Disease Markers

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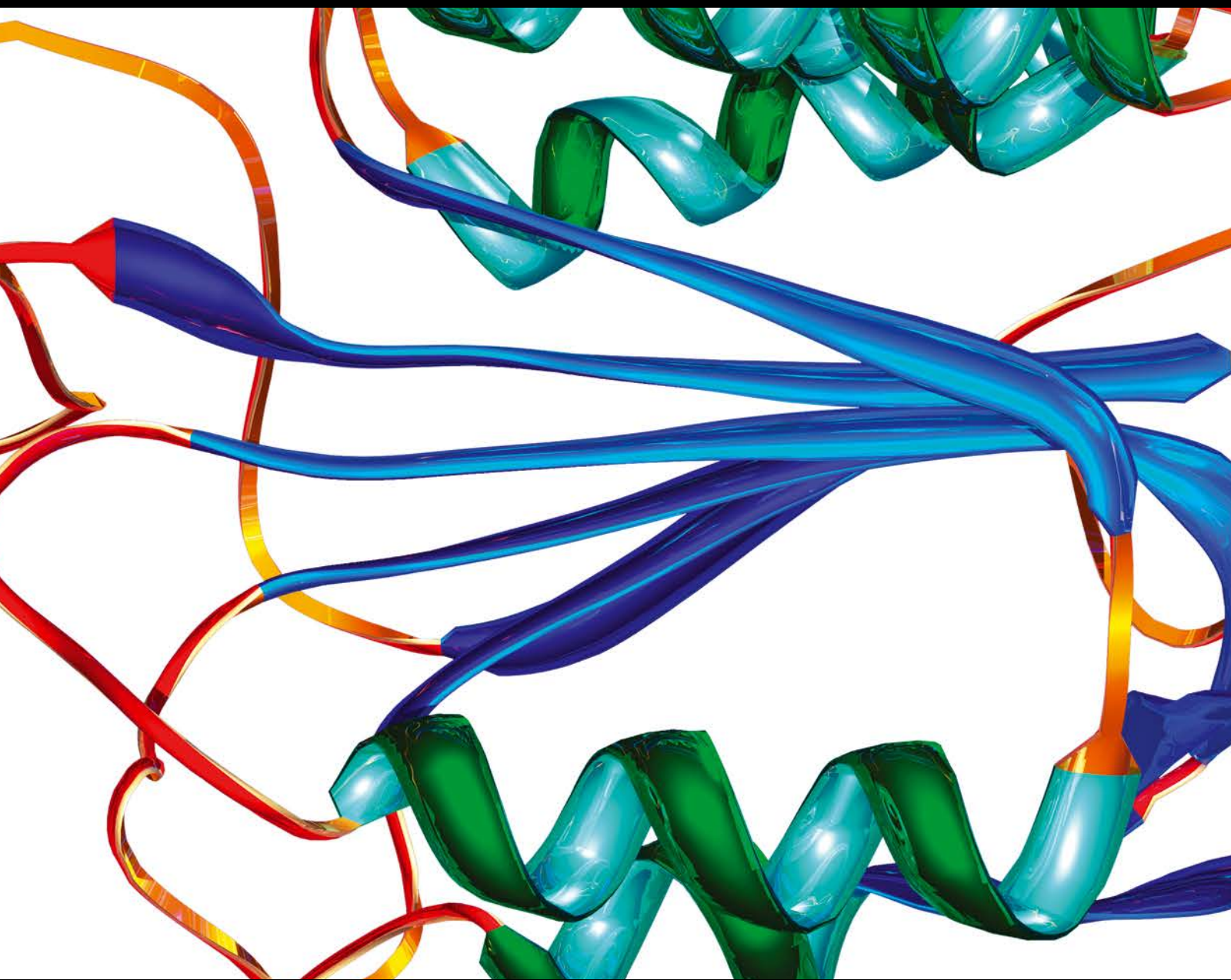
Guest Editors: Jacek Kurzepa, Fatma M. El-Demerdash, and Massimiliano Castellazzi



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Editorial Board

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Editorial

Matrix Metalloproteinases as a Pleiotropic Biomarker in Medicine and Biology

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The group of matrix metalloproteinases (MMPs), calcium- and zinc-dependent proteolytic enzymes, is responsible for extracellular protein degradation. Acting together, supported by intracellular processes they are able to digest any physiological extracellular protein. However, the biochemistry of extracellular matrix (ECM) is very complex, and proteolytic enzymes located in this compartment exert numerous pleiotropic effects beyond the characteristic for the degradation of structural elements. Therefore, MMPs are involved into several physiological and pathological processes [1].

Because of the ECM components' ability to model, as well as the influence on the activity of some biologically active compounds such as tumor necrosis factor α, chemokine CXCL-8, and transforming growth factor β, MMPs affect the pathogenesis of numerous diseases, mostly primarily associated with inflammation [2]. Therefore, the elevated level of particular MMP cannot be associated with failure of specific organ or tissue. In that case the MMPs can be biomarkers of disease? MMPs are sensitive and easily measurable, but due to their prevalence they are not specific for any tissue. For example, MMP-9 serum level is elevated in patients with relapsing remitting and secondary progressive multiple sclerosis (MS) compared to controls [3] and the MMP-9/TIMP-1 ratio may predict magnetic resonance image (MRI) activity during interferon-beta therapy [4]. However, despite the acknowledged involvement of some MMPs in MS pathogenesis and progression, the evaluation of these enzymes is not routinely recommended for MS diagnosis because their elevation is observed in numerous other diseases as stroke and bacterial and viral infections and even in smokers [5]. Nevertheless, the higher activity of individual MMPs in connection with patients' clinical status can help to predict the risk, diagnosis, or progress of the disease. For example, the MMP-9 serum level does not correlate with the risk of stroke but MMP-9 C(-1562)T polymorphism seems to be significantly associated with risk of stroke in patients with and without type 2 diabetes mellitus [6]. Also remaining MMPs possess the ability to predict the clinical status. The overexpression of MMP-7, MMP-10, and MMP-12 in colon cancer patients' sera correlates with a dismal prognosis [7] and high serum MMP-1 level showed a trend for short overall survival in non-small cell lung cancer patients [8].

