

Oxidative stress markers are increased in patients with mastocytosis

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Abstract

Background: Mastocytosis is characterized by clonal proliferation of mast cells limited to the skin (cutaneous mastocytosis: CM and mastocytosis in the skin: MIS) and/or involving internal organs (systemic mastocytosis: SM). Oxidative stress occurring in various inflammatory and neoplastic disorders causes molecular damage with the production of advanced oxidation protein products (AOPPs) and advanced glycation end products (AGEs). We evaluated these markers of oxidative stress in patients with CM/MIS and SM and correlated their levels with the presence of symptoms related to mast cell activation.

Methods: Serum levels of AOPPs and AGEs in 34 patients with mastocytosis (23 CM/MIS and 11 SM) and 27 healthy controls were measured by spectrofluorimetric and spectrophotometric methods. Serum tryptase levels were measured by immunofluorescence.

Results: Serum AOPPs, but not AGEs, were significantly higher in patients with mastocytosis as compared to healthy controls. While serum tryptase levels were higher in patients with SM as compared to those with CM/MIS, there was no difference in AOPP and AGE concentrations between these two groups of patients. Patients with recurrent mediator-related symptoms had lower AOPPs and AGEs as compared to patients without symptoms. AOPPs and AGEs were inversely correlated with the severity of symptoms, and in patients with symptoms, AOPPs correlated with tryptase levels.

Discussion: Our data show that mastocytosis is associated with a state of increased oxidative stress that, in patients with mediator-related symptoms, correlates with mast cell burden as assessed by tryptase. Patients with symptoms presumably have an adaptive response resulting in lower blood levels of AOPPs and AGEs.

Mastocytosis is a clinically heterogeneous disease characterized by clonal proliferation of mast cells (MC) in the skin and/or any other organ. Most patients with mastocytosis have an indolent course of the disease and a benign prognosis, whereas <5% of them have more aggressive forms (1). The currently used WHO classification identifies several disease variants including cutaneous mastocytosis (CM), indolent systemic mastocytosis (ISM), SM with an associated

clonal hematological non-MC-lineage disease (AHNMD), aggressive SM, and MC leukemia (2, 3). Proliferation of MC in CM is limited to the skin, whereas in SM, the cells accumulate in internal organs such as bone marrow, liver, spleen, lymph nodes, and gastrointestinal tract (2–4).

Regardless of cutaneous or systemic involvement, most patients with mastocytosis suffer from signs and symptoms due to the acute or chronic release of MC-derived mediators.

MC secrete a variety of proinflammatory and immunoregulatory molecules including histamine, heparin, neutral proteases such as tryptase and chymase, lipid mediators, and a wide range of cytokines and chemokines. These mediators exert major effects on peripheral and central cardiovascular system, gastrointestinal and respiratory tract, and bone remodeling (5). The typical skin manifestation of mastocytosis is urticaria pigmentosa, which is characterized by red/brown macules or papules that generate a wheal-and-flare reaction when scratched (Darier's sign). Other skin manifestations include classical urticaria, angioedema, and flushing (6). Gastrointestinal mediator-related symptoms include gastritis, peptic ulcer, diarrhea, and abdominal pain. Up to 60% of patients with mastocytosis have osteoporosis, which is thought to be related to the chronic release of mediators influencing bone resorption (7). Finally, patients with mastocytosis have major cardiovascular symptoms including hypotension, dizziness, and recurrent anaphylaxis (8, 9).

Oxidative stress is the result of imbalance between endogenous production of free reactive oxygen species (ROS) and reduction of antioxidant defense mechanisms. This imbalance can worsen inflammation and injury by enhancing proinflammatory cytokine release and altering enzymatic functions (10). Oxidative stress occurs in many inflammatory, neoplastic, and immunologic diseases, including asthma, allergic rhinitis, obesity, and leukemia (10–12). There is indirect evidence that oxidative stress might play a role in mastocytosis and/or contribute to clinical manifestations of this disease. For example, *in vitro*-induced oxidative stress activates MC degranulation from RBL-2H3 cells, a widely used model of mast cell activation (13). Furthermore, dogs with MC tumors show high levels of oxidative stress and reduced antioxidant activity *in vivo* (14), while activation of MC in rats is associated with the induction of oxidative stress that may be prevented by the inhibition of MC degranulation (15). However, no data are available on biomarkers of oxidative stress in patients with mastocytosis.

