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Tumor microenvironment in diffuse large B-cell lymphoma: Matrixmetalloproteinases activation is mediated by osteopontin overexpression

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ABSTRACT

Non-Hodgkin lymphomas (NHL) are a heterogeneous group of lymphoproliferative malignancies with variable patterns of behavior and responses to therapy. NHL development and invasion depend on multiple interactions between tumor cells and non-neoplastic cells. Such interactions are usually modulated by several cytokines. Accordingly, it was demonstrated that matrix-metalloproteinase (MMP)-2 and MMP-9 were activated in human lymphoid cell lines by interleukin-6 (IL-6). The activation of these enzymes is associated with tumor invasion and metastasis in human cancers. MMPs are also activated in several cancers by osteopontin (OPN), a secreted glycoprotein that regulates cell adhesion, migration, and survival. However, it is still unclear if MMPs play a role in NHL development and if their activation is determined by OPN and/or IL-6. In the present study, two groups of 78 NHL patients and 95 healthy donors were recruited for the analysis of OPN, MMP-2, MMP-9 and IL-6.

Significant higher circulating levels of MMP-2, MMP-9, OPN and IL-6 were observed in NHL patients when compared to healthy donors. Similar data were obtained by analyzing the activity of both MMP-2 and MMP-9. The multivariate regression model indicates that, in both NHL cases and healthy donors, OPN is associated with the increase of MMP-2 and MMP-9 levels independently of IL-6. These data were first confirmed by "in silico" analyses and then by "in vitro" experiments conducted on peripheral blood mononuclear cells randomly selected from both NHL patients and healthy donors.

Overall, our data suggest that the activation of MMPs in NHL development is mostly associated with OPN. However, IL-6 may play an important role in the lymphomagenesis through the activation of other molecular pathways.

1. Introduction

Non-Hodgkin lymphomas (NHL) are a heterogeneous group of lymphoproliferative malignancies with variable patterns of behavior and responses to treatment. Although at present a significant number of high grade lymphoma patients can be cured with intensive regimens of cytotoxic and immunotherapeutic drugs, still these patients may relapse or become resistant to treatment [1–5].

Diffuse large B cell lymphoma (DLBCL) is the most common form of lymphoma and accounts for 30–40% of all lymphomas in adults. Clinical outcome is extremely various, with 5 year survival rates between 30% and 80% related to the biological heterogeneity of this B-cell neoplasia.

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In fact, several genetic abnormalities play a role in DLBCL pathogenesis and progression.

NHL development and invasion depend on multiple interactions between tumor cells and non-neoplastic cells and on their interaction with the surrounding stroma/matrix environment [6]. The tumor microenvironment is a dynamic system, largely orchestrated by inflammatory cells, that includes cancer cells, stromal tissue (immune cells, fibroblasts, cytokines, and vascular tissue), as well as the surrounding extracellular matrix [6]. Perturbations of this network may increase the potential for chronic B-cell division and, ultimately, lymphomagenesis. Such perturbations may be modulated by several cytokines.

Accumulating evidence indicates that osteopontin (OPN), a matricellular protein found in the tumor microenvironment and expressed by host and cancer cells, may regulate tumorigenesis, cancer progression, and metastasis [7,8]. Several studies have showed that OPN levels are up-regulated in a variety of cancers [9–14], including HCV-associated B-cell lymphomas [15]. The role of OPN in cancer development may be related to its ability to facilitate extracellular matrix invasion, cell adhesion and migration [8]. Proteolysis and remodeling of the extracellular matrix (ECM) represent one of several initiating events that allow cancer cells to invade into the surrounding stroma [7,16,17]. Accordingly, OPN regulates the activity of matrix metalloproteinases (MMPs) which are a family of zinc-dependent proteinases whose enzymatic activity is directed against components of the ECM [8,18,19].