The low tissue specificity of isolated MMPs causes that single enzyme may not play a role of a good biomarker. However, some MMPs could be useful constituents of biomarker panels but only in combination with other biochemical parameters. The multiplex panel composed of MMP-7, CA125, CA72-4, and human epididymis protein 4 is suitable for the early detection of ovarian cancer [9]. The simultaneous evaluation of MMP-1, TIMP-1, CD40 ligand, and myeloperoxidase seems to be a novel promising diagnostic panel in timely diagnosis of acute aortic dissection [10]. Also, some products of MMPs catalysis were considered as the potential biomarkers. Citrullinated and MMP-degraded

vimentin (VICM) simultaneously and in combination with others markers revealed good potential to differentiate ulcerative colitis form noninflammatory bowel diseases [11].

Finally, last but not least, preanalytical conditions must be taken into account before starting MMPs analysis in body fluids. In fact, if in one hand the release of MMPs during clotting could affect their concentrations [12], on the other hand the use of some calcium-chelating anticoagulants could interfere with MMPs activity [13].

In conclusion, the enzymes from among MMPs evaluated individually cannot be considered as the specific biomarkers of the particular disease or pathological process. However, the sudden change in their body fluid level can act as an alarm siren informing on the upcoming threat which combined with clinical state of the patient may help in the diagnosis, treatment, or prognosis.

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Fatma M. El-Demerdash
Massimiliano Castellazzi

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Research Article

Interplay between Matrix Metalloproteinase-9, Matrix Metalloproteinase-2, and Interleukins in Multiple Sclerosis Patients

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Matrix Metalloproteases (MMPs) and cytokines have been involved in the pathogenesis of multiple sclerosis (MS). However, no studies have still explored the possible associations between the two families of molecules. The present study aimed to evaluate the contribution of active MMP-9, active MMP-2, interleukin- (IL-) 17, IL-18, IL-23, and monocyte chemotactic proteins-3 to the pathogenesis of MS and the possible interconnections between MMPs and cytokines. The proteins were determined in the serum and cerebrospinal fluid (CSF) of 89 MS patients and 92 other neurological disorders (OND) controls. Serum active MMP-9 was increased in MS patients and OND controls compared to healthy subjects ($p < 0.001$ and $p < 0.01$, resp.), whereas active MMP-2 and ILs did not change. CSF MMP-9, but not MMP-2 or ILs, was selectively elevated in MS compared to OND ($p < 0.01$). Regarding the MMPs and cytokines intercorrelations, we found a significant association between CSF active MMP-2 and IL-18 ($r = 0.3$, $p < 0.05$), while MMP-9 did not show any associations with the cytokines examined. Collectively, our results suggest that active MMP-9, but not ILs, might be a surrogate marker for MS. In addition, interleukins and MMPs might synergistically cooperate in MS, indicating them as potential partners in the disease process.

1. Introduction

Multiple sclerosis (MS) is a disease of the central nervous system (CNS) of supposed autoimmune origin, characterized by inflammation, demyelination, and neurodegeneration [1]. Although the pathological features of the disease are heterogeneous, a common event is thought to be the reactivation within the CNS of infiltrating myelin-specific T cells which, in turn, trigger the recruitment of innate immunity cells mediating demyelination and axonal loss [2]. The perivascular transmigration and accumulation of inflammatory cells within the CNS are mainly mediated by two events: the production of leukocyte-attracting chemokines and the blood-brain barrier

Table 1: Demographic and clinical characteristics of healthy controls and OND and RRMS patients.

	Healthy controls ($n = 40$)	OND ($n = 92$)	MS ($n = 89$)
Age	37.0 ± 7.5; 35.5 (30.3–44.0)	42.4 ± 13.7; 41.5 (33.0–49.0)	39.2 ± 10.9; 37.0 (30.5–48.0)
Sex: female/male	24/16	57/35	53/36
Disease duration (yrs)	—	—	5.9 ± 7.1; 3 (1–7.7)
EDSS	—	—	3.8 ± 1.9; 3.5 (2.5–4.4)
Clinically active MS: *n*/total (%)	—	—	32/40 (80%)
Clinically stable MS: *n*/total (%)	—	—	8/40 (20%)

EDSS: expanded disability status scale; MS: multiple sclerosis; RRMS: relapsing-remitting multiple sclerosis; OND: other neurological disorders.

(BBB) breakdown [3]. The production of chemokines may be important for the regulation of the inflammatory cells influx to sites of tissue damage. Within the chemokine family, particularly studied members in the course of MS are the monocyte chemotactic proteins (MCPs), with MCP-1 and MCP-2 being selectively expressed at high levels in active lesions, while MCP-3 was mostly observed in the extracellular matrix surrounding the vascular elements [4]. In addition to the establishment of a chemokine gradient, the BBB has to be disrupted in order for the leukocytes to infiltrate within the CNS [5]. This event is mediated by the action of matrix metalloproteinases (MMPs), a family of Zn^{2+}-dependent and Ca^{2+}-requiring endopeptidases involved in the modeling of the extracellular matrix in both physiological and pathological conditions. Among all MMPs, MMP-9 and MMP-2 have been extensively studied in MS given their ability to degrade the components of the basal lamina and to mediate BBB damage [6–8].

Notably, growing experimental evidence suggests the involvement of MMP-9 in the pathogenesis of MS, where its circulating levels in serum and cerebrospinal fluid (CSF) were found to be upregulated in MS patients compared with noninflammatory neurological disorders (NIND) and healthy controls [9–13]. On the contrary, the implication of MMP-2 in the pathogenesis of MS is more controversial, since this enzyme had demonstrated both protective [7] and detrimental actions [14]. Besides MMPs, inflammatory cytokines, in particular the interleukins belonging to the Th17 axis, IL-23 and IL-17, might also play a role in MS [15].

