

Chromogranin A is not a biomarker of mastocytosis



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Clinical Implications

- Chromogranin A is not a useful marker to detect mast cell activation in patients with mastocytosis. Elevated serum levels were exclusively associated with proton pump inhibitor use and did not correlate with mast cell burden or activation.

TO THE EDITOR:

Mastocytosis is characterized by the proliferation of clonal mast cells and its clinical features include flushing, pruritus, abdominal pain, diarrhea, hypotension, and anaphylaxis. The predominant form of cutaneous mastocytosis (CM) is urticaria pigmentosa (UP, also referred to as maculopapular CM). Other forms of CM include diffuse CM and mastocytomas.¹ Systemic mastocytosis (SM) is characterized by multifocal mast cell infiltrates in the bone marrow and other organs, and is subcategorized following World Health Organization (WHO) criteria.¹ One minor criterion for the diagnosis of SM is a serum tryptase >20 ng/mL, which generally reflects mast cell expansion and is a useful marker of mast cell activation by established criteria.² Other mediators where no such criteria have been proposed include heparin, histamine, and prostaglandin D₂ and some authors have suggested that chromogranin A (CgA) should be among these markers based on limited data.^{2,3}

In particular, serum levels of CgA have been reported “as fairly specific” to mast cells when evaluating patients for mast cell activation disorder (MCAD) when other causes of elevated chromogranin levels are excluded.³ CgA is a 439-residue granin family protein (48-60 kD) found in the secretory vesicles of neuroendocrine tissues and is a biomarker for the assessment of neuroendocrine tumors.⁴ Proton pump inhibitor (PPI) use is associated with an increase in CgA levels, as acid suppression by PPIs promotes hypergastrinemia that leads to increased CgA via gastrin-regulated enterochromaffin-like cells and decreases over time after the drug is discontinued.^{5,6} These effects are generally not as pronounced in patients treated with H₂ receptor antagonists.^{5,7} Patients with mastocytosis are frequently treated with these agents to control symptoms related to mast cell-driven acid hypersecretion.

We thus prospectively determined serum CgA, gastrin, and tryptase levels in 20 adults and 17 pediatric patients diagnosed with mastocytosis based on WHO criteria¹ (see Table E1, available in this article's Online Repository at www.jaci-inpractice.org). All patients had symptoms consistent with mast cell activation, such as flushing, pruritus, abdominal pain, diarrhea, hypotension, and anaphylaxis.

Serum CgA, gastrin, and tryptase levels were measured at the Mayo Clinic Laboratories (Rochester, Minn) with a normal reference of <93 ng/mL, <100 pg/mL, and <11.5 ng/mL, respectively. Bone marrow, skin, and small intestinal biopsies were obtained from adult patients with indolent systemic mastocytosis (ISM). Samples were fixed and stained for tryptase and CgA. HMC1.1, HMC1.2, and LAD2 human mast cell lines and the pancreatic beta islet cell carcinoma line, QGP-1, were used to determine relative quantitative expression of CgA using RT-PCR and western blotting (see the Methods and Materials section in this article's Online Repository at www.jaci-inpractice.org).