Biomarkers of oxidative stress can be classified as molecules that are modified by interactions with ROS in the microenvironment and molecules of the antioxidant system that change in response to increased redox stress. Advanced oxidation protein products (AOPPs) and advanced glycation end products (AGEs) are a family of compounds generated in body fluids by the transformation of macromolecules, including proteins, used as markers of oxidative stress and inflammation in several diseases (12, 16–19). These compounds are often functionally inactive and display either increased or reduced sensitivity to protease degradation. AOPPs are protein derivatives, predominantly albumin, and its aggregates mainly formed by chlorinated oxidants. AGEs, for example, pentosidine and carboxymethyllysine, are formed via autooxidation of sugars as well as other glycation intermediates and via lipoperoxidation of polyunsaturated fatty acids. These modified molecules may be employed more efficiently than other biomarkers to monitor disease progression and outcome. Stress-induced alterations in the conformation/structure of proteins lead to either their dysfunction or inhibition of their degradation. Modified

proteins can subsequently induce cellular dysfunctions, tissue damage, and disease onset/progression. AOPPs and AGEs are relatively stable molecules that can be measured by fast and inexpensive techniques, which makes them suitable markers to monitor oxidative stress (17, 18, 20). The aim of this study was to examine the serum levels of AOPPs and AGEs in patients with mastocytosis and to define whether these molecules are useful markers of oxidative stress in these patients.

Materials and methods

Patients

Thirty-four patients with mastocytosis (17 males and 17 females; 10 patients <18 years old and 24 > 18 years old; age range: 1–68 years; median age 35 years) were recruited in this study. Bone marrow biopsy was performed in 12 adult patients and it confirmed systemic mastocytosis in 11 of these patients. The biopsy in the other adult patients was not performed either because of low serum tryptase level or because patients declined the procedure. According to a recent proposal (4), adult patients that did not undergo bone marrow biopsy are indicated as 'mastocytosis in the skin' (MIS). None of the pediatric patients underwent bone marrow biopsy. In the case of two children with serum tryptase levels higher than 100 µg/L, their parents declined the biopsy. Therefore, the population under study included 23 patients with CM or MIS and 11 patients with SM.

Twenty-seven healthy age- and sex-matched individuals served as controls.

Among the patient population, 17 had only urticaria pigmentosa without any other mediator-related symptom and 17 had one or more of the following clinical manifestations: recurrent urticaria, angioedema, flushing, diarrhea, osteoporosis, or recurrent anaphylaxis. Symptom grading was determined according to the criteria reported by Valent et al. (1). Table 1 reports the patients' characteristics, the disease variant, the tryptase values, and symptom grading in the two groups of patients without (Table 1A) or with mediator symptoms (Table 1B). Almost all patients with SM (10/11) had mediator-related symptoms, whereas only one-third (7/23) of patients with CM or MIS had such symptoms.

None of the patients was on drug treatment at the time of blood sampling. In patients with recurrent anaphylaxis, blood was withdrawn at least 2 weeks after the episode to avoid potential interference of hypotension or drugs used to treat anaphylaxis, such as epinephrine, on the levels of oxidative stress markers.

Sera collection

Written informed consent according to the declaration of Helsinki was obtained from all patients and controls. Blood was withdrawn from the antecubital vein and allowed to clot at room temperature for 2 h. Serum was separated by centrifugation at 1200 g for 15 min and stored at –80°C until used.