IL-23, a member of the IL-12 cytokine family, is a heterodimeric protein with the ability to support the polarization and expansion of T cells toward a Th17 phenotype [16, 17]. Its involvement in the pathogenesis of MS has been suggested by evidence from the animal model of the disease, the experimental autoimmune encephalomyelitis (EAE). Indeed, this cytokine has proven to be essential for the development of EAE [18] and the transfer of Th17 cells, polarized and expanded by IL-23, was able to induce the disease in animals [19]. Th17 cells are strictly connected to the pathogenesis of MS through, but not limited to, the production of several proinflammatory cytokines including IL-17 (A and F), which has been found upregulated in chronic lesions of MS patients [20] and in the serum of Interferon-β (IFN-β) nonresponding patients [21]. In addition to the abovementioned factors, IL-18, another cytokine important in Th1 response in the course of MS [22], has been found increased in serum and CSF of MS patients compared to noninflammatory controls, with the levels of the molecule being higher in those with MRI gadolinium enhancing lesions [23]. Nonetheless, several animal and *in vitro* evidence connected both MMPs to IL-18 [24] and to the IL-17/IL-23 axis [25], demonstrating a general stimulating effect on the enzymes production, whereas other reports suggested a regulation of MMP-2 on MCP-3 activity [26] showing an anti-inflammatory effect [27]. However, to the best of our knowledge none of the previous studies evaluated the possible interrelationships between the active forms of MMP-9 and MMP-2 and the most common cytokines involved in MS pathogenesis. Therefore, in the present study our aim was to measure the levels of active MMP-9 and MMP-2, IL-17, IL-18, IL-23, and MCP-3 in the serum and CSF of MS patients and controls in order to investigate the contribution of these molecules to MS pathogenesis. Moreover, we aimed to explore possible interrelationships between cytokines, MMPs, and clinical variables.

2. Material and Methods

2.1. Patients Selection. For this study, we recruited 89 consecutive patients affected by definite relapsing-remitting MS (RRMS), according to McDonald criteria [28], who presented at the Neurology Clinic of the University of Belgrade. Evidence of a relapse at admission was considered clinical disease activity [29]. The data were available for a total of 40 patients out of 89. Accordingly, 32 patients were clinically active, whereas 8 patients were clinically stable. Patient disease severity was measured by Kurtzke's Expanded Disability Status Scale (EDSS) [30]. Disease duration was scored and expressed in years. At the time of sample collection, none of the patients had fever or other signs of acute infection, nor had they been receiving any disease-modifying therapies (DMTs) during the 6 months before the study. A total of 92 controls with other neurological disorders (OND) were also included in the study (Table 1). OND patients were free of immunosuppressant drugs, including steroids, at the time of sample collection. In addition, a total of 40 age- and sex-matched healthy controls (HC) were used. Informed consent was given by all patients before inclusion in the study and the study design was approved by the Ethics Committee of the School of Medicine, University of Belgrade.

2.2. CSF and Serum Sampling. Cerebrospinal fluid and serum samples were collected under sterile conditions and stored

in aliquots at −80°C until assay. "Cell-free" CSF samples were obtained after centrifugation at room temperature of specimens taken by lumbar puncture performed for diagnosis purposes. Serum samples were derived from centrifugation of blood specimens withdrawn by puncture of an anterocubital vein at the same time of CSF extraction. Paired CSF and serum samples from RRMS and OND patients were stored and measured under exactly the same conditions. For the healthy controls, only the serum was available.

2.3. Assay of Interleukins in Serum and CSF. IL-17A, IL-23, and MCP-3 levels were simultaneously measured in sera and CSF of patients, twofold diluted with dilution buffer or undiluted, respectively, by a multiplex sandwich enzyme-linked immunosorbent assay (ELISA) system based on chemiluminescence detection (Aushon SearchLight chemiluminescent assay kits, Tema Ricerca, Italy) according to the manufacturer's recommendations. All samples were analyzed in duplicate. The interleukin levels are reported as pg/mL. The lower concentration of each standard curve was 0.78 pg/mL for IL-17A, 19.5 pg/mL for IL-23, and 0.78 pg/mL for MCP-3.

IL-18 was measured in CSF and serum samples, twofold diluted, with commercially available ELISA (Boster Immunoleader cod. EK0864). Samples were assayed in duplicate. A standard curve was generated in each plate and the lower standard concentration was 15.6 pg/mL.

2.4. Assay of Active MMP-9 in Serum and CSF. Serum and CSF levels of circulating active MMP-9 were determined using a commercially available activity assay system (Human Active MMP-9 Fluorokine E Kit, R&D systems; Cat. Number F9M00) following the manufacturer's instructions. All the reagents were included in the kit. For the determinations, a standard curve in the range of 16–0.125 ng/mL was used; serum and CSF samples were diluted 100 times and 2 times, respectively, with the calibrator diluent (RD5-24) included in the kit. According to the manufacturer's data, the minimum detectable dose was 0.005 ng/mL and the range of intra-assay and interassay coefficient of variation (CV) was 3.9–4.8% and 8.0–9.3%, respectively.

2.5. Assay of Active MMP-2 in Serum and CSF. Serum and CSF levels of circulating active MMP-2 were determined using a commercially available activity assay system (MMP-2, Biotrak Activity Assay System, GE Healthcare; Cat. Number RPN2631) following the manufacturer's instructions. All the reagents were included in the kit. For the determinations, a standard curve in the range of 4–0.125 ng/mL was used; serum and CSF samples were diluted 25 times and 2 times, respectively, with the assay buffer included in the kit. According to the manufacturer's data, the sensitivity was 0.190 ng/mL and the range of intra-assay and interassay coefficient of variation (CV) was 4.4–7.0% and 16.9–18.5%, respectively.

2.6. Statistical Analysis. Normality of distribution was checked by Shapiro-Wilk test. Since the variables were not normally distributed, group comparisons were performed using Kruskal-Wallis followed by Mann-Whitney U tests, with Bonferroni correction for multiple comparisons. Bivariate correlations were performed by Spearman's rank test and frequency distributions were examined using the Chi-square test. To assess the association between abnormal MMPs and ILs values measured in serum or CSF and the MS pathology, a binary logistic regression analysis was performed. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Active MMP-9 and MMP-2 in Serum and CSF of MS Patients and Controls. Active MMP-9 and MMP-2 were detectable in 100% of serum samples and in 100% and in 93% (85 OND and 84 MS) of CSF samples for MMP-9 and MMP-2, respectively. As reported in Figure 1(a), the levels of active MMP-9 were different among the groups. In particular, we found a higher concentration of active MMP-9 in the serum of both MS patients and OND controls compared to healthy subjects ($p < 0.001$ and $p < 0.01$, resp.). On the contrary, active MMP-2 serum levels were similar in MS, OND, and healthy subjects (Figure 1(b), Kruskal-Wallis $H(2) = 1.009$, $p = 0.604$). Then, we compared the amounts of both active gelatinases measured in the CSF of MS patients and OND controls. As depicted in Figure 1(c), MS patients showed almost a doubled concentration of active MMP-9 compared to OND controls ($p = 0.009$), whereas the levels of active MMP-2 did not differ (Figure 1(d), median (interquartile range): 4.7 (2.3–11.4) and 5.1 (2.7–10.4) for OND and MS patients, resp.; $p = 0.713$). When patients were grouped according to clinical disease activity, there were no statistical differences between MS patients with and without clinical evidence of disease activity, for both serum and CSF active MMP-9 and MMP-2 (data not shown).