The adult cohort consisted of 10 female and 10 male patients with ISM, with a median age of 52.8 years and a tryptase level of 32.0 ng/mL (Table E1, available in this article's Online Repository at www.jaci-inpractice.org). Serum CgA median, 25th and 75th interquartile range (IQR) were 65.0, 38.3, and 135 ng/mL, respectively. Because patients with ISM are often treated with PPIs and H₂ antagonists,⁵ we divided the patients according to medication use. The median, 25th, and 75th IQR CgA serum values for those taking neither H₂ antagonists or PPIs were 40.5, 32.5, and 59.8 ng/mL, respectively (n = 6); for those on H₂ antagonists alone were 68.0, 46.0, and 107 ng/mL, respectively (n = 9); and for those taking H₂ blockers and PPIs were 508, 300, and 824 ng/mL, respectively (n = 5). We determined a significant difference in CgA serum values when comparing the no medication group ($P < .001$) and the H₂ antagonist group ($P < .05$) with those taking both PPIs and H₂ antagonists but not when comparing the no medication with those taking H₂ antagonists alone (Figure 1, A). Tryptase levels and D816V allelic frequency were not associated with H₂ antagonist or PPI use (Figure E1, available in this article's Online Repository at www.jaci-inpractice.org). These data are consistent with the conclusions that adult patients with mastocytosis not taking PPIs have serum CgA levels within the normal reference range and that the serum levels of CgA are significantly influenced by the use of PPIs.⁸ Serum gastrin levels correlated with CgA levels ($P = .020$, $r = 0.51$) (Figure 1, B).⁵ However, CgA and tryptase levels did not correlate (Figure 1, C). CgA levels were similarly measured in patients with pediatric mastocytosis (n = 17, age range: 3.6-15.6 years) (Table E1). All had mediator-related symptoms and CgA levels within the normal range (Figure 1, D). Also consistent with adult data, we found a positive correlation of CgA with gastrin ($P = .029$, $r = 0.83$) (Figure 1, E) but not between tryptase and CgA (Figure 1, F).

Representative marrow, duodenum, and skin biopsy staining for CgA were essentially negative compared with positive controls (Figure 2; Figure E2, available in this article's Online Repository at www.jaci-inpractice.org). Bone marrow staining with tryptase revealed focal mast cell aggregates with corresponding negative staining for CgA (compared with control; Figure E2). Skin biopsy staining of UP (Figure 2) indicated the presence of infiltrating dermal mast cells with corresponding weak to absent staining for CgA (compared with positive staining of an epidermal Merkel cell; Figure E2). Tryptase staining of the duodenum in a patient with severe gastrointestinal (GI) symptoms who is also treated by PPI highlighted the presence of mast cells in the lamina propria with no significant CgA staining (Figure 2).