MMPs, especially MMP-2 and MMP-9 play important role in OPN-induced tumor invasion and in metastatic process in human cancer cells [8,20–22]. Numerous studies have suggested that over expression of MMP-2 and MMP-9 are associated with an aggressive phenotype [23–29]. It was also appreciated their role in the dissemination of hematologic malignancies, such as human NHL and acute myelogenous leukemia (AML) [30–34]. In addition to OPN, the expression and activation of MMP-2 and MMP-9 can be influenced by different cytokines, such as interleukin-6 (IL-6) [35,36]. IL-6 is a multifunctional cytokine playing an important role in the tumor microenvironment. It is a potent lymphoid growth and differentiation factor, produced by various types of cells, including reactive and neoplastic B- and T-lymphocytes, monocytes/macrophages, fibroblasts, and hepatocytes [37,38].

IL-6 and its related signaling pathways have been identified to contribute to proliferation, migration and invasion of tumor cells [39–44]. Intriguingly, IL-6 levels are associated with a poor prognosis in diffuse large cell NHL [45,46]. Proinflammatory cytokines, detected in the tumor microenvironment, can actively participate in ECM destruction by up regulation of MMPs, such as MMP-2 and MMP-9 [47,48]. It was demonstrated that in human lymphoid cell lines IL-6 activates MMP-2 and MMP-9 [35].

Since OPN was also detected in several lymphoproliferations, it remains unclear if the activation of MMPs is determined by OPN itself and/or by IL-6. On this ground, such activation was assessed by both experimental and bioinformatics approaches.

2. Materials and methods

Biological samples were collected from 78 B-cell NHL patients (49 male and 29 female; median age 56 years) and from 95 healthy donors as control (61 male and 34 female; median age, 51 years old).

All samples were recruited for the purpose of this study at the Department of Biomedical and Biotechnological Sciences, University of Catania (Catania, Italy), in a time range of 5 years, and stored at −80 °C until analysis.

All B-cell NHL cases were classified as diffuse B-cell large lymphomas according to the World Health Organization (WHO) classification [5] and enrolled at the time of the first diagnosis and prior of any medical treatment.

All subjects gave written informed consent for the study, and local ethical committee approval was obtained. NHL patients infected with human immunodeficiency virus (HIV) or hepatitis C virus (HCV) were excluded from the study. Patients with previous diagnosis of cancer and/or with any degenerative chronic diseases were also excluded.

2.1. Laboratory assays

Frozen 1-ml aliquots of plasma which had never been thawed were packed in dry ice and sent to the laboratory for the analyses. Laboratory personnel were blinded to the case/control status of the plasma samples. Samples from a case controls were always assayed on the same plate. Ten percent blinded duplicate aliquots were included to assess intra-batch and inter-batch variability.

OPN, IL-6, MMP-2 and MMP-9 plasma concentrations were measured by enzyme-linked immunosorbent (ELISA) assay (R&D Systems, Europe). The MMP-2 and MMP-9 assays recognized both pro and active forms.

Plasma samples were diluted and the immunoassay was done according to the manufacturer instructions. Marker concentrations for each sample were calculated from the standard curve. All tests were assayed in duplicate. A subset of samples was assayed four times in every ELISA plate for quality control. No significant cross-reactivity to or interference with various proteins was observed. The optical density was measured at 450 nm using a microplate reader (Thermo-Lab Systems).

2.2. Measurement of MMP-2 and MMP-9 activity

Circulating levels of MMP-2 and MMP-9 (both pro-active and active form) were measured by specific Biotrak assay kits (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. The appropriate standards were added in each assay. The activation of the pro-form of MMPs was performed using p-aminophenylmercuric acetate (APMA).

2.3. Cell cultures and treatments

Peripheral blood mononuclear cells (PBMCs) were obtained using a Ficoll gradient from a randomly selected groups of 10 out of 95 healthy controls and 20 out of 78 NHL patients. After isolation, PBMC pellets were collected and stored immediately at −80 °C until analysis. PBMCs (1×10^6 cells/mL) were cultured in RPMI 1640 medium supplemented with 1% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco Life Technologies Inc.). MMP-2, MMP-9, OPN and IL-6 was analyzed, by ELISA kit (R&D System), in supernatants of PBMCs from NHL patients and controls, respectively. Cell viability was assessed by exclusion of 0.05% Trypan Blue and was always above 95%.