3.2. Interleukin Levels in Serum and CSF of MS Patients and Controls. The levels of IL-17, IL-18, IL-23, and MCP-3 in the serum of OND and MS patients were detectable in 21% of samples for IL-17 (16 OND and 22 MS), 86% for IL-18 (79 OND and 77 MS), 71% for IL-23 (65 OND and 64 MS), and 61% for MCP-3 (55 OND and 55 MS). In the CSF, the values were detectable in 35% of samples for IL-17 (35 OND and 27 MS), 59% for IL-18 (50 OND and 56 MS), 19% for IL-23 (18 OND and 17 MS), and 53% for MCP-3 (46 OND and 50 MS). As reported in Figures 2(a)–2(d), we did not find any significant difference in the serum concentration of the measured cytokines. The same result was observed in the CSF, where the levels of the cytokines were not different between the OND controls and the MS patients (Figures 2(e)–2(h)). When patients were grouped according to clinical disease activity, we did not find any statistical differences between MS patients with and without clinical evidence of disease activity, for both serum and CSF IL-17, IL-18, IL-23, and MCP-3 levels (data not shown).

3.3. Correlations between Interleukin Levels and Active MMP-9 and MMP-2 in Serum and CSF of MS Patients and with Clinical Outcomes. We evaluated possible correlations between the levels of MMPs and interleukins measured in the serum of MS patients. As reported in Table 2, we observed significant positive correlations between MCP-3 and IL-17, between MCP-3 and IL-23, and between IL-17 and IL-23.

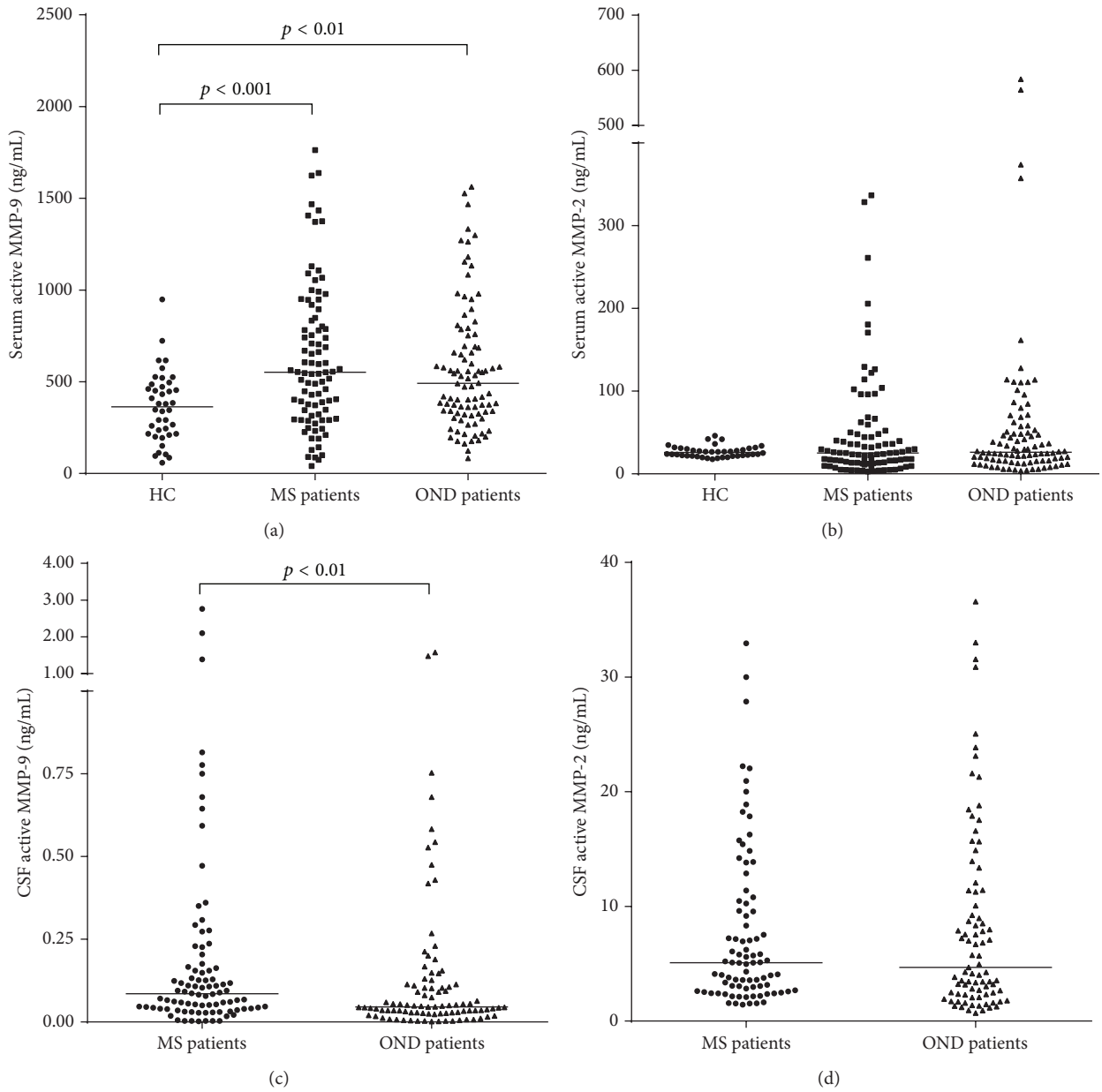

Figure 1: Median of serum total active MMP-9 and MMP-2, in RRMS patients, OND controls, and healthy donors and CSF active MMP-9 and MMP-2 in RRMS patients and OND controls. Serum levels of total active MMP-9 were statistically different among the groups (Kruskal-Wallis; $H(2) = 15.45$, $p < 0.0001$) and CSF active MMP-9 levels were elevated in RRMS patients compared to OND patients. (a) Serum concentrations of total active MMP-9 were not different among RRMS (median (IQR): 552 (318–841) ng/mL) and OND (492 (330–737) ng/mL) patients; whereas they were higher (Mann Whitney; $p < 0.001$ and $p < 0.01$) in RRMS and OND patients when compared to HC (363 (216–482) ng/mL). (b) Serum levels of active MMP-2 were not different between RRMS patients (25.2 (13.1–48.1) ng/mL), OND patients (26.2 (15.8–52.5) ng/mL), and HC (25.8 (21.8–30.7) ng/mL). (c) CSF amounts of active MMP-9 were more increased in RRMS (0.084 (0.040–0.165) ng/mL) than in OND (0.046 (0.027–0.113) ng/mL) patients (Mann Whitney; $p = 0.009$). (d) CSF levels of active MMP-2 were not different between RRMS (5.1 (2.7–10.4) ng/mL) and OND (4.7 (2.3–11.4) ng/mL) controls. IQR: interquartile range; HC: healthy controls; MMP: matrix metalloproteinase; RRMS: relapsing-remitting MS; OND: other neurologic disorders; CSF: cerebrospinal fluid.