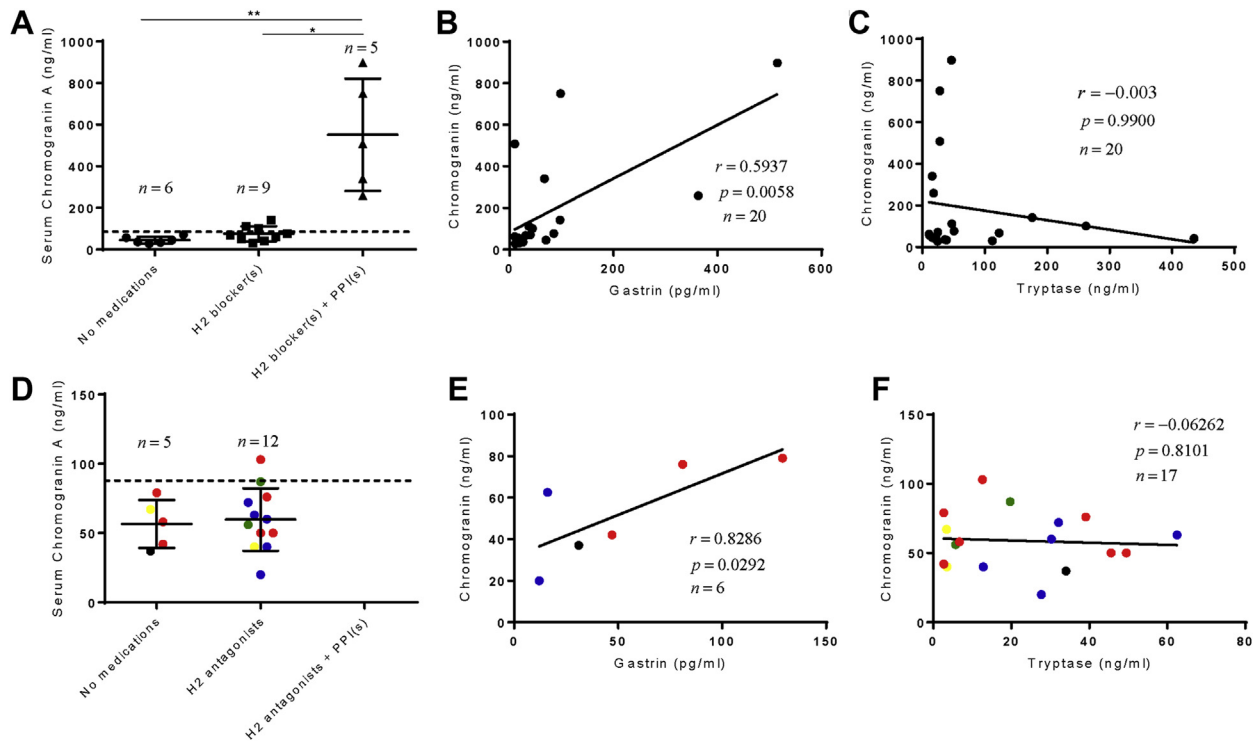


FIGURE 1. Serum chromogranin levels and correlations in adults and children with mastocytosis. Adults (upper row) CgA serum levels (A), correlation of CgA with gastrin (B) and tryptase (C). Pediatric patients (lower row) with parallel analysis shown in (D), (E), and (F). For D-F, red circle = UP, yellow circle = mastocytomas, green circle = DCM, blue circle = ISM, and black circle = children with an unknown diagnosis. Data indicate that all subjects with mastocytosis exhibit normal levels of CgA (excluding the use of PPIs), which correlates with gastrin but not tryptase. No patients were excluded from analysis. Patients were treated with H₂ antagonists alone, and H₂ plus PPI, for a median of 6.3 and 5.3 years, respectively. CgA, Chromogranin A; DCM, diffuse cutaneous mastocytosis; ISM, indolent systemic mastocytosis; PPI, proton pump inhibitor; UP, urticaria pigmentosa.

The relative expression of chromogranin by qPCR and western blotting in mast cell lines compared with positive control (QGP-1 cells) was low, even when cells were activated through the IgE receptor (Figure E3, available in this article's Online Repository at www.jaci-inpractice.org).

We thus determined that CgA is largely not identified in mast cell infiltrates in the bone marrow, skin, and GI tract (Figure 2), and serum levels are within the normal reference range in patients with pediatric and adult mastocytosis, except in those patients treated with PPIs (Figure 1, A and D), whereupon all of these patients exhibited an elevated CgA. Serum tryptase levels and D816V frequencies in peripheral blood, regardless of medication usage, did not associate with CgA levels (Figure E1, available in this article's Online Repository at www.jaci-inpractice.org).

There are reports that suggest serum levels of CgA aids in the diagnosis of patients with MCAD and SM.^{3,9} In one study, CgA was found to be elevated in 5 of 8 patients with SM,³ although reference and patients' CgA values were not reported, whereas in our study, a single CLIA (Clinical Laboratory Improvement Amendments)-approved assay was used in all 37 subjects. Our results are consistent with studies in patients with gastroesophageal reflux disease (GERD), which show that acid-suppressive therapy increases serum CgA levels independent of disease activity.⁵ CgA staining in areas of high mast cell density in bone marrow, skin, and GI biopsies was

interpreted as absent or unconvincing, which is consistent with a previous report.¹⁰

In summary, we found that the use of PPIs is the cause of elevated serum CgA in patients with mastocytosis. These results, coupled with the immunohistochemical data, demonstrate that mast cells are not a significant systemic source of serum CgA. Therefore, we recommend that serum CgA not be used as a biomarker of mast cell disease.

