

The prognostic significance of the activities of matrix metalloproteinases-2 and -9 in dogs for mammary gland neoplasia (pilot study)

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The aim of this study was to determine the activity of matrix metalloproteinase-2 and -9 in blood and tumour tissue of female dogs with benign and malignant mammary gland neoplasias. The increased activity of matrix metalloproteinase-2 and -9 was registered in 3.8–8.9% of canines with benign neoplasias (adenoma, mixed tumour) and in 32.5–63.5% of those with malignant neoplasias (carcinoma: mixed type, ductal, tubular, tubulopapillary). Neoplastic transformation is accompanied by blood level increase of both latent and active enzyme forms. The dogs with malignant mammary gland neoplasias were diagnosed with credible increase of matrix metalloproteinases-2 activity up to 1.59–1.96 in blood plasma and up to 21.57–24.84 in tumour tissue and the increase of matrix metalloproteinases-9 activity up to 2.16–2.67 and 29.53–35.26 respectively. For benign mammary neoplasms, the proportion of dogs with enhanced expression of matrix metalloproteinase-2 was higher than the number of patients in whom an increase in the level of matrix metalloproteinase-9 or both of these enzymes was registered by 1.7 times, for dogs with malignant tumours – in blood plasma – by 1.4–1.6 times, dogs with neoplastic changed functional tissue – 1.7 and 1.9 times, respectively. Histological type and metastatic foci presence did not correlate with enzymes' activity. The enzymes' activity figures in benign neoplasias fluctuated within those in clinically healthy animals. A positive characteristic of determining the plasma and tissue expression level of matrix metalloproteinases in dogs with mammary tumours is the low degree of invasiveness of the method against the background of the high informativeness of the results obtained in the preoperative period. The obtained results prove the possibility of using matrix metalloproteinases-2 and -9 to predict the course and to control the treatment of mammary neoplasia.

Keywords: tumours; predictors of neoplasia; metastasis; zymography.

Introduction

Matrix metalloproteinases (MMP) are zinc-dependent endopeptidases regulating the turnover of intercellular matrix components. While metastasizing, tumour cells should enter a blood vessel and degrade intercellular matrix (Hanifé et al., 2014). Blood vessels and extracellular matrix degradation occurs through MMP activity, thus MMP activity is crucial for determining the metastatic potential of a cancer cell (Mustafa et al., 2022). MMP-9 and MMP-2, synthesized by epithelial cancer cells and cancer-associated fibroblasts, play an important role in canine malignant mammary gland tumours (Aresu et al., 2011).

MMP-2 is expressed in 85% of non-metastatic tumours and in all metastatic mammary gland tumours in canines (Nowak et al., 2016).

The research by Santos et al. (2011) showed that MMP-2 expression in canine mammary tumours had no effect on lifetime and non-recurring survival period, but the research by Lamp et al. (2013) showed that higher MMP-2 expression is connected with higher risks of premature death and metastasizing. The research by Fathipour et al. (2018) shows that MMP-9 serum activity is higher in dogs with skin neoplasias compared with healthy ones. Besides, positive correlation between MMP-9 and Ki-67 expression in canine mammary gland adenocarcinomas (Nowak et al., 2008) had been proved alongside substantially lower survival time in dogs with high MMP-9 expression mammary tumours. Therefore, MMP-9 and Ki-67 are independent prognostic markers of malignant mammary tumours in dogs (Santos et al., 2013). MMP-9 activity is 2–6 times higher in benign mammary tumors and 4–26 times higher in canine mammary adenocarcinomas compared to normal mammary tissue (Yokota et al., 2001; Hirayama et al., 2002). However, MMP-9 expression had little

difference in canine malignant mammary tumours and mammary inflammatory carcinomas (Raposo et al., 2016). Dogs with mammary adenocarcinoma show higher serum MMP-9 activity (Santos et al., 2012). Tumour tissue MMP-9 activity positively correlates with plasma MMP-9 activity in dogs with mammary tumours (Shia et al., 2011).