Table 1 Characteristics of patients (A) without mediator symptoms, (B) with mediator symptoms

Patient no	Sex	Age	Disease category	Tryptase $\mu\text{g/l}$	Pediatric /adult	Symptom grading
(A)						
1	M	2	CM	4.6	Pediatric	0
2	M	3	CM	2.6	Pediatric	0
3	M	12	CM	10.6	Pediatric	0
4	F	16	CM	5.9	Pediatric	0
5	M	16	CM	11.2	Pediatric	0
6	F	37	CM	7.2	Adult	0
7	F	21	MIS	3.7	Adult	0
8	M	27	MIS	6.7	Adult	0
9	F	29	MIS	2.9	Adult	0
10	F	35	MIS	4.5	Adult	0
11	F	35	MIS	46.1	Adult	0
12	M	37	MIS	94	Adult	0
13	F	38	MIS	11	Adult	0
14	M	39	MIS	15.3	Adult	0
15	F	43	MIS	12.7	Adult	0
16	F	54	MIS	6.8	Adult	0
17	M	28	ISM	24.6	Adult	0
(B)						
18	F	1	CM	12.5	Pediatric	2
19	F	2	CM	102	Pediatric	2
20	M	8	CM	25.7	Pediatric	3
21	M	8	CM	3.5	Pediatric	1
22	M	10	CM	114	Pediatric	2
23	M	68	ISM	29.6	Adult	2
24	M	21	MIS	5.8	Adult	1
25	F	62	MIS	14	Adult	2
26	F	24	ISM	17.5	Adult	4
27	F	39	ISM	50.8	Adult	3
28	F	41	ISM	27.5	Adult	2
29	M	42	ISM	115	Adult	1
30	M	44	ISM	157	Adult	1
31	M	46	ISM	128	Adult	2
32	F	48	ISM	79.9	Adult	3
33	M	55	ISM	102	Adult	1
34	F	46	SM-AHNMD	9.5	Adult	3

CM, cutaneous mastocytosis; MIS, mastocytosis in the skin; ISM, indolent systemic mastocytosis; SM-AHNMD, systemic mastocytosis with an associated clonal hematological non-MC-lineage disease.

Determination of AGEs, AOPPs, and tryptase

AGEs and AOPPs were measured by spectrofluorimetric and spectrophotometric methods, respectively, as previously described (12, 16–18).

For AGE determination, serum was diluted 1 : 50 with phosphate-buffered saline (PBS; pH 7.4), and fluorescence intensity was recorded at maximum emission (~440 nm) upon excitation at 350 nm and expressed in arbitrary units (AU). The serum concentration of AGEs was normalized to the total protein amount determined by the Bradford assay and expressed in AU for protein gram.

For AOPP determination, 200 μl of blood serum was diluted 1 : 5 with PBS and 200 μl of chloramines T (0–100 mol/l) for calibration, and 200 μl of PBS as blank were applied on a microtitre plate. Ten microliters of 1.16 M KI and 20 μl of acetic acid were added, and absorbance at 340 nm was measured immediately. The serum concentration of AOPPs was normalized to the total protein amount determined by the Bradford assay and expressed as chloramine nmoles/mg of protein.

Each sample was analyzed in triplicate both for AGE and for AOPP determination. Serum tryptase was measured using a commercially available kit (Phadia).

Statistical analysis

Data are expressed as medians. Differences between groups were analyzed by Mann–Whitney test. Correlation between two variables was evaluated with Spearman's rho. The statistical analysis was performed with SPSS for Windows (version 17.0, SPSS, Chicago, Illinois, USA). The level of statistical significance was set at $P < 0.05$.

Results

Oxidative stress markers and tryptase in patients with mastocytosis

AOPP serum levels were threefold higher in patients with mastocytosis, either CM/MIS or SM, than in healthy donors (6.69 ± 0.72 vs 2.22 ± 0.33 nmol/mg prot, $P < 0.0001$; Fig. 1A), whereas there was no difference in AGE serum levels (242.47 ± 28.84 vs 259.89 ± 16.73 AU/g prot, $P = 0.977$) (Fig. 1B). Patients with SM showed higher levels of tryptase as compared to those with CM/MIS (102 ± 12.4 vs 9.5 ± 2.1 $\mu\text{g/l}$, $P < 0.0001$) (Fig. 2A). By contrast, there was no significant difference in AOPPs and AGEs between these two groups (AOPP: 6.56 ± 0.97 vs 6.72 ± 1 nmol/mg prot, $P = 0.782$; AGE: 299.78 ± 40.37 vs 246.43 ± 30.05 AU/g prot, $P = 0.985$) (Fig. 2B,C). There was no significant difference in AOPP and AGE serum levels between pediatric and adult patients with mastocytosis (Fig. S1A,B).