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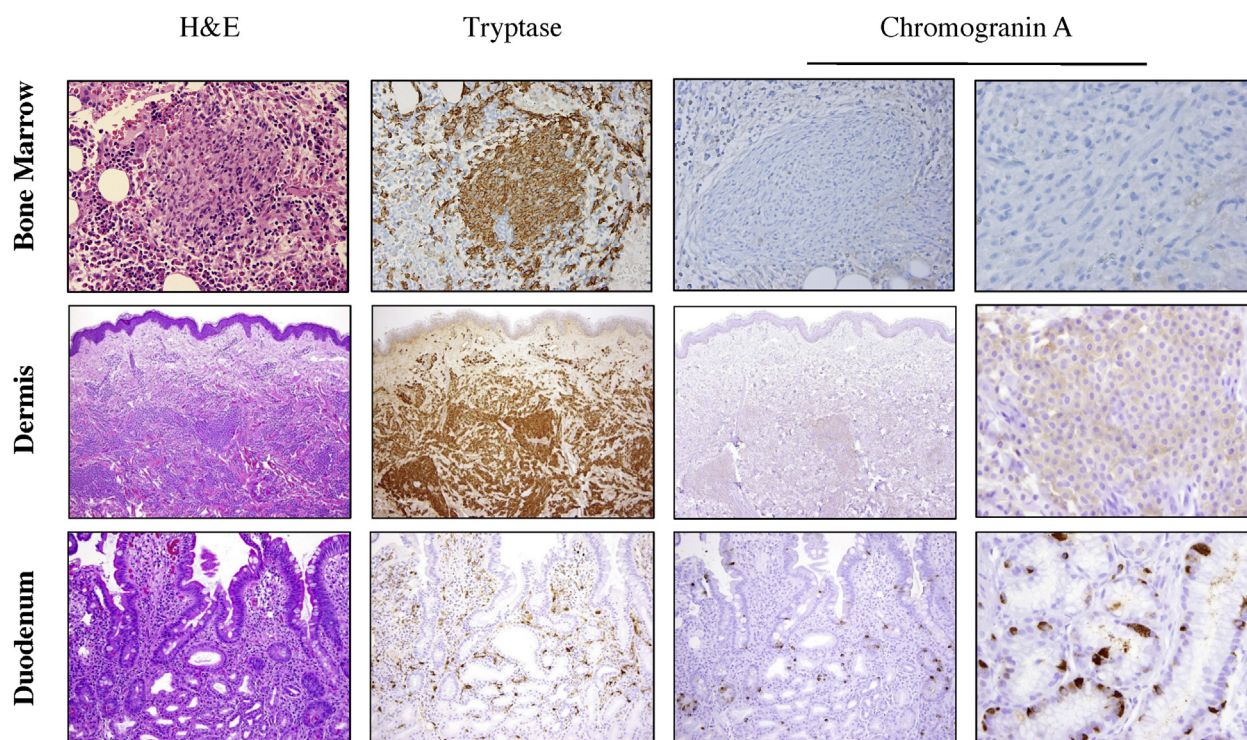


FIGURE 2. Chromogranin immunohistochemistry in adults with ISM. Top row: mast cell aggregate in bone marrow. From left to right: H&E and tryptase staining identify mast cell aggregate with the corresponding absence of CgA staining at $\times 200$ and $\times 600$. Middle row: mast cell infiltrate in highly vascularized UP lesion with parallel staining. CgA staining at $\times 100$ and at $\times 600$ is scant. Bottom row: duodenal section in patient with severe GI symptoms noting the absence of CgA in resident mast cells of lamina propria and positive staining of G cells at $\times 200$ and at $\times 600$. CgA, Chromogranin A; GI, gastrointestinal; H&E, hematoxylin and eosin; ISM, indolent systemic mastocytosis; UP, urticaria pigmentosa.

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METHODS AND MATERIALS

Study participants

Twenty adult patients with ISM and 17 pediatric patients with mastocytosis diagnosed based on World Health Organization criteria^{E1} were evaluated prospectively after informed consent, as approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board under protocol 02-I-0277. No patient had evidence of a neuroendocrine tumor based on clinical presentation and findings. All patients had symptoms consistent with mast cell activation, such as flushing, pruritus, abdominal pain, diarrhea, hypotension, anaphylaxis, and musculoskeletal pain.

Determination of tryptase, chromogranin A (CgA), and gastrin levels

Patient serum CgA, gastrin, and tryptase levels were measured at the Mayo Clinic (Mayo Clinic Laboratories, Rochester, Minn). CgA was measured using an automated immunofluorescent assay (normal reference range <93 ng/mL), gastrin was measured using the commercially available assay, Immulite 2000

Gastrin manufactured by Siemens Medical Solutions Diagnostics (normal reference range <100 pg/mL), and total serum tryptase levels were determined using a commercial assay (ImmunoCAP; Quest Diagnostics, Madison, NJ) with the normal reference range of 0.0–11.5 ng/mL.