PBMCs were incubated for 24 h in the presence or absence of recombinant human (rh)-OPN (1 μg/mL, R&D Systems, Abingdon, UK), and rh-IL-6 (100 ng/mL, R&D Systems), used both alone or in combination. In parallel, the anti-OPN monoclonal antibody (mAb) and the anti-IL-6 mAb at the final concentration of 10 μg/mL (R&D Systems) were used. The supernatants were, then, collected for the ELISA assay.

2.4. Gene expression data sets

Two publicly available gene expression data sets of lymphoma samples were analyzed. For the Hummel data sets [49] the samples were hybridized on Affymetrix U133A GeneChips. This dataset consists of 221 B-cell lymphomas. Probe level normalization was done using the calibration and variance stabilization method. Probe-set summarization was performed using the median polish method on the normalized data.

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Rosenwald dataset [50] included 240 patients with untreated diffuse large-B-cell lymphoma who had no previous history of lymphoma, according to a protocol approved by the National Cancer Institute institutional review board. Lymphochip DNA microarrays were composed of genes whose products are preferentially expressed in lymphoid cells and genes thought or confirmed to play a part in cancer or immune function. These microarrays were constructed from 12,196 clones of complementary DNA and were used to quantitate the expression of messenger RNA in the tumors.

Expression measurements of OPN, IL-6, MMP-2 and MMP-9 were extracted from each data set through their respective National Center for Biotechnology Information gene identifier. When more than one cDNA clone or probe set matched a given gene, all possible pairs were considered.

2.5. Statistics

Differences in MMP-2, MMP-9, OPN, and IL-6 between cases and controls were tested using the non-parametric Wilcoxon test. Correlations were evaluated through Spearman correlation coefficient (r). To evaluate the independent contribution of OPN and IL-6 to MMP-2 and MMP-9 levels, a multivariate regression model was estimated, including both OPN and IL-6 as predictors of MMP level. Log (IL-6) was considered in the regression model to account for its non-normal distribution. Concentrations of MMP-2/MMP-9 in PBMC were compared by means of paired t-test.

3. Results

3.1. Circulating plasma levels of MMP-2, MMP-9, OPN, IL-6 and MMPs activity

Fig. 1 shows the distribution of circulating plasma levels of OPN, IL-6, MMP-2, MMP-9, and MMPs activity, analyzed in NHL patients and healthy controls. Higher plasma levels of MMP-2, MMP-9, OPN and IL-6 were observed in NHL patients compared to controls ($p < 0.01$). Similar trend was observed analyzing the activity of MMP-2 and MMP-9 since their mean levels were significant higher in NHL group compared to controls ($p < 0.001$ — Fig. 1).

Positive correlations of MMP-2 and MMP-9 versus OPN and IL-6 were observed in NHL patients ($r > 0.60$) but not in healthy donors (Fig. 2). Similarly, positive correlation was observed for MMP-2 and MMP-9 activity versus OPN and IL-6 ($r > 0.65$ — Fig. 2).

The independent contribution of OPN and IL-6 to MMP-2 and MMP-9 levels was evaluated through a multivariate regression (Table 1). In both NHL cases and healthy donors, OPN increases the MMP-2 and MMP-9 levels ($\beta > 0$), independently from IL-6. Conversely, the effect of IL-6 on MMP-2 and MMP-9 levels was no longer significant when OPN was included in the regression model.

3.2. Computational analysis of OPN, MMPs and IL-6 in two datasets

Two publicly available gene expression data sets of lymphoma samples were considered. The Hummel data sets comprised 221 B-

