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# VEGF and MMP-9: biomarkers for canine lymphoma

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## Abstract

Vascular endothelial growth factor (VEGF) and metalloproteinase (MMP) 2 and 9 are useful biomarkers in human lymphoma. During cancerogenesis, transforming growth factor beta (TGF- $\beta$ ) stimulates VEGF and MMPs production. VEGF and TGF- $\beta$  plasma levels were tested by ELISA, MMP-2 and MMP-9 by gelatine zymography in 37 dogs with lymphoma, 13 of which were also monitored during chemotherapy. Ten healthy dogs served as control. Lymphoma dogs showed higher act-MMP-9 ( $P < 0.01$ ) and VEGF ( $P < 0.05$ ), and lower TGF- $\beta$  than controls, and a positive correlation between act-MMP-9 and VEGF ( $P < 0.001$ ). Act-MMP-9 and VEGF were significantly higher in T-cell lymphomas, and in stage V compared with stages III–IV disease, regardless of immunophenotype. VEGF was higher in high-grade compared with low-grade T-cell lymphomas. No correlation was found between cytokines levels at presentation and outcome. During chemotherapy, act-MMP-9 and VEGF decreased in B-cell lymphomas ( $P < 0.01$ ), suggesting a possible predictive role in this group of dogs.

## Introduction

In human and veterinary oncology, one area of major promise is the identification of molecular markers, which may predict response to chemotherapy and tumour relapse, thereby offering predictive information. It has been reported that canine non-Hodgkin's lymphoma (cNHL) shows overlapping features with human non-Hodgkin's lymphoma (hNHL), allowing for the formulation of a comparative classification system and for the consideration of the dog as a possible spontaneous model for this tumor. [1, 2] Cytokines play an important role in the pathogenesis of hNHL, and elevated plasma or tissue cytokine levels contribute to its progression. [3, 4] Cytokines exert their effects on neoplastic and reactive cells, providing growth advantages for tumour cells in either an autocrine or a paracrine fashion. [5] Angiogenesis plays a critical role in the initial development of cancer as well as in the metastatic spread. Vascular endothelial growth factor (VEGF) is one of the most potent and specific promoters of angiogenesis, and it acts by stimulating endothelial cells to form new vessels. [6] Previous reports have demonstrated that the expression of VEGF and VEGF receptor-2 promotes a signalling system that is centrally involved in tumour angiogenesis. [7]

Transforming growth factor beta (TGF- $\beta$ ) is a naturally occurring potent inhibitor of cell growth. [8] In early cancer progression, it acts as tumour suppressor; however, at later stages it becomes a tumour promoter. [9, 10] In fact, the increased production of TGF- $\beta$  by tumour cells contributes to cancer angiogenesis by inducing the expression of VEGF. [11] A further potent capability of TGF- $\beta$

is to suppress the immune system by hampering tumour identification and cytolysis. [12] TGF- $\beta$ 1 also upregulates matrix metalloproteinase (MMP)-2, which acts as an autocrine mediator of tumour cell invasion, and various proteinases that act in degradation. [13] Increased MMPs expression renders tumour cells capable of digesting essential tissue barriers, especially basement membranes that line blood vessels, thereby promoting cell motility. [14] MMP-2 (gelatinase A) and -9 (gelatinase B) are also able to regulate angiogenesis directly. [15]

Previous studies have shown that angiogenesis is increased in canine lymphoma, by highlighting the higher micro-vessel density in neoplastic lymph nodes compared with the normal lymph nodes [16]; however, the neovascularization was not correlated with VEGF immunoreactivity or overall survival time. Conversely, Gentilini *et al.* [17] measured circulating VEGF, MMP-9 and MMP-2 in cNHL and found a longer disease-free interval in dogs with a low VEGF plasma level at admission; however, no differences in VEGF levels were noticed before and after treatment in eight dogs obtaining complete remission. When the included dogs were grouped according to some of the known prognostic factors, a significantly higher VEGF level was found in symptomatic dogs (substage b) compared with asymptomatic dogs (substage a). However, no attempt was made to evaluate the relationship between the level of angiogenic factors in cNHL and immunophenotype, cytological subtypes or grading. [18] Moreover, activation of MMP-9 and MMP-2 was not evaluated.