Serum pro-MMP-2 activity is two times higher in malignant tumours than in benign mammary tumours and healthy animals. The presented results of previous research show the presence of MMP-2, E-cadherin and Ki-67 interconnected expression in mammary tumour tissue in female dogs. Still, no authentic differences between metastatic and non-metastatic neoplasias have been found, which justifies the necessity of further studying the role of the above-mentioned antigens in mechanisms of the disease progression (Nowak et al., 2015). Currently presented results of the studies on the expression of matrix metalloproteinases in female dogs with mammary neoplasia are contradictory. The possibility of their use as predictors of mammary gland cancer in dogs and their correlation with other biological tumor markers remain debatable. Based on the urgency of the problem, the goal of the study was to substantiate the prognostic significance of matrix metalloproteinases-2 and -9 expression in benign and malignant mammary neoplasias in female dogs.

Materials and methods

The study was conducted at the Chair of Veterinary Surgery and Reproduction of Dnipro State Agrarian and Economic University and "Best" Zaporizhzhia private veterinary hospital for the period of 2021–2022. The research was conducted following the requirements of the European Convention for the Protection of Vertebrate Animals (Strasbourg, 1986)

and the Law of Ukraine “On Protection of Animals from Cruelty” (2006), as confirmed by the conclusion of the Bioethics Commission of Dnipro State Agrarian and Economic University.

Female dogs with verified benign and malignant neoplasia of the mammary gland, as well as clinically healthy animals, served as the object. The subject of research was the level of matrix metalloproteinases-2 and -9 in blood plasma and tumour tissue of the mammary gland in clinically healthy and cancerous dogs.

Female dogs with stage 2–3 single tumours were selected for the study. According to histological classification: malignant are carcinomas of various types, adenomas and benign mixed tumors being benign.

The histological type of breast tumours was determined according to the classification of Goldschmidt et al. (2011), staging was based on criteria by Elston & Ellis (1991) in the modification of Peña et al. (2013).

The total number of examined animals was 79 with benign tumours and 167 with malignant ones. Expression of matrix metalloproteinases-2 and -9 was determined in plasma and neoplastically changed mammary gland tissue. Four groups of dogs were formed for the purpose of conducting research. The first (control) one included 10 clinically healthy animals, the second one – 11 patients with benign neoplasms, the third one – 25 bitches with malignant neoplasms. In animals of the second and third groups, the tumour process was characterized only by local changes in the mammary gland, secondary neoplastic foci in regional lymph nodes and distant tissues were not found. The fourth group included 16 patients diagnosed with metastasized malignant mammary neoplasms.

Zymography of matrix metalloproteases involved the use of dog plasma for research. The material was collected into the test tubes with 3.8% sodium citrate (Eximlab, China) during the preoperative examination. The tubes were centrifuged for 15 minutes at 3000 g (Thermo Heraeus Labofuge 400, USA). Plasma was collected into 50 µL Eppendorf tubes and frozen.

A pool of plasma from healthy dogs was used for the reference sample. Ten samples were collected from clinically healthy dogs before being placed in tubes with 3.8% sodium citrate (Eximlab, China). The test tubes were centrifuged for 15 minutes at 3000 g (Thermo Heraeus Labofuge 400, USA). Equal volumes of donor plasma were thoroughly mixed to a homogeneous mass, after which they were poured into 50 µL Eppendorf tubes and frozen.

The Detect MMP activity in conditioned media (abcam, United Kingdom) Gelatin zymography protocol was used for the study and adapted for use on plasma using the patent Method for determining gelatinases in blood plasma (patent UA 83196 U: MPK G01N33/49, G01N27/26, G01N33/84, G01N33/96. № 201303700).

Plasma samples and a plasma pool aliquot of subjects were incubated at 37 °C for 20 minutes followed by 20-times dilution with a buffered physiological solution. It was mixed in a 1:1 ratio with Laemmli buffer (0.4 M Tris-HCl, 5% SDS, 20% glycerol, 0.03% bromophenol blue, pH 6.8).

Electrophoretic fractionation was performed using vertical electrophoresis (Clever Scientific nanoPAC-300P, United Kingdom) in a cold chamber at 4 °C for about 4 hours at 20 mA. Electrophoresis of blood plasma samples was performed in a 7.5% polyacrylamide gel (PAAG) in the presence of 0.1% sodium dodecyl sulfate (SDS), 2 mg/ml gelatin, 1.5M Tris-HCl, pH 8.8, 0.05% ammonium persulfate, 0.005% TEMED. Each gel well was loaded with 20 µL of a prepared sample. A solution containing 0.025 M Tris, 0.192 M glycine, and 0.01% SDS (pH 8.3) was used as an electrode buffer.