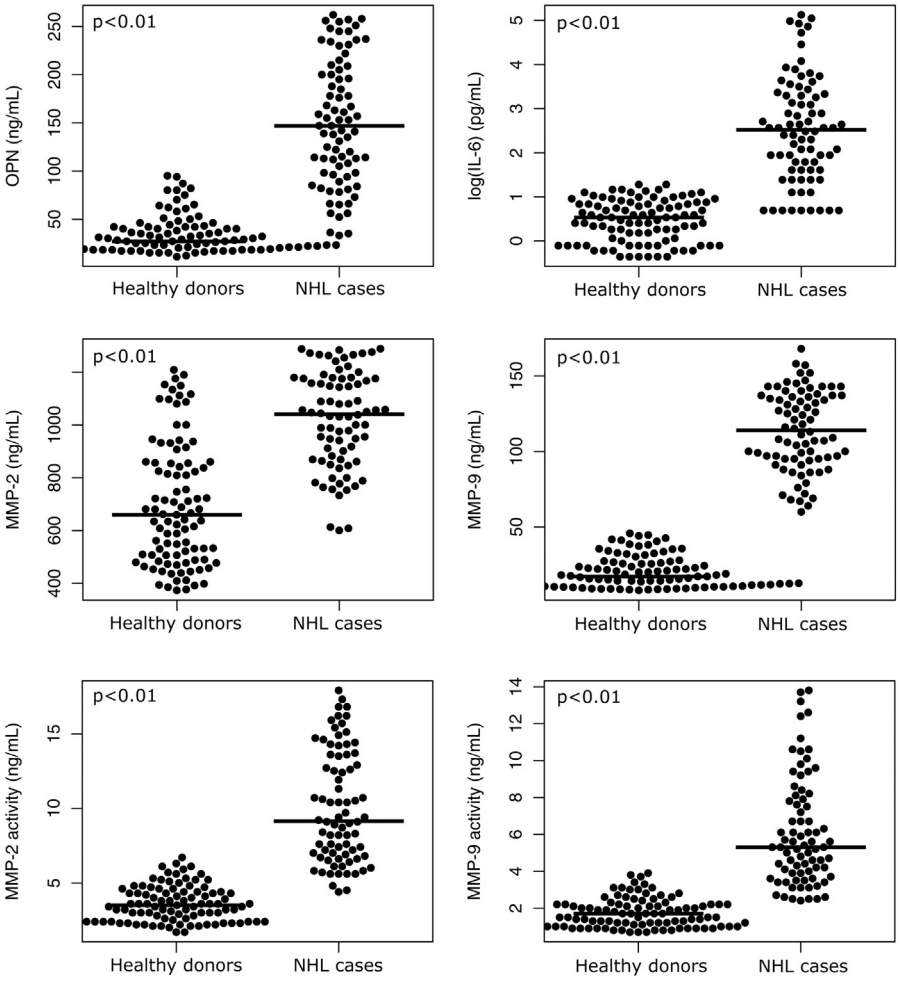

Fig. 1. Distribution of OPN, log(IL-6), MMP-2, MMP-9 and MMPs activity in healthy donors and NHL cases. Differences between healthy donors and NHL cases were evaluated through Wilcoxon test. OPN, osteopontin; IL-6, interleukin-6, MMP, matrixmetalloproteinase; and NHL, non-Hodgkin lymphoma.