Of note, we did not find any relation between the active forms of MMPs and the interleukins, although there was a tendency toward a significant negative correlation between serum active MMP-9 and IL-18 ($p = 0.076$).

Then, we evaluated the correlations between active MMPs and interleukins measured in the CSF of patients. The results are summarized in Table 3. Notably, we found a positive correlation between IL-18 and active MMP-2 and MCP-3 and IL-17 and between IL-18 and IL-23.

There were no significant correlations between disease severity scored by EDSS, disease duration, and serum and CSF levels of the measured proteins.

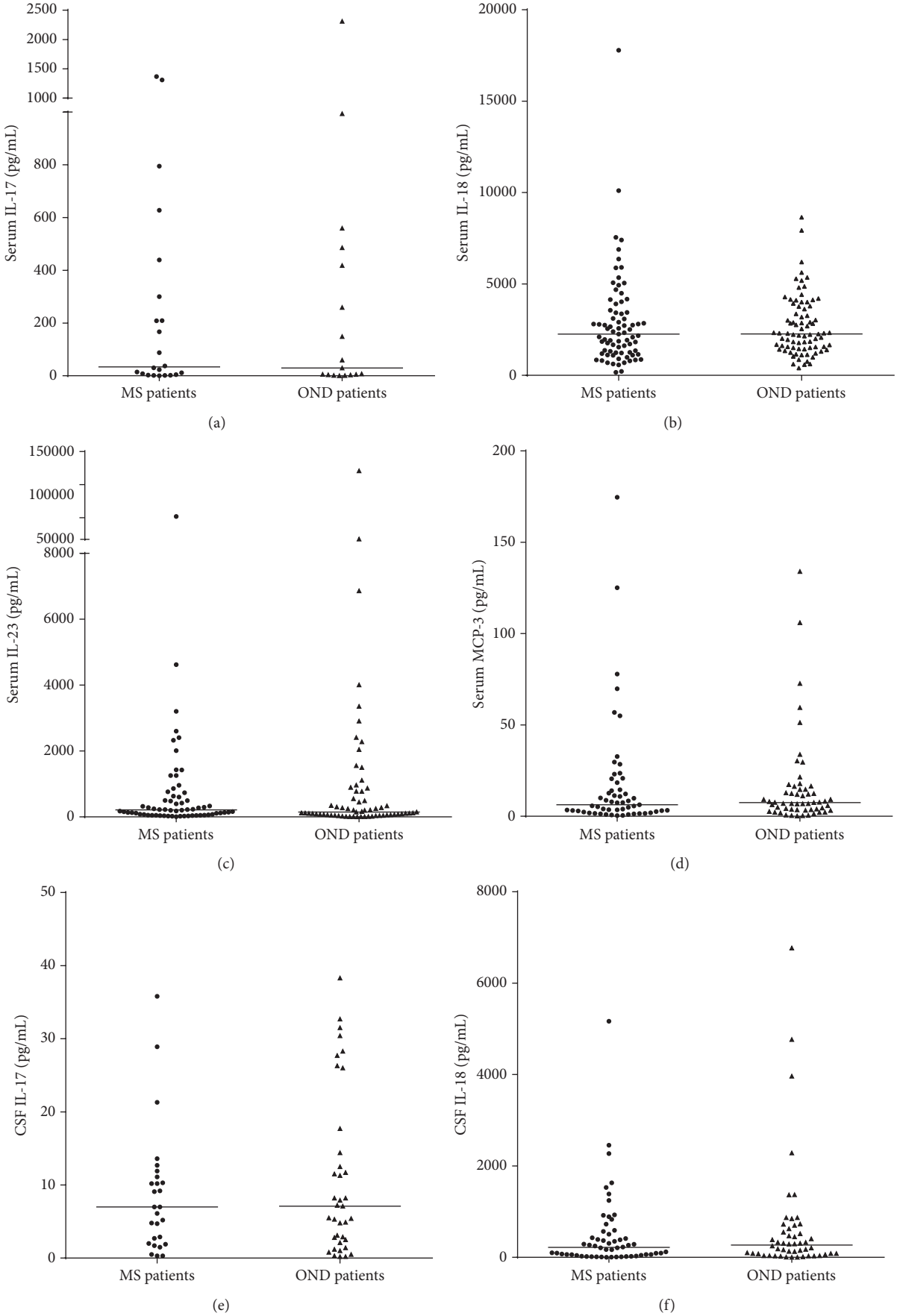

Figure 2: Continued.