By hypothesizing a relationship among cytokines and gelatinases, the aim of this work was the measurement of plasmatic pro-MMP-2, pro-MMP-9 and their activated forms, VEGF and TGF- $\beta$  by gelatine zymography and an enzyme-linked *immunosorbent* assay, respectively, in a cohort of cNHL subdivided based on cytological classification and immunophenotype. Additionally, in a smaller group of dogs undergoing treatment, we correlated biomarkers levels at presentation to clinical stage (to evaluate whether higher levels predicted a more advanced clinical stage), to immunophenotype (B versus T) and to remission status at the end of chemotherapy (to evaluate whether variation in the angiogenic pathway may be related to remission status).

## Materials and methods

### Patients and samples

Plasma was obtained from dogs with lymphoma at various clinical stages, and from breed-, age- and gender-matched healthy control dogs presenting for periodical examination. An informed consent was obtained from all owners according to the regulations of each institutional animal care committee. Dogs underwent complete staging work-up, including physical examination, complete blood cell count, flow cytometric and cytological analysis of nodal fine-needle aspirate, peripheral blood and bone marrow aspirate, thoracic radiography and abdominal ultrasound. Briefly, flow cytometric immunophenotype was determined as previously reported on fine needle aspiration of lymph nodes, peripheral blood and bone marrow samples. [19] The following monoclonal antibodies were used: CD45-PEb (clone YKIX716.13, Serotec, Oxford, UK), CD3-FITC (clone CA17.2A12, Serotec, T cells), CD4-FITC (clone YKIX302.9, Serotec, T-helper and neutrophils), CD8-PE (clone YCATE55.9, Serotec, T-cytotoxic/suppressor), CD5 (clone YKIX322.3, T-cell), CD21-PE (clone CA21D6 Serotec, mature B cells), CD34-PE (clone 1H6, Pharmingen, Becton Dickinson, San Jose, CA, USA, precursor cells), and CD79a (B-cells, clone HM57, Dako, Atlanta, GA, USA). Acquisition was performed with FACSCalibur (Becton Dickinson) and analysis was conducted by using a commercially available software (Cell Quest, Becton Dickinson). Lymphoma subtypes were classified based on the Kiel-updated cytological classification. [18] Plasma was processed by the Department of Veterinary Pathology, Hygiene and Health, University of Milan, and by the Department of Animal Pathology, University of Turin. At presentation, peripheral blood

was sampled in sterile EDTA tubes, and the plasma obtained by centrifugation was put in separated polypropylene tubes and stored at  $-20^{\circ}\text{C}$ . Each sample was centrifuged for 30 min at 1000 rpm before assaying. Owners of dogs with lymphoma were offered to treat their animals with multidrug chemotherapy, consisting of doxorubicin, vincristine, cyclophosphamide, l-asparaginase and prednisone. [20] In these dogs, plasma was collected at three standard times (at diagnosis, halfway through the treatment and at the end of chemotherapy). The remission status of the treated dogs was recorded at each recheck examination.

## **MMPs analysis using gelatine zymography**

MMP-2 and MMP-9 activity was studied by zymography, a technique revealing the gelatinase activity of latent pro-enzymes (zymogens) and mature MMPs. A 1:10 dilution was made from 10  $\mu\text{L}$  of plasma into sample buffer, and 60  $\mu\text{L}$  of the diluted sample was subjected to electrophoresis on an 8% SDS-PAGE gel co-polymerized with 0.1% gelatine. The protein concentration in the sample was adjusted to 1 mg/mL. Following electrophoresis, the gel was incubated for 1 h at room temperature in a 2.5% Triton X-100 solution and then at  $37^{\circ}\text{C}$  for 16 h in Tris-HCl buffer, pH 7.4, containing 10 mM  $\text{CaCl}_2$ . The gels were stained with 0.1% Coomassie Brilliant Blue R-250 and then de-stained with 30% methanol and 10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatine. Culture medium conditioned with A2058 melanoma cells was used as control to identify the pro-MMP-9 gelatinolytic band, while conditioned media from HT1080 fibrosarcoma cells was used to identify the active forms of MMP-2 and MMP-9 and small amounts of pro-MMP-2, as already described. [21, 22] The amount of MMP-9 in 20  $\mu\text{L}$  of the A2058 melanoma cell-conditioned media was defined as 100 arbitrary units (a.u.). The activity from 20  $\mu\text{L}$  of HT1080 fibrosarcoma cell-conditioned media was defined as 100 a.u. for MMP-2. The bands were quantified considering both the intensity and the extension area, using an image analyser system with GelDoc 2000 and Quantity One software (BioRad, Hercules, CA, USA).