Oxidative stress markers in patients with and without mediator-related symptoms

We next compared the levels of tryptase and concentrations of oxidative stress markers in the serum of patients with or without mediator-related symptoms. Serum levels of tryptase were about fourfold higher in patients with symptoms as compared to those without (29.6 vs 7.2 $\mu\text{g/l}$, $P = 0.002$). This was primarily due to the fact that in the group with symptoms, 10 of 17 patients had SM, whereas only 1 patient with SM was in the group without symptoms (Table 1). Surprisingly, patients with mediator-related symptoms had lower serum levels of AOPP and AGE as compared to patients without symptoms (AOPP: 5.48 vs 8.32 nmol/mg prot, $P = 0.024$; AGE: 184.23 vs 298.16 AU/g

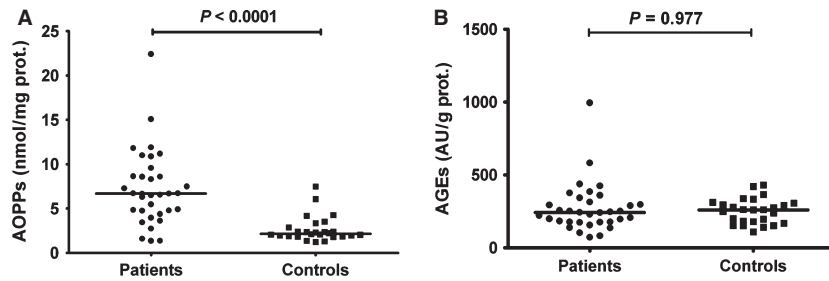


Figure 1 Serum level of advanced oxidation protein products (A) and advanced glycation end products (B) in patients with mastocytosis and in healthy donors (controls). Lines represent medians. Statistical analysis was performed using Mann–Whitney test.

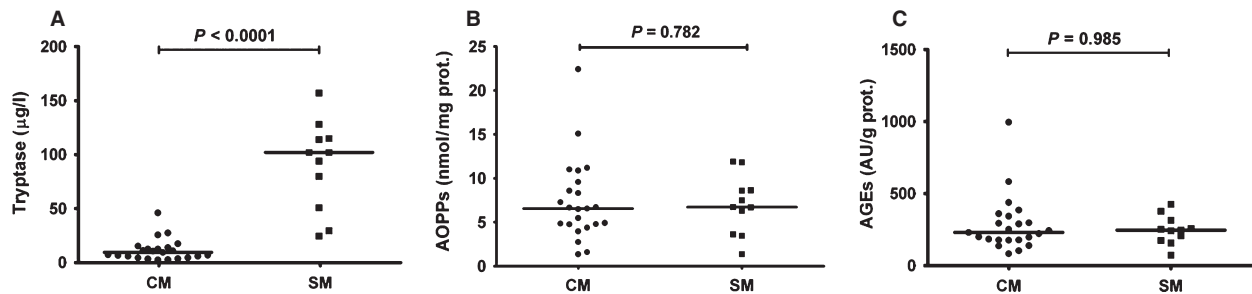


Figure 2 Serum levels of tryptase (A), advanced oxidation protein products (B), and advanced glycation end products (C) in patients with cutaneous mastocytosis or with mastocytosis in the skin (CM/MIS) and patients with systemic mastocytosis (SM). Lines represent medians. Statistical analysis was performed using Mann–Whitney test.

prot, $P = 0.003$), (Fig. 3A,B). Evaluation of AOPPs and AGEs in patients stratified according to symptom grading (0 = no symptoms to 4 = severe symptoms requiring frequent hospitalization) showed a significant inverse correlation between oxidative stress markers in serum and severity of symptoms (Fig. 4A,B).