After the electrophoresis was completed, the gels were washed two times for 30 minutes each with a washing buffer containing: 2.5% Triton X-100, 50 mM Tris-HCl, 5 mM CaCl₂, 1 µM ZnCl₂ (pH 7.5).

After washing, the gels were washed for 5–10 minutes with incubation buffer (1% Triton X-100, 50 mM Tris-HCl, 5 mM CaCl₂, 1 µM ZnCl₂, pH 7.5) at 37 °C with constant shaking followed by a 24 hour incubation in a fresh buffer at a temperature of 37 °C.

At the end of the incubation period, the incubation buffer was drained off and a staining solution gel (40% methanol, 10% acetic acid, 50% distilled water, 0.5% Coomassie blue) was poured in and kept for 1 hour. After staining, the gels were washed out with distilled water and filled with a developing solution (40% methanol, 10% acetic acid, 50% distilled

water) until the bands of metalloproteinase activity were well visualized. Processing of the materials received. Gels were scanned and digital images processed using ImageJ (Wayne Rasband and contributors National Institutes of Health, USA, 1998).

MMP activity in tissues was determined according to the method described by Bencsik et al. (2017) with some modifications.

Tumour and normal mammary tissue samples were cut into 0.3 cm³ pieces and placed in 1.5 mL of solubilization buffer. The samples were homogenized on ice using a BioSpec Tissue Tearor (model 985-370), then centrifuged at 2000 g for 45 minutes at 4 °C. The supernatant was selected, and total protein concentration was determined using a Stanbio LiquiColor kit (#0345; Stanbio Laboratories) according to the manufacturer's instructions on a Minray MR A reader at 550 nm. Samples were stored at –80 °C until further analysing.

To carry out zymography, the samples were diluted with Laemmli buffer to a protein concentration of 20 mg/mL and zymography was performed according to the Detect MMP activity in conditioned media Gelatin zymography protocol (abcam, United Kingdom) as described for blood plasma.

MMP activity in a pool of normal mammary gland tissue obtained from 10 clinically healthy female canines was used as a reference.

Statistical analysis and graphic processing of the results were performed using the Statistica 10 software (StatSoft inc., USA, 2011), where pool plasmas were used as a standard, the gelatinase activity of which was taken as 100%. ANOVA (analysis of variance) with Bonferroni's correction was used to correctly compare mean values between groups. The obtained indicator was considered statistically significant at P < 0.05.

Results

The conducted studies showed that a statistically significant increase in the activity of matrix metalloproteinases was registered in mammary neoplasms, regardless of their histological type. However, if for malignant neoplasias their level exceeded the indicators of clinically healthy animals in 100% of cases, then for benign neoplasias the indicators were exceeded within 14% of patients. Compared to clinically healthy animals, high activity of matrix metalloproteinases in patients with benign neoplasias was registered in blood plasma of 3.8–6.3% and in tumour tissues of 3.8–8.9% of female canines (Table 1). Both in blood plasma and in pathologically altered functional tissue, the number of patients diagnosed with an increase in the level of matrix metalloproteinase-2 was greater than the proportion of dogs with increased expression of matrix metalloproteinase-9 and both of them (MMP-2 & MMP-9) by 1.7 times.

Table 1

Activity of MMP-2 and MMP-9 in bitches with benign mammary tumors (N = 79)

Enzyme	Number of animals with high activity MMP-2 & MMP-9 in blood plasma, %	Number of animals with high activity MMP-2 & MMP-9 in tumour tissue, %
MMP-2	6.3	8.9
MMP-9	3.8	5.1
MMP-2 & MMP-9	3.8	5.1

The difference between its activity in tissues and blood plasma was: MMP-2 – 1.4 times; MMP-9 and both metalloproteinases – 1.3 times. In malignant tumours (Table 2), an increase in MMP-2 activity in blood plasma was found in 58.1% of animals, in neoplastic tissue – in 63.5% of animals; MMP-9 – 41.9% and 36.5%, respectively. In a third of cases, activation of both matrix metalloproteinases was registered. In malignant neoplasms, an increase of MMP-2 level (compared to MMP-9) was more often registered: by 1.4 times in plasma and by 1.8 times in tumour focus, of both enzymes (MMP-2 & MMP-9) – 1.7 and 1.9 times, respectively.