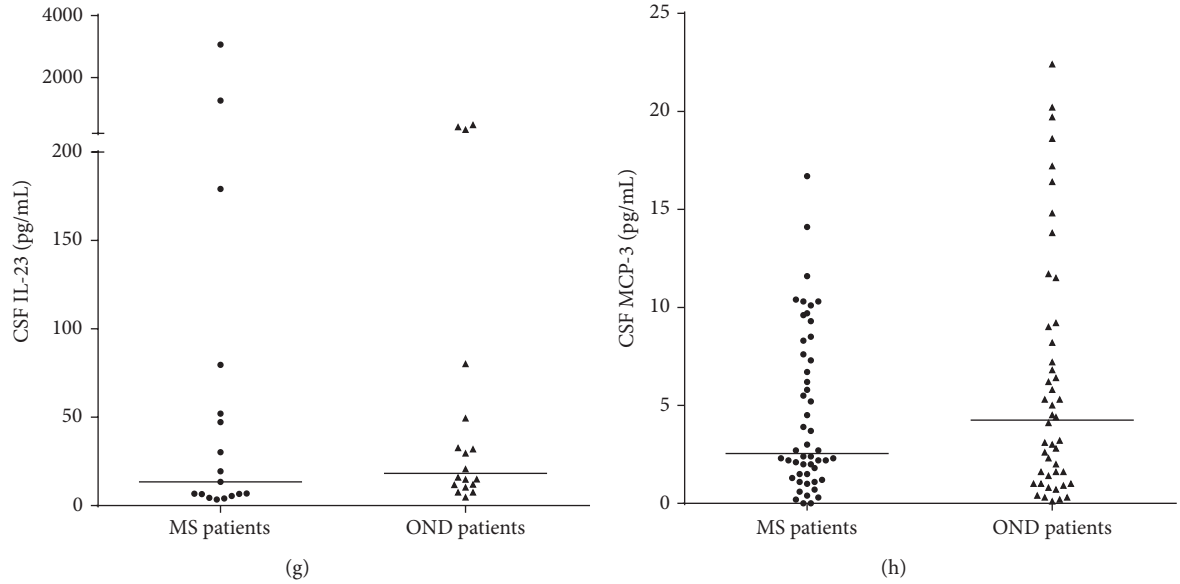

Figure 2: Median of serum and CSF IL-17, IL-18, IL-23, and MCP-3 concentrations in RRMS patients and OND controls. None of the examined cytokines/chemokines was different between RRMS patients and OND patients in either the serum or CSF. (a) Serum levels of IL-17 in RRMS (median (IQR): 33.7 (4.2–335.0) pg/mL) and OND (44.8 (4.1–468.0) pg/mL) patients. (b) Serum levels of IL-18 in RRMS (2259 (1232–3505) pg/mL) and OND (2266 (1509–3766) pg/mL) patients. (c) Serum levels of IL-23 in RRMS (212.3 (64.9–625.3) pg/mL) and OND (148.9 (54.6–774.9) pg/mL) patients. (d) Serum levels of MCP-3 in RRMS (6.3 (2.6–18.4) pg/mL) and OND (7.5 (3.5–14.7) pg/mL) patients. (e) CSF levels of IL-17 in RRMS (7.0 (2.0–11.1) pg/mL) and OND (7.1 (2.3–16.1) pg/mL) patients. (f) CSF levels of IL-18 in RRMS (218 (49–551) pg/mL) and OND (269 (71–644) pg/mL) patients. (g) CSF levels of IL-23 in RRMS (13.4 (5.9–65.9) pg/mL) and OND (18.2 (11.4–57.1) pg/mL) patients. (h) CSF levels of MCP-3 in RRMS (2.6 (1.5–7.8) pg/mL) and OND (4.3 (1.3–9.1) pg/mL) patients. IQR: interquartile range; CSF: cerebrospinal fluid; IL: interleukin; MCP: Monocyte Chemoattractant Protein; RRMS: relapsing-remitting MS; OND: other neurological disorders.

Table 2: Correlation matrix of active MMP-9, active MMP-2, and interleukins measured in the serum of MS patients.

Variables	(1)	(2)	(3)	(4)	(5)	(6)
(1) Active MMP-9	—					
(2) Active MMP-2	−0.166 (89)	—				
(3) IL-17	−0.207 (22)	0.290 (22)	—			
(4) IL-18	−0.204 (77)	0.132 (77)	0.387 (22)	—		
(5) IL-23	−0.196 (64)	−0.017 (64)	0.466 (22)*	−0.138 (63)	—	
(6) MCP-3	−0.128 (55)	0.028 (55)	0.922 (21)**	0.212 (55)	0.468 (48)**	—

Values in brackets represent the degrees of freedom. * $p < 0.05$; ** $p < 0.01$.

3.4. Evaluation of Abnormal MMPs and Interleukin Levels in Serum and CSF of MS Patients and Controls. Based on the median values of the active MMPs and cytokines measured in the serum or CSF from the whole population, we determined in all subjects whether the active MMP-9 or cytokines were increased or active MMP-2 was decreased. These values were considered abnormal. In addition, the cytokine abnormal values were merged in one category (Table 4, *combined interleukins*), including subjects with at least one abnormal value of IL-17, IL-18, IL-23, or MCP-3.

As shown in Table 4, we did not find any difference in the frequency of abnormal levels of either interleukins or MMPs in serum. The same result was observed when we analyzed the frequency of patients with abnormal CSF levels of the considered proteins, with the exception of the active MMP-9. Indeed, there was a higher proportion of MS patients with abnormally increased levels of the enzyme compared to OND controls (Pearson Chi-square (1): 9.335, $p = 0.002$). Then, we searched for possible association of abnormal levels of MMPs and interleukins with MS by employing a binary logistic regression analysis, considering the diagnosis (MS or OND) as the outcome variable and entering the abnormal levels of MMPs or cytokines alone or in combination (*combined interleukins*) as predictors. From this analysis, no association emerged between serum active MMP-9, active MMP-2, or the cytokines and MS pathology. On the contrary when we analyzed the proteins measured in the CSF, we found that only the abnormal values of active MMP-9 were associated with an increased likelihood of being affected by MS (Odds Ratio: 2.52, 95% Confidence Interval: 1.39–4.59, $p = 0.002$). Of note, the inclusion of the other covariates did not improve the reliability of the model (data not shown).

Finally, we compared the levels of serum or CSF active MMP-9 and MMP-2 measured in MS patients, grouped

Table 3: Correlation matrix of active MMP-9, active MMP-2, and interleukins measured in the CSF of MS patients.

	(1)	(2)	(3)	(4)	(5)	(6)
(1) Active MMP-9	—					
(2) Active MMP-2	0.244 (84)	—				
(3) IL-17	−0.306 (27)	−0.129 (25)	—			
(4) IL-18	−0.076 (56)	0.300 (53)*	0.437 (19)	—		
(5) IL-23	0.075 (17)	−0.344 (16)	0.450 (7)	0.248 (10)	—	
(6) MCP-3	−0.127 (50)	0.237 (47)	0.485 (20)*	0.454 (33)**	0.575 (13)*	—

Values in brackets represent the degrees of freedom. * $p < 0.05$; ** $p < 0.01$.

Table 4: Percentage of MS patients and OND controls with abnormal serum and CSF values.