In agreement with these data, patients with CM/MIS and mediator-related symptoms had lower serum levels of AOPPs and AGEs as compared to patients with CM/MIS without symptoms (AOPPs: 4.36 vs 7.28 nmol/mg prot, $P = 0.017$; AGEs: 157.84 vs 289.54 AU/g prot, $P = 0.005$), (Fig. S2). This analysis could not be made in patients with SM because there was only one patient without mediator-related symptoms in this group. In patients with mediator-related symptoms, serum AOPPs correlated with tryptase levels ($\rho = 0.564$; $P = 0.018$) (Fig. 5).

Discussion

Accumulation of AOPPs and AGEs can be detected in a variety of acute and chronic diseases (17, 21–24). In this study, we found higher levels of AOPPs, but not AGEs, in the serum of patients with mastocytosis as compared to healthy donors. There was no statistically significant difference in AOPP or AGE levels between patients with SM and CM/MIS or between pediatric and adult patients. The different behaviour observed for AGEs and AOPPs is very likely due to the activation of specific biochemical pathways related to the pathological condition under study.

tosis and in healthy donors (controls). Lines represent medians. Statistical analysis was performed using Mann–Whitney test.

MIS) and patients with systemic mastocytosis (SM). Lines represent medians. Statistical analysis was performed using Mann–Whitney test.

Our data indicate that mastocytosis, a disease characterized by mast cell proliferation either in the skin or in extracutaneous organs, is associated with a state of increased oxidative stress. AOPPs are generated through a complex process from free radical-mediated protein oxidation. Thus, the higher serum AOPP levels measured in patients with mastocytosis are likely consequent to an increased ROS production from mast cells.

Several biomarkers have been reported in the blood or urine of patients with mastocytosis. Some of these markers, such as serum tryptase and urinary histamine metabolites, are currently used as diagnostic test markers for mastocytosis and to monitor disease progression (1, 4). Other cytokines, such as IL-6 (25) and IL-31 (26), are significantly elevated in the serum of patients with mastocytosis as compared to healthy individuals. The levels of IL-6 and IL-31 correlate with disease severity and with MC proliferation in the bone marrow (25, 26). Interestingly, blood serotonin levels lower than those in healthy controls have been reported in patients with mastocytosis and mediator symptoms such as fatigue, headache, flushing, diarrhea, and psychiatric disorders (27). Given the heterogeneity of mastocytosis, searching for biomarkers able to identify subsets of patients with different phenotypes of the disease may be useful to better define the clinical variants of the disease. Our results suggest that AOPPs and AGEs are potentially useful markers to monitor the level of oxidative stress and to define the severity of mediator-related symptoms in patients with mastocytosis.

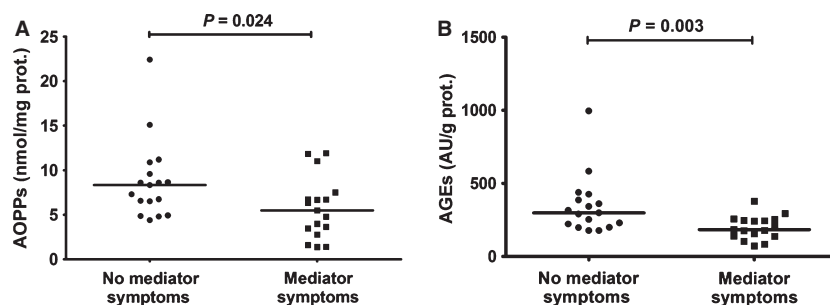


Figure 3 Serum levels of advanced oxidation protein products (A) and advanced glycation end products (B) in patients with or without

mediator symptoms. Lines represent medians. Statistical analysis was performed using Mann–Whitney test.