Visual confirmation of more active expression of matrix metalloproteinases-2 and -9 and their latent forms (pro-MMP-2 and pro-MMP-9) in dogs with mammary gland neoplasms is presented in Figure 1.

The most common histological types of mammary gland neoplasias, the development of which was accompanied by a high level of metalloprotease activity, were selected to determine the possible correlation of the histological type with the expression level of matrix metalloproteinases.

(Tables 3 and 4). Among benign neoplasms of the mammary gland, high activity of matrix metalloproteinases-2 & -9 was registered in adenoma and benign mixed tumours, the number of which was 45.5% and 54.5%, respectively. Malignant neoplasms, which were characterized by a local pathological focus, were represented by carcinomas: mixed type in 36% of cases, ductal in 28%, tubular in 24%, tubulopapillary in 12%. Metastatic foci were diagnosed according to the indicated histological types of carcinomas in 43.8%, 31.2%, 12.5% and 12.5% of patients.

Table 2
Frequency of enhanced activity of matrix metalloproteinases-2 & -9 in malignant neoplasms of the mammary gland (N = 167)

Enzyme	Number of animals with high activity MMP-2 & MMP-9 in blood plasma, %	Number of animals with high activity MMP-2 & MMP-9 in tumour tissue, %
MMP-2	58.1	63.5
MMP-9	41.9	36.5
MMP-2 & MMP-9	35.3	32.9

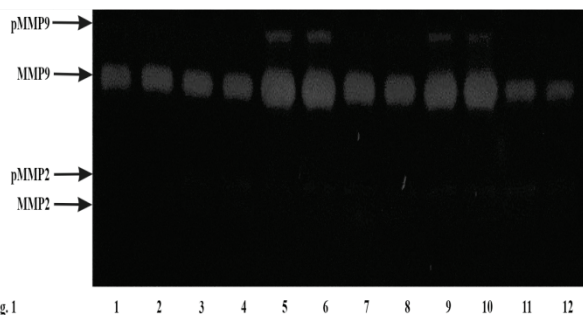


Fig. 1

Fig. 1. Gelatin zymography of plasma samples of bitches with mammary gland tumours

Table 3
The structure of benign mammary gland tumours in bitches with high activity of MMP-2 & -9 (N = 11)

Histological type	n
Adenoma	5
Benign mixed tumour	6
Total	11

Table 4
Histological type of malignant neoplasms of the mammary gland from increased expression of MMP-2 & -9

Histological type	Without metastases	With metastases
Mixed type carcinoma	9	7
Ductal carcinoma	7	5
Tubular carcinoma	6	2
Tubulopapillary carcinoma	3	2
Total	25	16

The results of the research made it possible to determine the "basic" level of promatrix and matrix metalloprotease-2 and -9 activity in clinically healthy dogs (Table 5). It should be noted that there is no significant difference between the latent and active forms of MMP-2 and MMP-9: the former were 0.60 ± 0.17 and 0.66 ± 0.21 , the latter were 0.71 ± 0.14 and 0.79 ± 0.29 , respectively. The development of benign mammary neoplasms did not significantly affect changes in serum pro-MMP-2 & -9 and MMP-2 & -9 parameters. Their fluctuations were within statistical error.

Significant activation of promatrix and matrix metalloproteinases was detected in bitches with malignant mammary neoplasms. During the non-metastatic stages of oncogenesis, the activity of pro-MMP-2 and MMP-9 increased significantly ($P < 0.001$) by 3.1 and 2.7 times (from 0.60 ± 0.17 to 1.60 ± 0.18 and 0.66 ± 0.21 to 2.07 ± 0.26 , respectively) compared to clinically healthy animals. Progression of the disease with the dissemination of tumour cells and the formation of metastatic foci was followed by the activation of matrix metalloproteinases: pro-MMP-2 – 1.90 ± 0.11 ; pro-MMP-9 – 2.42 ± 0.13 ; MMP-2 – 1.96 ± 0.25 ; MMP-9 – 2.67 ± 0.19 ,

which exceeds the value of healthy female dogs ($P < 0.001$) by 3.4, 2.8, 3.7 and 3.2 times, respectively.