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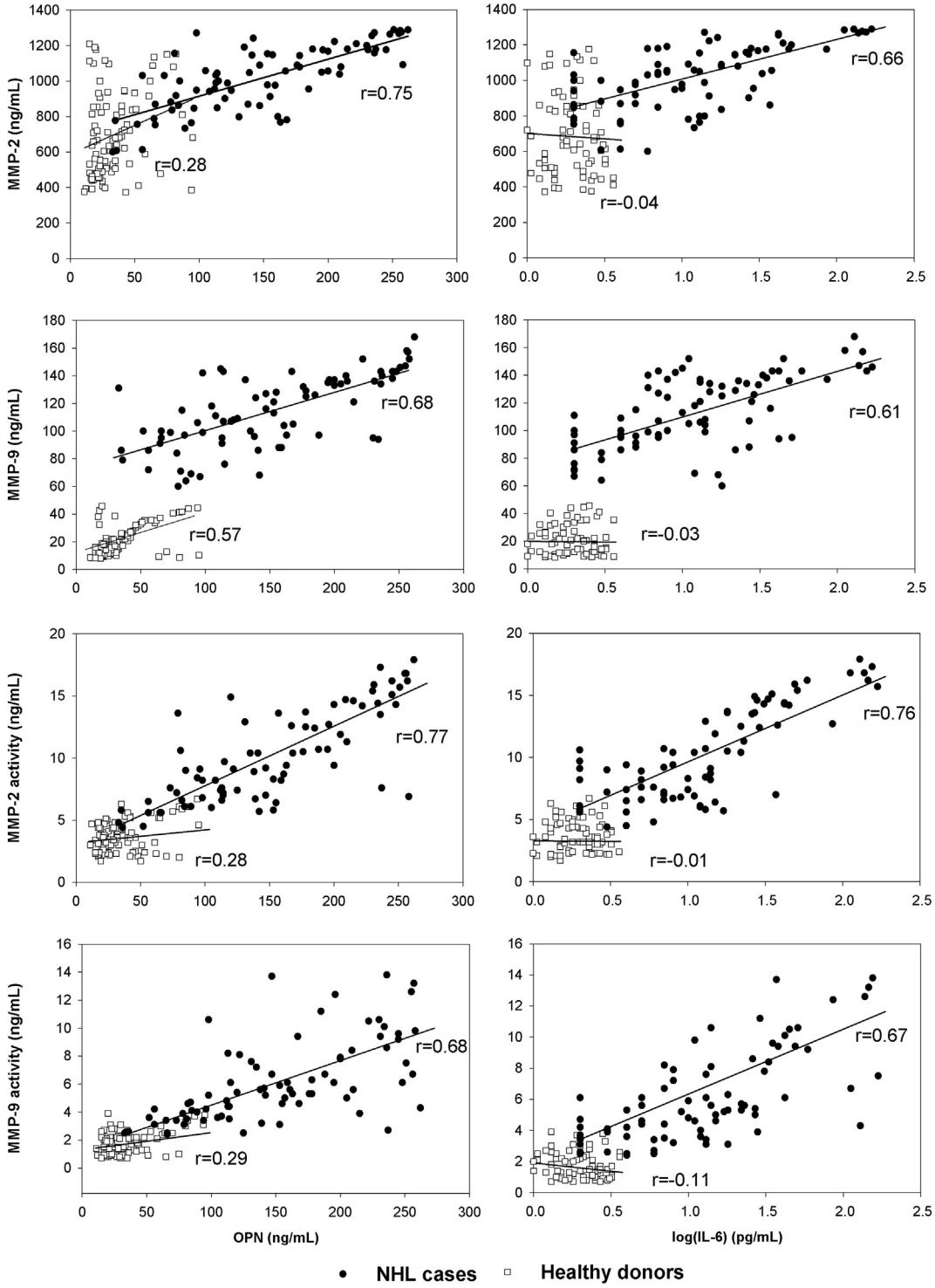

Fig. 2. Scatter plots for both total and activity of MMP-2 and MMP-9 versus OPN and log(IL-6) in NHL cases and healthy donors. Correlations were evaluated through Spearman correlation coefficient r. MMP, matrixmetalloproteinase; OPN, osteopontin; IL-6, interleukin-6; and NHL, non-Hodgkin lymphoma.

cell lymphomas, while the Rosenwald dataset included 240 patients with untreated diffuse large-B-cell lymphoma. Data from Hummel series confirmed that OPN relative mRNA expression increased the expression of MMP-2 and MMP-9 independently from IL-6. Among Rosenwald case series, the effects was confirmed only for MMP-2 (Table 2).

3.3. Induction of MMP-2 and MMP-9 in PBMC

To examine the effects of OPN and IL-6 on MMP-2 and MMP-9 activation, the PBMCs from NHL patients and healthy donors were treated with rhOPN (1 μg/mL) and rhIL-6 (100 ng/mL) alone and in combination. Increased levels of MMP-2 and MMP-9 were observed after 24 h in the supernatants of PMBCs from NHL patients compared to the

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Table 1
Regression coefficients (β) for MMP-2 and MMP-9 in NHL cases and healthy donors.