## **Measurements of cytokine levels**

Circulating VEGF and TGF- $\beta$  levels were quantitatively analysed by an enzyme-linked immunosorbent assay (ELISA), using specific canine commercial kits (R&D Systems, Minneapolis, MN, USA) following manufacture instruction. Each sample was tested in duplicate. Spectrophotometer readings at 450 nm (wavelength correction set to 570 nm) were performed using a Thermo Labsystems Multiskan Ascent Photometric plate reader (American Instrument Exchange, Haverhill, MA, USA). The lower limits of detection for VEGF and TGF- $\beta$ 1 are less than 19.5 and 4.61 pg/mL, respectively. Samples were prepared with HCl/NaOH/HEPES-activation solution, as recommended by the manufacturer (R&D Systems), to activate latent TGF- $\beta$ 1 into the immunoreactive form, the only one detectable by the kit.

## **Statistical analysis**

The data were expressed as the mean  $\pm$  SEM. Comparison among cytokines and MMPs levels in dogs with different lymphoma subtypes and healthy controls was calculated by Student's *t*-test or analysis of variance (ANOVA). Differences in frequencies among all the cytokines were determined by  $\chi^2$  analysis. Standard regression analysis, using Pearson and Spearman correlation coefficients, were used to determine the relationships between MMP, VEGF and TGF- $\beta$ 1 values. All tests were performed using NCSS 2000 software (Kaysville, UT, USA). Statistical significance was set at  $P < 0.05$ .

## Results

Thirty-seven dogs with lymphoma and 10 healthy controls were evaluated. Among the lymphoma cases, 21 were of B-cell immunophenotype (19 high-grade, 1 low-grade and in 1 case no lymph node glass smear was available) and 16 of T-cell immunophenotype (9 high-grade and 7 low-grade). The main B-cell subtype was centroblastic polymorphic (17 of 21), whereas in T-cell lymphoma (including high- and low-grade) no predominant cytological subtype was detected.

Thirty-four dogs underwent complete staging work-up: 20 lymphomas were of stages III–IV, whereas 14 of stage V. Twenty-nine of the 37 dogs were treated and received the same CHOP-based chemotherapeutic protocol. [19] At the end of the 12 weeks of treatment, 12 (41.4%) dogs were in complete remission, 3 (10.3%) obtained partial remission, 3 (10.3%) dogs had stable disease and 11 (38.0%) dogs experienced progressive disease. When considering immunophenotype and outcome, among the 18 treated dogs with B-cell lymphoma, 9 obtained complete remission, 2 partial remission, 2 stable disease and 5 experienced progressive disease. Among the 11 treated dogs with T-cell lymphoma, three obtained complete remission, one partial remission, one stable disease and six experienced progressive disease.

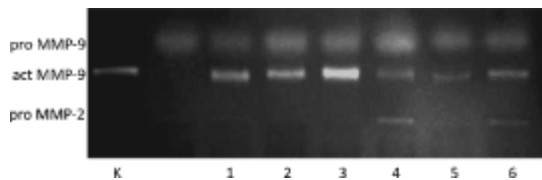
### Correlation between MMPs and cytokines profiles at admission and disease status, clinical stage and immunophenotype

The quantification of gelatinases through gel zymography showed similar values of pro-MMP-9 in tumour and control dogs (B-cell lymphomas =  $93.1 \pm 6.4$  a.u.; T-cell lymphomas =  $97.9 \pm 9.4$  a.u. and control dogs =  $92.3 \pm 2.5$  a.u.). However, dogs with lymphoma showed a significantly higher catalytic activity of MMP-9 ( $P < 0.01$ ) compared with healthy controls ( $91.7 \pm 4.8$  a.u. versus  $12.4 \pm 2.1$  a.u., respectively; Table 1). When considering the cytokine levels and clinical stage, statistically significant higher expression of act-MMP-9 was found in stage V B-cell lymphomas ( $95.3 \pm 3.4$  a.u.) than in stage III/IV B-cell lymphomas ( $83.4 \pm 4.7$  a.u.;  $P < 0.01$ ). Higher expression of act-MMP-9 was found in stage V T-cell lymphomas ( $113.7 \pm 3.2$  a.u.) than in stage III/IV T-cell lymphomas ( $94.7 \pm 2.1$  a.u.;  $P < 0.05$ ).