Immunohistochemistry of patient tissue samples

Bone marrow biopsy specimens were fixed either in B-5 fixative or B-Plus fixative, embedded in paraffin, and processed by using standard procedures. Immunohistochemical studies with antitryptase (Cell Marque, Hot Springs, Ark) and CgA antibodies (Ventana Medical Systems, Tucson, Ariz) were performed by using immunoperoxidase staining procedures and an automated immunostainer (Ventana Medical System), according to the manufacturer's instructions.

Biopsies of skin and small intestine were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin tissue blocks, and sectioned at 5 μ m for routine histology and immunohistochemistry. Immunohistochemistry was performed using the Roche Ventana BenchMark ULTRA slide stainer (Roche Ventana Medical Systems) following automated staining protocols: 5 μ m tissue sections were

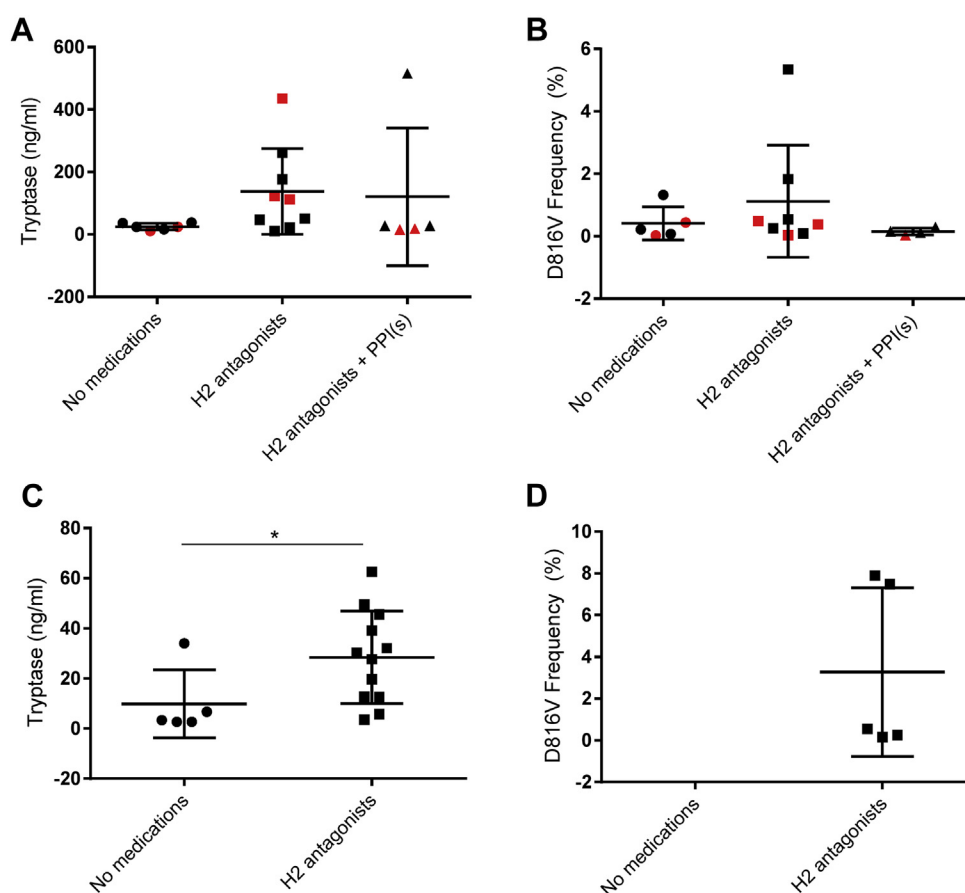


FIGURE E1. Tryptase and KIT D816V mutation frequency levels in patients segregated by H₂ antagonist or PPI medication use. (**A, C**) Serum tryptase levels versus medication use in adults and children, respectively; (**B, D**) KIT D816V allelic frequency in peripheral blood versus medication use in adults and children, respectively. All subjects in (**D**) with KIT D816V were treated with H₂ antagonists. Red dots indicate patients with anaphylaxis. No statistically significant differences were found between adult patients, based on medication use, in tryptase levels or D816V allele burden. Tryptase levels were significantly elevated in pediatric patients treated with H₂ antagonists ($P = .0351$), as determined by the Mann-Whitney test. PPI, Proton pump inhibitor.