	OND (%)	MS (%)
Serum		
High active MMP-9	46.7	53.9
Low active MMP-2	53.3	49.4
High IL-17	8.7	12.4
High IL-18	43.5	42.7
High IL-23	32.6	38.2
High MCP-3	30.4	30.3
Combined interleukins	*63.0*	*69.7*
CSF		
High active MMP-9	40.2	62.9**
Low active MMP-2	51.8	47.6
High IL-17	20.7	13.5
High IL-18	28.3	30.3
High IL-23	9.8	9.0
High MCP-3	28.3	24.7
Combined interleukins	*45.7*	*44.9*

The cut-off values used for the determination of the frequency of abnormal values were as follows.
Serum: active MMP-9, 534 ng/mL; active MMP-2, 25.2 ng/mL; IL-17, 33.6 pg/mL; IL-18, 2262 pg/mL; IL-23, 181 pg/mL; MCP-3, 7.2 pg/mL.
CSF: active MMP-9, 0.055 ng/mL; active MMP-2, 5.06 ng/mL; IL-17, 7 pg/mL; IL-18, 237 pg/mL; IL-23, 15.9 pg/mL; MCP-3, 3 pg/mL.
** $p < 0.01$.

according to the abnormal level of cytokines alone or in combination. This analysis did not show any difference in the concentrations of the two MMPs between the patients with normal or high values of ILs either in serum or in CSF.

4. Discussion

There is evidence connecting both MMPs and Th17/Th1-related cytokines to the pathogenesis of MS. Indeed, both families of proteins have been advocated as markers of disease activity [31–33], for therapeutic response [34] and as active players in the MS disease course [15, 35]. In particular, MMPs are involved in both BBB disruption and formation of MS lesions [36], whereas cytokines and chemokines may play important roles in the recruitment of leukocytes into the CNS [4] and in the initiation of the autoimmune tissue inflammation [15]. Nevertheless, MMPs and cytokines/chemokines may also cooperate in the opening of the BBB, a key event that can further support the leukocyte migration within the CNS [37]. Notwithstanding the accumulating evidence that might suggest a possible interplay between MMPs and cytokines, there is still a lack of clinical studies exploring possible associations between the mentioned molecules in the serum and CSF of MS patients.

In light of the above considerations, we set out the present study with the aim to evaluate the contribution of MMPs, namely, active MMP-9 and MMP-2, and the cytokines/chemokines IL-17, IL-18, IL-23, and MCP-3 to the pathogenesis of MS. More importantly, for the first time we evaluated the possible intercorrelations involving these two classes of molecules. In agreement with previous studies [31, 38], we found that serum active MMP-9 was higher in patients with MS and OND compared to healthy controls, whereas the CSF active MMP-9 was selectively elevated in MS patients. On the contrary, our finding of the lack of association between serum and CSF levels of active MMP-2 and MS disagrees with previous observations [32]. In our view, divergences in patient selection or genetic and environmental factors [39] might partially explain these conflicting results.

The lack of difference we found in the serum and CSF cytokine concentrations also appears in contradiction with previous reports showing higher serum levels of IL-23 and IL-18 in MS patients compared to healthy controls [23, 34, 40]. Moreover, other studies showed increased [40] but also unchanged [34] levels of IL-17 in the serum or PBMC [41] of MS patients compared to controls. This apparent discrepancy might be due to a different selection in the control group, since, at variance of ours, most of the studies compared MS patients with heathy donors, and to the low detectable rate of cytokines in both serum and CSF. Of note, to the best of our knowledge, there are no data in literature about MCP-3 circulating levels.

The observed strong correlations between IL-17, IL-23, and MCP-3 in the serum and between MCP-3, IL-17, IL-18, and IL-23 in the CSF of MS patients suggest that, though not massive, the MS pathology might be characterized by a general overproduction of cytokines and chemokines. However, if the overproduction occurs it remains within the normal values measured in OND controls, since we did not find any difference in the proportion of abnormal cytokine levels between the two groups. Collectively, these results suggest that, at least in our cohort, cytokines might represent poor surrogate markers of the disease.

On the contrary, active MMP-9, which was selectively elevated in the CSF of MS patients, could be considered as appropriate indicator of ongoing inflammation in MS. Consistently, we found a higher proportion of MS patients with abnormal CSF levels of active MMP-9 compared to OND patients, with an increased likelihood of being affected by MS (Odds Ratio: 2.52). However, the missed correlation between active MMP-9 and cytokines in either the serum or CSF (although likely due to the low detection rate of cytokines) suggests that the activation cascade of the enzyme might not act in concert with these soluble proinflammatory factors in the course of MS.

On the other hand, the significant positive correlation between active MMP-2 and IL-18 suggests that this proinflammatory cytokine might be able to modulate the activation cascade of MMP-2. In line with this hypothesis, a recent *in vitro* study on neuron-like cells reported an upregulation of MMP-14, the physiological activator of MMP-2, upon treatment with increasing amounts of IL-18 [42]. However, this finding seems in contradiction with the supposed major role of active MMP-2 in the resolution phase of the disease, where its levels were lower in patients with MRI evidence of disease activity [32] indicating a possible anti-inflammatory action [26]. Of note, we did not find any difference in both MMPs and cytokine levels between clinically active and inactive MS patients, suggesting that these molecules do not seem correlated to clinical exacerbations. However, it is well known that MRI is superior on clinical examination in measuring MS disease activity [43], and thus we cannot exclude that the real contribution of these proteins to the disease pathogenesis has been underestimated. Indeed, in previous reports [32, 38] we observed differences in active MMPs only when patients were categorized in active or inactive disease based on MRI findings.

This study was not without its limitations. First, the small sample size may have weakened the consistency of our data, making it difficult to draw any definitive conclusion about the possible use of the analyzed molecules as reliable biomarkers of the disease. Second, the design of the study was cross-sectional, thereby precluding our ability to establish any real cause/effect relationship between the cytokines and MMPs. A longitudinal approach could be more suitable. Third, the lack of complete data on the clinical activity of the disease may have mined the ability to detect real differences between clinically active and stable MS patients. However, in previous studies we did not find significant differences when patients were grouped according to clinical evidence of disease activity [31, 32]. Fourth, the lack of MRI examinations in our study could have affected our findings. Finally, the number of patients and controls with detectable levels of cytokines was low, limiting the reliability of our results. Consequently, a replication of the data in larger cohorts of MS patients and controls is warranted.

In conclusion, although with limitations, our study confirms that active MMP-9 could be a potential surrogate marker for monitoring MS disease, whereas cytokines and chemokines seem not able to discriminate between MS patients and controls. Nonetheless, our results also highlighted that MMPs and cytokines might synergistically cooperate in MS, indicating them as potential partners in the disease processes. Further studies in a larger number of patients are needed to verify the effective nature and role of this cooperation in the modulation of the inflammatory responses operating in MS.