Table 1. Baseline values of MMP-9 (pro and act), VEGF and TGF- $\beta$  in controls, B-cell lymphomas and T-cell lymphomas (expressed as the mean  $\pm$  SEM)

	Controls	Lymphoma B	Lymphoma T
1. * $P < 0.05$ . ** $P < 0.01$ versus controls.			
Pro-MMP9 (a.u.)	$92.3 \pm 2.5$	$93.1 \pm 6.4$	$97.9 \pm 9.4$
Act-MMP9 (a.u.)	$12.4 \pm 2.1$	$89.2 \pm 8.8^{**}$	$103.3 \pm 2.9^{**}$
VEGF (pg/mL)	$40.1 \pm 10.4$	$55.4 \pm 21.4^*$	$67.4 \pm 19.1^*$
TGF- $\beta$ (pg/mL)	$19185.5 \pm 7843.4$	$11377.6 \pm 3412.7$	$10343.4 \pm 5430.9$

When examining the immunophenotype, T-cell lymphomas presented a higher concentration of act-MMP-9 at the time of diagnosis ( $103.3 \pm 2.9$  a.u.) than B-cell lymphomas ( $89.2 \pm 8.8$  a.u.), and this was statistically significant ( $P < 0.05$ ). Neither lymphoma nor control dogs showed expression of act-MMP-2. Plasma levels of pro-MMP-2 were not significantly increased in lymphoma dogs ( $104.3 \pm 10.2$  a.u.) with respect to control dogs ( $98.2 \pm 3.9$  a.u.; Fig. 1; Table 1).



**Figure 1.**

Representative MMP zymography from seven samples. Lanes 1, 2 and 3 are T-cell lymphomas. Lanes 4, 5 and 6 are B-cell lymphomas.

Plasma VEGF levels in dogs with lymphoma were higher than that in controls ( $59.3 \pm 14.3$  pg/mL versus  $40.1 \pm 10.4$  pg/mL, respectively;  $P < 0.05$ ), and this was statistically significant ( $P < 0.05$ ; Table 1). Also, VEGF plasmatic levels were significantly higher ( $P < 0.01$ ) in stage V B-cell lymphomas ( $58.3 \pm 4.5$  pg/mL) than in stage III/IV B-cell lymphomas ( $49.1 \pm 2.1$  pg/mL), and in stage V T-cell lymphomas ( $78.1 \pm 3.7$  pg/mL) than in stage III/IV T-cell lymphomas ( $63.1 \pm 4.5$  pg/mL).

In dogs with B-cell lymphoma, the mean VEGF value at diagnosis was lower when compared with dogs with T-cell lymphoma ( $55.4 \pm 21.3$  pg/mL versus  $67.4 \pm 19.1$  pg/mL, respectively). A positive correlation between concentrations of act-MMP-9 and VEGF plasma levels was found in all lymphoma dogs ( $r = 0.78$ ,  $P < 0.001$ ).

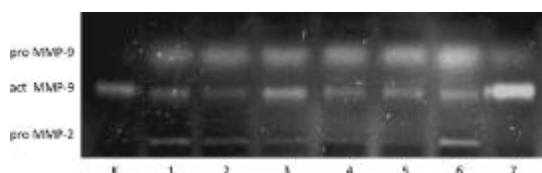
Concerning plasma TGF- $\beta$  levels, the median values were similar between B- and T-cell lymphoma ( $11377.6$  pg/mL and  $10343.4$  pg/mL, respectively). Although not statistically significant, the mean plasma TGF- $\beta$  value in control healthy dogs ( $19185.5$  pg/mL) was higher when compared with lymphoma dogs (Table 1). At the time of diagnosis, VEGF levels were significantly lower in low-grade ( $59.7 \pm 3.3$ ) compared with high-grade ( $76.4 \pm 4.8$ ) T-cell lymphomas ( $P < 0.05$ ). No such differences were observed for MMP-2, MMP-9 and TGF- $\beta$ .