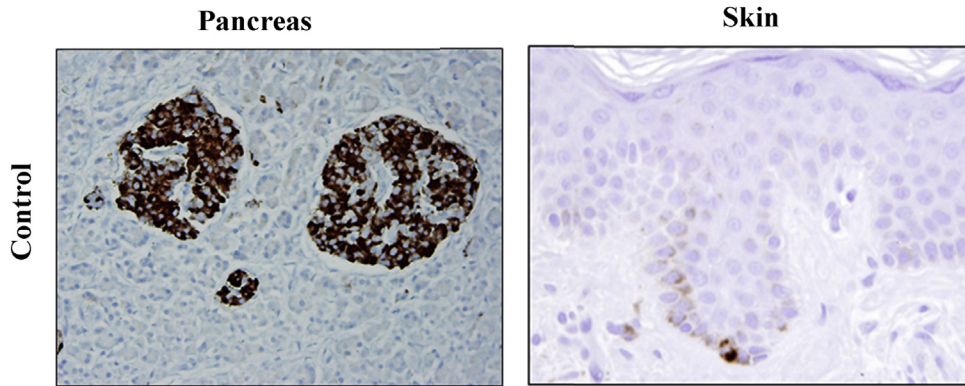


FIGURE E2. Chromogranin A controls for immunohistochemical staining. The left image shows chromogranin staining for beta islet cells in pancreas at $\times 200$ magnification. The right image shows chromogranin staining in the Merkel cell of epidermis at $\times 600$ magnification.^{E4}