	MMP-2		MMP-9	
Variable	β	p-Value	β	p-Value
NHL cases (n = 78)				
Female gender	−4.415	0.881	14.348	0.001
Age (5 years)	6.289	0.271	−0.816	0.331
OPN	1.844	<0.001	0.2111	<0.001
Log(IL-6)	17.894	0.348	5.474	0.053
Healthy donors (n = 95)				
Female gender	59.092	0.242	−4.224	0.032
Age (5 years)	−8.434	0.349	−0.009	0.980
OPN	3.401	0.006	0.286	<0.001
Log(IL-6)	−24.562	0.621	−0.696	0.717

MMP, matrixmetalloproteinase; OPN, osteopontin; and NHL, non-Hodgkin lymphoma.

Table 2
Regression coefficients (β) for MMP-2 and MMP-9 in NHL cases from Hummel and Rosenwald series.

	MMP-2		MMP-9	
Variable	β	p-Value	β	p-Value
Hummel series (n = 221)				
Female gender	0.040	0.682	0.133	0.362
Age (5 years)	0.012	0.221	0.029	0.055
OPN	0.260	<0.001	0.250	<0.001
Log(IL-6)	2.151	0.003	0.359	0.737
Rosenwald series (n = 240)[a]				
OPN	0.192	0.007	0.071	0.244
Log(IL-6)	1.218	0.447	0.885	0.523

[a] Information on gender and age was not available in the Rosenwald series; MMP, matrixmetalloproteinase; OPN, osteopontin; NHL, non-Hodgkin lymphoma.

untreated cells (Fig. 3). Similar trend was observed analyzing the supernatant from PBMC derived from healthy donors (Supplementary Fig. 1). To determine if the increase of MMP-2 and MMP-9 levels were due to the OPN and/or IL-6 stimulation, we first neutralized OPN and then IL-6 in cultured PBMCs. The release of MMP-2 and MMP-9 strongly decreased after the inactivation of OPN in the presence of IL-6 stimulation compared to the cells treated with OPN and anti-IL-6; on the contrary, the increase in MMP-9 and MMP-2 secretion rate was about 3 and 2.6 folds, respectively when the cells were treated with rh-OPN in combination with anti-IL-6. These results strongly support an independent role of OPN on MMPs activation (Fig. 3).

4. Discussion

The hematopoietic microenvironment is constituted by highly organized structure (stromal cells, extracellular matrix proteins and cytokines), which serves to regulate the location, proliferation, and function of the hematopoietic cells [51–53]. Their alterations are associated with the malignant transformation.

A growing number of studies showed the crucial role of MMPs in the development and progression of several cancers, including hematopoietic malignancies [54–56]. The mechanism of MMPs expression and activation may be regulated by different pathways as result of cytokines and growth factors stimulation [18,57,58]. For instance, OPN may activate MMPs [8,18] as well as IL-6 may induce the induction of gelatinases (MMP-2 and MMP-9) in human lymphoma cells [35]. However, it was unclear if the activation of MMPs in NHL was determined by OPN and/or IL-6. Our results demonstrated a strong increase of MMP-2, MMP-9, OPN and IL-6 plasma levels in NHL patients when compared with controls. Our data are in agreement with previous results in which MMP-2 and MMP-9 are associated with NHL progression [31–33,59].

Usually, the overexpression of MMP-9 and MMP-2 is linked with invasive behavior of human cancer cells [20,60,61]. Furthermore, the expression levels of MMP-2 and MMP-9 were analyzed in relation to the expression of IL-6 and OPN detected in NHL patients. As expected, positive correlations of MMP-2 and MMP-9 versus OPN and IL-6 were observed in NHL patients. However, a multivariate regression model revealed that the activation of MMP-2 and MMP-9 is mediated by OPN and not by IL-6, suggesting that OPN regulates the bioavailability of MMPs. These observations were further validated by bioinformatics and experimental approaches. In the Hummel dataset, the increased relative mRNA expression of MMP-2 and MMP-9 was associated with the increased expression of OPN independently from IL-6. While, by analyzing the Rosenwald dataset, the effects was confirmed only for MMP-2.

Our in vitro experiments indicate that the increase in MMP-9 and MMP-2 secretion rate was mostly associated with OPN stimulation. The observation that IL-6 plasma levels were higher in NHL patients compared to controls indicates that IL-6 may play an important role in the lymphomagenesis through the activation of other molecular pathways.