### **Correlation between MMPs analysis and cytokines profiles at admission and remission status at the end of chemotherapy**

Plasma was obtained at admission in 29 dogs undergoing the same chemotherapeutic protocol. MMP and cytokines profiles did not differ between dogs obtaining remission (complete and partial) or experiencing progressive disease, thereby not being useful in predicting treatment response.

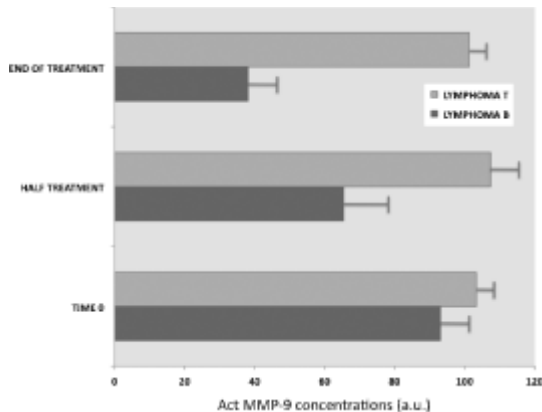
### **MMPs analysis and cytokines profiles during chemotherapy and follow-up**

Plasma was serially obtained from 13 dogs (10 B-cell and 3 T-cell lymphomas) undergoing chemotherapy. In these dogs, pro-MMP-9, pro-MMP-2 and act-MMP-2 levels did not change at the three standard times. However, act-MMP-9 was significantly decreased in all B-cell lymphoma dogs at the end of chemotherapy ( $38.1 \pm 12.3$  a.u.;  $P < 0.01$ ; Fig. 3). In contrast, no evident modifications of the quantitative activity of act-MMP-9 were observed in the T-cell lymphoma dogs ( $101.3 \pm 10.0$  a.u.; Figs 2 and 3).



## Figure 2.

Representative MMP zymography from three dogs during chemotherapy. Lanes 1, 2 and 3: dog 6, B-cell lymphoma, respectively, at time 2, time 1 and time 0. Lanes 4, 5 and 6: dog 17, B-cell lymphoma, respectively, at time 2, time 1 and time 0 in. Lane 7: T-cell lymphoma in dog 35.



## Figure 3.

Plasma concentrations of act-MMP-9 (mean values) in B- and T-cell lymphomas at the three standard times during the chemotherapy protocol.

In dogs undergoing chemotherapy, 8 of 9 cases of B-cell lymphoma showed decreased VEGF values at the end of treatment in the order of 19.47 pg/mL. The mean value of the reduction, excluding one case of anaplastic B-cell lymphoma that presented a rise of VEGF levels, was 20.7 pg/mL ( $P < 0.05$ ). In the three dogs with T-cell lymphoma undergoing treatment, the mean value of the rise of plasma VEGF was 15.6 pg/mL at the end of treatment.

## Discussion

Cytokines act as important key regulators of the tumour microenvironment, by influencing survival and proliferation of neoplastic and vascular cells. In people with NHL, angiogenic factors are emerging as a powerful prognostic tool. [3, 4, 23] To our knowledge, few studies are available in veterinary oncology on the role of VEGF and MMPs [17, 22, 24-26] and the clinical impact of early and serial monitoring of these cytokines has not been extensively studied.

Here, we identified possible plasma biomarkers having predictive relevance in canine lymphoma. Specifically, our results show that VEGF and act-MMP-9 levels were significantly higher (1) in dogs with lymphoma when compared with controls, (2) in T-cell lymphomas compared with B-cell lymphoma at admission and that (3) VEGF was higher in high-grade compared with low grade T-cell lymphoma.