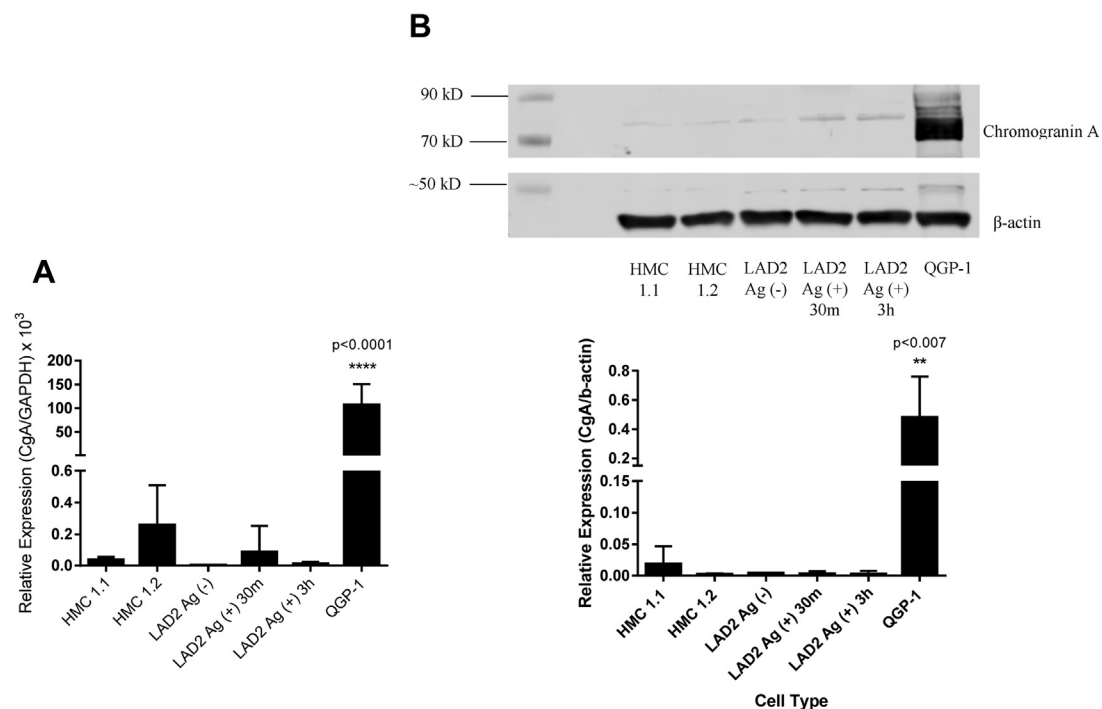


FIGURE E3. CgA message and protein expression in mast cell lines. **(A)** CgA mRNA expression was significantly greater (>1000 -fold) in QGP-1 cells^{E5} than in all mast cell lines, with no significant differences in expression among mast cell lines, including LAD2 cells stimulated through the IgE receptor. **(B)** Significantly lower protein expression at the predicted size of CgA (at 70 kD) in mast cell line lysates than in QGP-1 lysates ($P < .007$), with no significant differences in expression among mast cell lines and minimal protein expression in mast cells.^{E6} All lysates were normalized to b-actin. Blot is representative of 3 experiments. No CgA was detected in cell supernatants. CgA, Chromogranin A.

prepared and placed on poly-L-lysine-coated glass slides. After antigen retrieval, tissue sections were incubated for 32 minutes with a monoclonal mouse antihuman mast cell tryptase antibody (Dako, Catalog No. M7052, clone AA1) at a dilution of 1:400, and detection using the prediluted Ventana Ultra view reagent. Tissue sections were counterstained with hematoxylin. For CgA staining, after antigen retrieval, tissue

sections were incubated for 20 minutes with a monoclonal mouse antihuman chromogranin antibody (Roche, Catalog No. 760-2519, clone LK2H10) at factory prediluted concentrations, and detection using the prediluted Ventana Ultra view reagent. Tissue sections are counterstained with hematoxylin. Human pancreas was used as a positive control; the pancreatic islet cells are positive for chromogranin.

TABLE E1. Study population characteristics

Characteristics	Adults (n = 20)	Pediatric (n = 17)
Mastocytosis diagnosis		
Indolent systemic	20	5
Diffuse cutaneous	0	2
Urticaria pigmentosa	0	7
Mastocytoma	0	2
Unknown	0	1
Median patient age (y)	52.8 (range: 30.4-67.8)	10.9 (range: 3.6-15.6)
Gender		
Female	10 (50%)	7 (41%)
Male	10 (50%)	10 (59%)
Race		
Caucasian	19 (95%)	14 (82%)
African American	1 (5%)	0
Asian	0	1 (6%)
Multiracial	0	1 (6%)
Unknown	0	1 (6%)
Ethnicity		
Hispanic/Latino	2 (10%)	1 (6%)
Not Hispanic/Latino	18 (90%)	16 (94%)
D816V <i>KIT</i> mutation		
Positive	18 (90%)	5 (29%)
Negative	2 (10%)	12 (71%)
Median serum tryptase (ng/mL)	32.0 (IQR: 19.0-96.7)	19.7 (IQR: 4.60-36.6)
Median creatinine* (mg/dL)	0.73 (range: 0.55-0.91 in females) 0.92 (range: 0.81-1.17 in males)	0.47 (range: 0.25-0.83)
Median estimated glomerular filtration rate (eGFR) [†] (mL/min/1.73 m ²)	99.25 (range: 69.0-118.5)	Not measured

IQR, Interquartile range.

All *creatinine and [†]eGFR values were within normal limits.

Cell culture

Mast cell lines, HMC1.1 and HMC1.2, obtained from Dr. Joseph H. Butterfield (Mayo Clinic), were cultured in Iscove's Dulbecco modified Eagle medium supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL). LAD2 cells were cultured in StemPro-34 SFM (serum-free medium) supplemented with StemPro-34 supplement, L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL), and human recombinant stem cell factor (SCF) (100 ng/mL). Detailed instructions for culture of HMC and LAD2 cell lines have been previously published.^{E2} The pancreatic beta islet cell carcinoma line, QGP-1, was obtained from (JRCB Cell Bank, Osaka, Japan) and received as a kind gift from Dr. Electron Kebebew (Center for Cancer Research, National Cancer Institute). QGP-1 cells were cultured in RPMI-1640 medium supplemented with FBS (10%) and penicillin-streptomycin (100 U/mL), and were passaged weekly with trypsin-EDTA treatment. All cell lines were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