Table 5
Activity of matrix metalloproteinases-2 & -9 in blood plasma of tumour-carrying bitches ($x \pm SD$)

Indicator	Clinically healthy (n = 10)	Neoplasm	
		benign (n = 11)	malignant
		without metastases (n = 25)	with metastatic foci (n = 16)
pro-MMP-2	0.60 ± 0.17	$1.27 \pm 0.38^{**}$	$1.60 \pm 0.18^{***}$
pro-MMP-9	0.66 ± 0.21	$1.05 \pm 0.27^{**}$	$2.07 \pm 0.26^{***}$
MMP-2	0.71 ± 0.14	0.45 ± 0.22	$1.59 \pm 0.14^{***}$
MMP-9	0.79 ± 0.26	0.88 ± 0.11	$2.16 \pm 0.23^{***}$
			$2.67 \pm 0.19^{***}$

Note: ** – $P < 0.05$, compared to clinically healthy dogs, *** – $P < 0.001$, compared to clinically healthy dogs.

It should be noted that the activity of the latent and active forms of the corresponding matrix metalloproteinases-2 and -9 in clinically healthy dogs and bitches with benign and malignant neoplasms of the mammary gland was at the same level.

The activity of matrix metalloproteinases-2 & -9 in the neoplastically changed areas of the mammary gland increased significantly (Table 6). Moreover, compared to normal tissue, the excess during malignant transformation was ($P < 0.001$): 15–18 times for MMP-2; 14–17 times for MMP-9. As in blood plasma, activity levels of matrix metalloproteinases in tumour tissue did not correlate with metastasis.

Table 6
Activity of matrix metalloproteinases in canine mammary gland tumour tissue ($x \pm SD$)

Gene	Clinically healthy (n = 10)	Neoplasm	
		benign (n = 11)	malignant
		without metastases (n = 25)	with metastatic foci (n = 16)
MMP-2	1.41 ± 0.23	2.03 ± 0.47	$21.57 \pm 3.90^{***}$
MMP-9	2.09 ± 0.16	2.61 ± 0.39	$29.53 \pm 3.11^{***}$
MMP-2 & MMP-9	3.53 ± 0.19	4.52 ± 0.41	$51.10 \pm 3.27^{***}$
			$60.10 \pm 3.56^{***}$

Note: see Table 5.

Discussion

Unlike normal tissue, during neoplastic transformation of the mammary gland, the formation of the NGAL/MMP-9 complex (neutrophil gelatinase associated lipocalin/matrix metalloproteinase 9) occurs. Therefore, MMP-9 expression activation alongside presence of a heterodimer with tissue damage biological marker (NGAL) in malignant types of mammary neoplasms indicates an increased invasion and risk of metastasizing (Chen et al., 2019).

Santos et al. (2016) found that the vascular endothelial growth factor receptor (VEGFR-2) in malignant mammary neoplasms was positively associated with stromal matrix metalloproteinase-9, which suggests the existence of a correlation between the activation of endothelial cells and the secretion of proteins that destroy the matrix. High expression of VEGFR-2 and MMP-9 may be one of the molecular pathways of tumour aggression.

The obtained results are consistent with the report of Gramulia et al. (2016) regarding increased expression of matrix metalloproteinase-9 (MMP-9) in most cases of malignant mammary neoplasms against the background of the absence of statistically significant differences between histological types (anaplastic, simple, mixed, solid, simple papillary carcinomas) or histopathological degrees according to indicators: positivity, intensity and character of cytoplasmic staining.

These results confirm the data of Shia et al. (2011), who proved the relationship between the levels of matrix metalloproteinases-2 and -9 with mammary tumour malignancy in canines. The expression of MMP-2 and MMP-9 was significantly higher in neoplastic tissues compared to normal

tissues ($P < 0.05$). Their plasma level in malignant types of mammary neoplasms was significantly higher than in bitches with benign tumours and non-neoplastic changes (MMP-9: $P = 0.15$ and $P < 0.05$; MMP-2: $P = 0.54$ and $P < 0.01$, respectively). However, a high level correlation in tumour tissue and plasma was registered only for MMP-9.