MMP-9 and VEGF are two of the most potent factors involved in angiogenesis. It is well known that, in addition to its role in proteolytic degradation, MMP-9 can also release angiogenic factors that bind to ECM, such as VEGF. MMP-9 is also a functional component of the angiogenic switch during multistage carcinogenesis, as it increases the availability of angiogenesis inducers. [15, 27]

In this study, we found a correlation between the levels of VEGF and act-MMP-9 and lymphoma immunophenotype, the explanation of which could rely on the more aggressive biological behaviour and rapid spread that characterize T-cell lymphoma. Indeed, it has been documented that



canine T-cell lymphoma usually harbours a poor prognosis [28]: in the early stage, high-grade nodal lymphoma does not destroy tissue boundaries, thereby not showing an invasive growth pattern. However, as the tumour progresses, it may become locally invasive and tends to disseminate, as partially shown by our predictive correlations between plasma levels of VEGF and MMP-9. However, it is a matter of fact that canine lymphoma encompasses a wide range of distinct entities showing different biologic behaviour; as a consequence, not all T-cell lymphomas carry the same prognosis. [18]

For this reason we grouped our cases based on their morphological aspect as well, and, interestingly, we found that the low-grade T-cell lymphomas had a significant lower level of VEGF, being in accordance with results obtained in human medicine. [29] On the basis of these findings it may be hypothesized that a different angiogenic pathway occurs in low- and high-grade T-cell lymphomas. Because B-cell lymphomas were mainly of high-grade in our series, it was not possible to discriminate the VEGF data. In hNHL, VEGF and MMP-9 expressions correlate with: (1) subtype, (2) grade, (3) clinical course and (4) survival. [29, 30] Patients with elevated VEGF and MMP-9 levels have a higher likelihood of recurrence or death than patients with low-angiogenic NHL.

We also analysed the role of VEGF and act-MMP9 in different stages of lymphoma (III–IV versus V). Their levels were found to be different between lower and higher stages, thereby suggesting a possible role of cytokine levels to differentiate between advanced and early cancer.

According to our results, cytokines levels at presentation did not correlate to remission status obtained at the end of chemotherapy. The reason behind this finding may reside in the low number of treated cases or in distinct characteristics of the dog groups studied. It may be possible that group stratification according to clinical stage and immunophenotype will eventually lead to outcome correlation. Intriguingly, by serially determining cytokines level during and after chemotherapy, we recognized plasma VEGF and MMP-9 as a dynamic follow-up parameter in B-cell lymphoma.

Indeed, both biomarkers decreased significantly from admission to the end of treatment in these dogs, thereby being in agreement with several human studies indicating that VEGF and MMP-9 might predict treatment response. [3, 4, 30] At midterm plasma check, the biomarkers levels were not significantly different when compared with the levels obtained at admission (data not shown). As a consequence, it may be assumed that the most relevant changes in the production of these markers are reflected at the end of treatment, being attributable to the persistence of high circulating levels of VEGF and MMP-9 during chemotherapy. Conversely, in T-cell lymphomas, VEGF levels increased at the end of the treatment, whereas MMP-9 showed no changes. More data are needed to evaluate the plasma kinetics of these two biomarkers in dogs with lymphoma to predict a possible correlation between their synthesis and the responses to chemotherapy.

On the other hand, the role of MMP-2 seems to be irrelevant in the lymphoma microenvironment. Pro-MMP-2 levels in dogs with lymphoma were not significantly increased, regardless of the immunophenotype.

TGF- $\beta$  showed lower plasma levels in lymphoma dogs compared with controls; however, there was a high individual variability at diagnosis. Furthermore, we found no correlation between plasma TGF- $\beta$  levels and lymphoma immunophenotype, and there was no correlation at the three standard treatment times. It may be hypothesized that a reduction of TGF- $\beta$  reflects the ability of the tumour cells to acquire resistance to the anti-proliferative signals of TGF- $\beta$ . In NHL and other haematological malignancies, the aberrant expressions of receptors (types I, II and III) and mutations in TGF- $\beta$  signalling cascade have been described, demonstrating that cancer cells

frequently acquire resistance to the anti-proliferative signals of TGF- $\beta$ . [31] The same may hold true for dogs with lymphoma; however, additional studies with larger patient numbers are warranted to verify these observations.

In conclusion, VEGF and act-MMP-9 levels were significantly higher in dogs with lymphoma compared with healthy controls. Furthermore, VEGF and act-MMP-9 levels were higher in T-cell lymphomas and in dogs with a more advanced disease, thereby providing a new tool that might help oncologists in predicting outcome. Future studies need to be conducted to highlight the potential role of anti-angiogenic agents in the treatment of different subtypes of canine lymphoma.

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