LAD2 mast cell stimulation and degranulation studies

For all experiments, FcεRI on LAD2 mast cells was cross-linked overnight with biotinylated human myeloma IgE (100 ng/mL). Cells were then challenged with streptavidin (100 ng/mL) for 30 minutes and 3 hours. Samples were then prepared for and

analyzed by quantitative RT-PCR or western blotting as described below. β-Hex release was measured as a reflection of cell degranulation as described.^{E3}

Quantitative RT-PCR

RNA was extracted from 1.5×10^6 cells of each cell line using the RNeasy Mini kit (QIAGEN, Germantown, Md). RNA quality and content was analyzed using the NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific, Waltham, Mass). Reverse transcription was performed on 500 ng of RNA from each sample using the SuperScript III First-Strand Synthesis System (ThermoFisher Scientific), according to the manufacturer's instructions. RT-PCR analysis was then performed in triplicate using the Bio-Rad C1000 Touch Thermocycler (Hercules, Calif). Briefly, for each reaction, 2 µL cDNA template was mixed with 7 µL nuclease-free water, and then added to 1 µL $\times 20$ Assays-on-Demand Primers and probes (Hs00174938_m1 for CgA, Hs99999905_m1 for GAPDH [glyceraldehyde-3-phosphate dehydrogenase]) and 10 µL $\times 2$ TaqMan Universal PCR Master Mix (Applied Biosystems) in a 96-well plate, for a total of 20 µL per reaction. RT-PCR was performed under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The expression level of the target gene was normalized to internal GAPDH, and levels were calculated using the comparative Ct method.

Western blotting

Total cell lysates of all cell lines were prepared for protein expression analysis by the following method. A total of 1×10^6 cells of each type were collected and centrifuged at $150 \times g$ for 5 minutes. Cells were then washed once with PBS and lysed in 50 μ L RIPA (radioimmunoprecipitation assay) buffer. Lysates were incubated at 4°C for 30 minutes and then centrifuged at $10,000 \times g$ for 5 minutes. Supernatants of lysates were collected in new microcentrifuge tubes, and 50 μ L $\times 2$ SDS sample buffer was added and mixed by pipetting. Samples were boiled for 5 minutes before use or storage at -80°C . Before use, lysates were centrifuged at $10,000 \times g$ for 5 minutes. Lysates were then loaded and resolved onto 4% to 12% SDS-PAGE gels (Novex Bis-Tris Gels, Invitrogen). Proteins were then transferred onto nitrocellulose membranes (ThermoFisher Scientific). Blots were blocked in a TBS-based blocking buffer (LI-COR Odyssey Blocking Buffer) and incubated overnight at 4°C with primary antibody with the following dilutions in TBS and blocking buffer: 1:10,000 for mouse monoclonal β -actin (Cell Signaling Technology), and 1:200 for rabbit polyclonal CgA (Santa Cruz Biotechnology). Blots were then washed in TBS-T wash buffer $\times 3$ for 8 minutes each wash. Blots were then incubated with antimouse and antirabbit secondary antibodies for 1 hour at room temperature. Membranes were washed 3 times with TBS-T for 5 minutes per wash, and then twice more with TBS for 5 minutes per wash. Blots were imaged and quantified relative to β -actin using Image Studio 5.2 on the LI-COR Odyssey CLx.

CgA band was predicted to appear at 70 kD according to the manufacturer's instructions.

Statistical analysis

All statistics were performed in GraphPad Prism 6. The Kruskal-Wallis test with multiple comparisons was performed in GraphPad Prism 6 for the analysis of differences between serum CgA and gastrin levels based on H₂ antagonist or PPI use. For correlations between serum CgA, gastrin, and tryptase values, nonlinear regression (curve fit) was performed. Correlations were found using column analysis with an r value generated for x versus every y data set, using a nonparametric Spearman correlation, and a 2-tailed P value. For comparison of mRNA and protein expression, ordinary 2-way ANOVAs with Tukey's tests for multiple comparisons were performed.

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