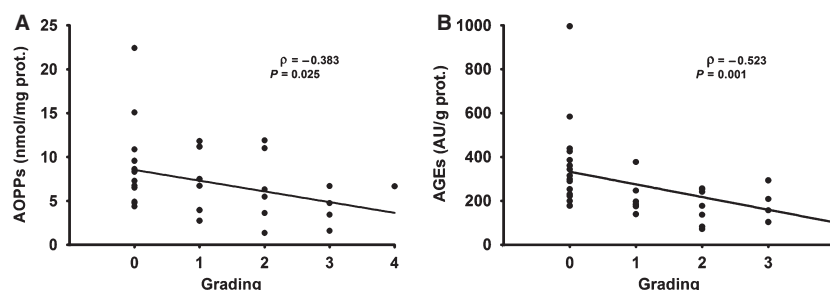


Figure 4 Serum levels of advanced oxidation protein products (A) and advanced glycation end products (B) in patients with mastocytosis stratified according to the severity of mediator-related symptoms: 0: no symptoms; 1: mild, infrequent, no therapy required; 2:

mild/moderate, frequent, kept under control with therapy; 3: severe, frequent, difficult to control; and 4: severe adverse event, requiring hospitalization (1). Statistical analysis was performed using Spearman's rho.

Our findings in patients with mastocytosis are in line with observation in other clonal hematological diseases such as multiple myeloma and other myeloproliferative or lymphoproliferative diseases. Myeloproliferative disorders are characterized by proliferation of one or more hematopoietic lineages and progress with different probability to blast crisis or fibrosis. Increased levels of AOPPs without significant changes in circulating concentrations of AGEs, similar to those found in patients with mastocytosis, have been detected in patients with polycythemia vera (18). These data support the hypothesis that an increased production of AOPPs may be a characteristic of patients suffering myeloproliferative diseases or abnormal growth and accumulation of one or more hematopoietic lineages. Furthermore, we reported that blood levels of AGEs were increased in patients with essential thrombocythemia complicated by thrombotic events. Thus, AGEs, a biomarker known to be intimately involved in the pathophysiology of cardiovascular disease (28), appears to be associated with a higher thrombotic risk (18).

Interestingly, patients with mastocytosis and recurrent mediator-related symptoms have lower circulating levels of AOPPs and AGEs as compared to patients without symptoms. A potential explanation of this finding is that a higher level of oxidative stress may impair MC activation *in vivo* resulting in minor or no mediator-derived symptoms. Oxidized proteins are often functionally inactive and can demonstrate either increased or reduced sensitivity to protease

degradation. MC are known to express receptors for advanced glycation end products (RAGE) (29). Sick et al. (30) showed that AGEs can activate rat mast cells, suggesting that they may contribute to a vicious cycle involving generation of ROS, increased formation of AGEs, activation of RAGE, and increased low-grade inflammation typical of chronic diseases. However, RAGE is a key regulator in the delicate balance between cell survival and cell death depending on the intensity and/or duration of its activity, and RAGE signaling triggered by low concentrations of its ligands may support tissue homeostasis and concur to tissue repair and inflammation resolution. It has also been suggested that RAGE activation may be not restricted to pathological statuses, the receptor being involved in tissue homeostasis and regeneration/repair upon acute injury and in resolution of inflammation (31). Although no specific AOPP receptor has been identified to date, the existence of several conventional and scavenger receptors for oxidized and glycated proteins and inhibitory receptors that could regulate the immune response have been demonstrated in phagocytes (32). There is growing evidence that increased oxidative stress negatively modulates cellular signaling pathways to ultimately induce endogenous defense mechanisms and inhibitory responses (33). Interestingly, exposure to AOPPs has resulted in a decreased free radical generation from activated bovine neutrophils and in a loss of their viability (32). Also, on the basis of these observations, we could

