been reported and, to our knowledge, no investigation has been done on PC so far. In detail, we found a single study by Ribatti et al[81], that analyzed the correlation between the number of chymase-positive MCs and angiogenesis. They analyzed primary gastric adenocarcinoma tissues from 30 patients by means of immunohistochemistry with anti-CD31 antibody to stain endothelial cells, and anti-chymase antibody to stain MCs. The results demonstrated that the number of chymase-positive MCs was strongly correlated with MVD and malignancy grade.

Summing up, several experimental data have more times demonstrated the role of tryptase-positive MCs in tumor angiogenesis, compared to the very little knowledge on the pro-angiogenic role of chymase-positive MCs. Moreover, no data have been published in scientific literature about the correlation between angiogenesis and the area of chymase-positive MCs.

In the present research paper, we studied, by means of immunohistochemistry and image analysis system, the relationship between MCs area positive to chymase and angiogenesis in a population of 52 PC patients who had undergone potential radical surgery. We analyzed and cor-

related to each other four parameters, MCAPC, MCDPC, MVD and EA in PDAT. In our opinion, the evaluation of the area and density parameters might be more representative of the enzymatic (MCDPT and MCAPC) and angiogenic (EA and MVD) activities, rather than the single tissue parameter.

In accordance with the literature data, our study confirms the pivotal role of MCs in tumor angiogenesis. Simultaneously, it supports the evidence that chymase-positive MCs are strongly correlated with tumor angiogenesis in PC. In addition, it clearly shows the key role of chymase-positive MCs, in terms of both density and area, in PC angiogenesis.

Currently, only a few therapeutic options are available for PC, which still remains a disease with a very poor prognosis. Hence, the medical need for new different treatment strategies. Based on the role of MCs proteases in PC angiogenesis, a phase III clinical trial has investigated on the efficacy of masitinib, a C-KitR tyrosine kinase inhibitor able to inhibit MCs degranulation, in PC patients, giving interesting clinical results[44,48,82-84].

Moreover, we have already suggested the possibility to inhibit MCs tryptase with specific inhibitors, such as Gabexate or Nafamostat mesylate[85-88].

No less, the results of the present study suggest that another interesting therapeutic strategy to pursue might be the use of chymase inhibitors in PC patients, also in combination therapies. For example, Bowman-Birk inhibitor is a soybean-derived serine protease inhibitor able to suppress MCs' chymase activity[89-91], by blocking both angiotensin II formation and MMP-9 activation by chymase, thus preventing angiogenesis[90,91].

## Conclusions

A detailed revision of the literature has demonstrated that MC's chymase has never been studied in PC tissue. To the best of our knowledge, we have demonstrated for the first time that both MCDPC and MCAPC correlate significantly with tumor angiogenesis in PC, in terms of MVD and EA. In our opinion, the study of both parameters, density and area, might be more representative of the enzymatic (MCDPT and MCAPC) and angiogenic (EA and MVD) activities rather than the single tissue parameter. Our findings highlight a new angiogenetic pathway in PC that might be evaluated as a new possible therapeutic target. This evidence might be useful to start new clinical trials evaluating the safety and efficacy of already known anti-chymase molecules for the treatment of PC patients.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

### Funding

This research received no external funding.

### Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of "Mater Domini" Hospital, "Magna Graecia" University of Catanzaro (protocol code 242, date of approval: 22 December 2016).

### Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

### Authors' Contribution

Conceptualization, G.R. and C.L.; methodology, M.A. and F.C.; software, N.Z.; validation, G.R., M.A. and C.D.G.; formal analysis, R.T.; investigation, C.L. and M.L.; resources, A.F.Z., N.Z. and D.L.; data curation, C.L.; writing—original draft preparation, C.L.; writing—review and editing, M.L.; visualization, N.Z. and R.T.; project administration, M.A., C.L. and G.R.; supervision, G.R..; funding acquisition, not applicable. All authors have read and agreed to the published version of the manuscript.

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