These findings are in line with previous studies showing that inhibition of OPN decreased the expression and activity of MMPs in human cancer [18,19,57,58,62]. While, the activation of OPN enhances the secretion of MMP-2 and MMP-9 [63,64]. It is also known that NHL lymphoma development may be associated with chronic inflammation that may be linked with a history of viral infections [15,65].

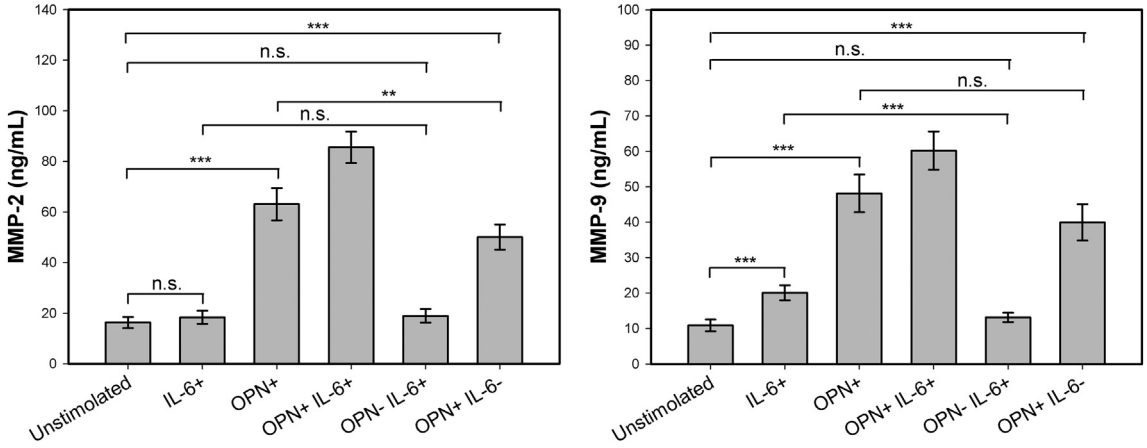

Fig. 3. MMP-2/MMP-9 concentrations expressed as mean and 95% confidence intervals in PBMCs stimulated with IL-6 and/or OPN in NHL cases. Concentrations were compared by means of paired t-test. **: $p < 0.01$; ***: $p < 0.001$; n.s.: not significant. MMP, matrixmetalloproteinase; OPN, osteopontin; IL-6, interleukin-6, NHL, non-Hodgkin lymphoma; OPN+, treated with the recombinant OPN; OPN-, treated with the monoclonal antibody anti-OPN; IL-6+, treated with the recombinant IL-6; and IL-6-, treated with the monoclonal antibody anti-IL-6.

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In this context, it was demonstrated that MMPs are involved in inflammation and cancer processes through pro-inflammatory cytokines, chemokines, and other proteins [47,66]. A relationship was found between inflammatory cell infiltration and the expression, activity of MMP, and the degradation of ECM in tumor progression [67,38]. Similarly, it was shown that OPN is overexpressed in HCV-associated lymphoproliferations [15].

The molecular mechanism by which OPN mediates MMPs activation in tumor conferring aggressiveness remains poorly understood; however, previous studies have indicated that OPN stimulates the secretion of urokinase-type plasminogen activator, which regulates pro-MMP-9 activation, cell motility, invasion, and tumor growth [62,63]. Furthermore, OPN was implicated in the regulation of phosphoinositide-3-kinase–dependent Akt phosphorylation, cell adhesion and migration in prostate cancer cells, and activation of protein kinase Cα/nuclear factor-inducing kinase/nuclear factor κB-dependent signaling cascades.

Overall, these data suggest that the involvement of tumor microenvironment in high-grade B-cell lymphomas plays an important role on the activation of molecular pathway. The identification of a correlated expression between OPN and MMPs in NHL suggests that OPN/MMPs pathway activation is involved in the pathogenesis of human B-cell lymphomas. This may also lead to the development of new therapeutic strategies for the treatment of these disorders.

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