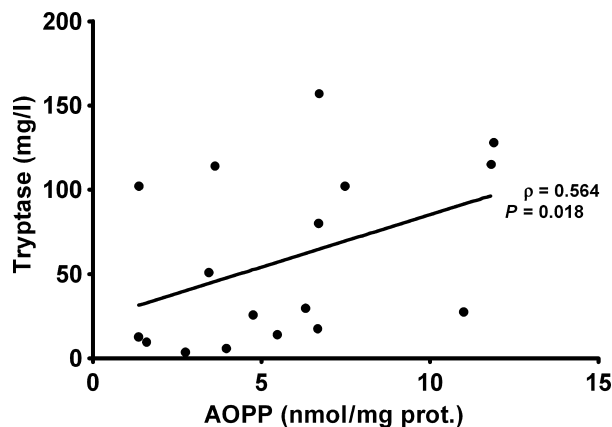


Figure 5 Correlation between advanced oxidation protein products and tryptase serum levels with mediator-related symptoms. Statistical analysis was performed using Spearman's rho.

hypothesize an adaptive mechanism that could explain why patients with higher oxidative stress may be less exposed to symptoms due to MC activation. Thus, in patients with mastocytosis, the repetition of graduated stresses may induce a multiform adaptive response able to block MC activation and/or functions. An alternative explanation for the lower levels of stress markers in patients with mediator-derived symptoms is that continuous secretion of MC could prevent accumulation of stress-modified products. For example, MC-derived heparin has been shown to prevent oxidative stress

and tissue damage in a model of testicular torsion (34). Finally, the possibility that increased oxidative stress may reduce sensitivity of target organs (skin, GI tract, and cardiovascular system) to mediators released by MC cannot be excluded.

In conclusion, this is the first study showing the possible involvement of AOPPs and AGEs in mastocytosis. In fact, in patients with CM/MIS and SM, serum AOPPs and AGEs, besides being good biomarker of oxidative stress, are probably involved in regulating in a complex fashion MC functioning and activation. The precise role of AOPPs and AGEs in the clinical presentation and progression of mastocytosis remains to be determined.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

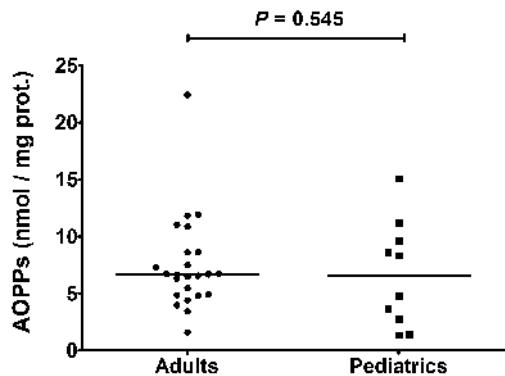
Figure S1 AOPP (A) and AGE (B) serum levels in pediatric (<18 years old) and adult patients (>18 years old) with mastocytosis; lines represent medians. Statistical analysis was performed using Mann–Whitney test.

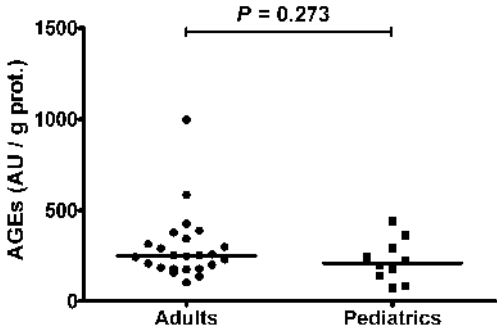
Figure S2 AOPP (A) and AGE (B) serum levels in CM patients with and without mediator-related symptoms; lines represent medians. Statistical analysis was performed using Mann–Whitney test.

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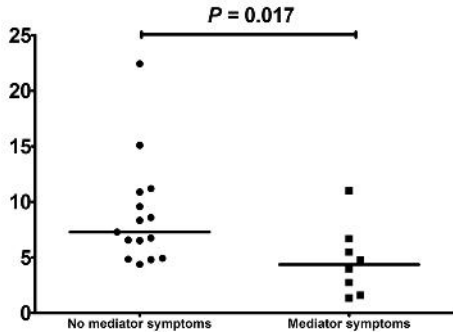
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A

AOPPs (nmol / mg prot.)

**B**

AGEs (AU / g prot.)