The published work by Saxena et al. (2016) proves the overexpression of matrix metalloproteinases -2 and -9 in a wide range of cancers and their important role in tumour cell proliferation, invasion and metastasis, thereby confirming our results. In particular, the tumour tissue showed an increase in MMP-2 in 58.3%, MMP-9 in 41.6%, and both metalloproteinases in 33.3% of cases.

Akkoc et al. (2012) also demonstrated increased levels of MMP-9 and MMP-2 in tumour samples using Western blot analysis; protein band analysis revealed a 1.9- to 3-fold MMP-9 increase in neoplastic tissue (neoplastic mammary epithelial cells, stromal fibroblasts and inflammatory cells) as well as in metastatic cancer cells in lymphatic vessels. Unlike these results, Pisamai et al. (2017) demonstrated the correlation of expression of matrix metalloproteinases (MMP-2; -7; -9) and their tissue inhibitors (TIMP-1; -2) with different clinical stages and histological grade of mammary tumours in canines. Compared with the control samples, a significant decrease in MMP-7 and TIMP-2 levels was observed for all types of hyperplasia; an increase in the histological grade was accompanied by an increase in the expression of MMP-2, MMP-9 and TIMP-1.

Krupakaran et al. (2016) using gelatin zymography found that MMP-2 active form expression and other bands intensity depend on tumour grade and stage. In particular, histiocytomas and malignant neoplasms showed 3–4 times higher MMP-2 and MMP-9 intensity bands than other groups. However, the difference in expression of matrix metalloproteinases with or without metastases and correlation with the stage of the disease was not determined.

Ferreira et al. (2021) claim an established difference in MMP-9 activity depending on the histomorphological type of mammary gland neoplasms in bitches. Increased immunoeexpression of MMP-9 was determined in epithelial and stromal cells in tubulopapillary and complex carcinomas.

The key role of MMP-9 in disease progression and neoplastic cells dissemination is proved by the information published by Hallett et al. (2013): specific suppression of matrix metalloproteinase-9 expression by anti-matrix metalloproteinase-9 DNzyme (AM9D) significantly ($P < 0.05$) leads to a decrease in the growth rate and size of the neoplasm, inhibition of angiogenesis and increased apoptosis.

The importance of matrix metalloproteinase-2 in tumour invasion is proved by a credible ($P < 0.01$) decrease in its activity after mastectomy and postoperative chemotherapy (Divya et al., 2019).

In malignant types of mammary tumours, MMP-9 overexpression leads to greater release of transforming growth factor beta (TGF- β) from its latent form with subsequent activation of TGF- β /SMAD signaling and increased aggressiveness (Dong et al., 2019).

Thus, high activity levels of matrix metalloproteinases-2 & -9 reflect the invasiveness of malignant neoplasms, compared to benign ones, against the background of the lack of correlation with the histological type and the degree of dissemination of tumour cells.

Conclusions

Activation of matrix metalloproteinases-2 & -9 occurs in all female dogs during malignant transformation of functional mammary tissue (mixed-type carcinoma, ductal, tubular, and tubulopapillary ones) and in 10% of benign changes (adenoma, mixed tumours). For malignant neoplasms, high levels of MMP-2 are registered in 58.1–63.5%, MMP-9 – in 36.5–41.9%, both metalloproteinases – in 32.9–35.3% of patients, benign – in 6.3–8.9%, 3.8–5.1% and 3.8–5.1% of dogs respectively.

Compared with the parameters of clinically healthy animals, the vascular and tissue level of matrix metalloproteinase-2 and -9 latent and active forms increased credibly ($P < 0.001$) only during the malignant course of the disease. Neoplasm histological type and metastatic foci presence had no influence on their activity.

The activity of MMP-2 and MMP-9 should be used as an important prognostic factor to assess the risk of mammary neoplasm progression in female dogs and to control the effectiveness of their treatment. Blood le-

vels of MMP-2 and MMP-9 are important predictors in the preoperative period, when clinical signs of malignancy (ulcers, fistulas, severe pain reaction, edema, etc.) are absent.

The presented research is part of the scientific topic of the Department of Veterinary Surgery and Reproduction of the Dnipro State Agrarian and Economic University "Clinical correction of ecological destruction of anthropogenic origin in domestic animals of the Dnipro industrial region" (state registration number 0115U002143).

The authors consider that there is no conflict of interest.

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