Competing Interests

The authors declare that they have no conflict of interests.

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Review Article

A Tale of Two Joints: The Role of Matrix Metalloproteases in Cartilage Biology

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Matrix metalloproteinases are a class of enzymes involved in the degradation of extracellular matrix molecules. While these molecules are exceptionally effective mediators of physiological tissue remodeling, as occurs in wound healing and during embryonic development, pathological upregulation has been implicated in many disease processes. As effectors and indicators of pathological states, matrix metalloproteinases are excellent candidates in the diagnosis and assessment of these diseases. The purpose of this review is to discuss matrix metalloproteinases as they pertain to cartilage health, both under physiological circumstances and in the instances of osteoarthritis and rheumatoid arthritis, and to discuss their utility as biomarkers in instances of the latter.

1. Introduction

Matrix metalloproteinases are a family of zinc-dependent endopeptidases collectively capable of degrading all components of the extracellular matrix. The actions of these enzymes are potent and highly catabolic, and as such physiologic expressions of the genes coding for matrix metalloproteinases are strictly regulated and reserved for instances where dramatic tissue remodeling is required, as occurs during wound healing [1] and embryonic development [2]. Their versatility and efficacy also render them potent effectors of pathological processes, and this is where much interest in their activity is garnered. Ectopic overexpression matrix metalloproteinase activity has been implicated in a wide array of disease states, including tumor initiation and metastasis, atherosclerosis, osteoarthritis, and rheumatoid arthritis. The purpose of the present review is to discuss matrix metalloproteinases as they relate to articular cartilage homeostasis.

2. The Role of Matrix Metalloproteinases in Healthy Cartilage

Seven matrix metalloproteinases have been shown to be expressed under varying circumstances in articular cartilage—matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-3 (MMP-3), matrix metalloproteinase-8 (MMP-8), matrix metalloproteinase-9 (MMP-9), matrix metalloproteinase-13 (MMP-13), and matrix metalloproteinase-14 (MMP-14). Of those seven, four have been found to be constitutively expressed in adult cartilage, presumably serving roles in tissue turnover, and upregulated in diseased states—MMP-1, MMP-2, MMP-13, and MMP-14 [3]. The presence of the MMP-3, MMP-8, and MMP-9 in cartilage appears to be characteristic of pathologic circumstances only.

MMP-1 (interstitial collagenase) is involved in the degradation of collagen types I, II, and III. In embryonic development its expression is restricted to areas of endochondral and intramembranous bone formation and is especially abundant in the metaphyses and diaphysis of long bones. During that time, it is expressed in hypertrophic chondrocytes (immediately preceding terminal differentiation in endochondral ossification) and osteoblasts only [4]. Expression levels are low under healthy circumstances, but significant upregulation is observed in arthritic cartilage and may play an active role in collagen degradation in this tissue but is evidently absent in the instance of synovitis [5].

MMP-2 (gelatinase A) is involved in the breakdown of type IV collagen and is most commonly expressed early in the process of wound healing [6]. Expression in adult cartilage is

weak and attributable to normal (very low) collagen turnover, and, similar to MMP-1, it is upregulated in arthritic states [7].

MMP-3 (stromelysin-1) is capable of degrading a wide array of extracellular molecules, including collagen types II, III, IV, IX, and X, fibronectin, laminin, elastin, and various proteoglycans. In addition, it has been found to have transcription factor-like activity, apparently being able to upregulate the expression of other matrix metalloproteinases [8]. It is involved in wound healing, expression being typical in fibroblasts and epithelial cells following expression to inflammatory compounds [9], possibly explaining the presence of high MMP-3 levels in osteoarthritic cartilage and the synovium in osteoarthritis [10] and absence in normal joint tissues and showing promise for this enzyme as a candidate marker for osteoarthritis [11].

MMP-8 (neutrophil collagenase) is the principal collagenase found in human dentin, being involved in turnover and remodeling in that tissue [12], and it is expressed in a wide array of cell types, including neutrophil precursors and epithelial cells [13]. Consistent with most other matrix metalloproteinases, it is involved principally in wound healing, mostly in wounds of an acute character [14]. Its expression in arthritic tissue is clearly beneficial; genetic deficiencies of MMP-8 exacerbate inflammation in arthritis through downregulation of neutrophil apoptosis and clearance, subsequently causing hyperinfiltration of joints with neutrophils [15].

MMP-9 (gelatinase B), similar to MMP-1, is most active during embryonic development, being essential to angiogenesis in the growth plate and apoptosis of hypertrophic chondrocytes in utero [16]. It has also been demonstrated to be highly expressed in the early stages of wound healing [17], perhaps due to its involvement in angiogenesis. In the joint capsule it is produced by monocytes and macrophages; production by chondrocytes appears minimal [18], though these cells do appear to play a considerable regulatory role in leukocyte MMP-9 expression. Inhibition of leukocyte release by chondrocytes appears to be lifted in an arthritic state, apparently in response to increased MMP-3 activity (possibly via transcriptional regulation) and MMP-13 expression [19].

MMP-13 is by far the most studied of the matrix metalloproteinases in terms of its role in cartilage, as it is considered the major catabolic effector in osteoarthritis and other forms of arthritis, owing to its robust ability to cleave the type II collagen that predominates in articular cartilage. Having such a unique and dramatic effect on said tissue, it is obvious why MMP-13 is frequently employed as the matrix metalloproteinase of choice in the detection and study of osteoarthritis. Particularly telling and supporting its utility as a biomarker in osteoarthritis is the tendency of external influences to act upon the joint through upregulation of MMP-13 specifically, as will be discussed in further detail later in this review. Though as is mentioned it is involved principally in the degradation of type II collagen, the enzyme also targets other matrix molecules such as types IV and IX collagen, perlecan, osteonectin, and proteoglycan [20], and it is likely involved in matrix turnover in healthy cartilage.

MMP-14 (membrane-type 1 matrix metalloproteinase) is involved in aggrecan degradation and cadherin cleavage and has been shown to be involved in inhibition of tumor angiogenesis [21]. It has been shown to have a significant role in postnatal bone formation through promotion of osteogenesis and chondrogenesis [22]. MMP-14 is upregulated in arthritic cartilage and furthermore appears to have the ability to activate MMP-2 [23] and MMP-13 [24], potentially compounding its influence in arthritis.

The careful modulation of matrix metalloproteinases is required for maintenance of cartilage health. The presence of these enzymes alone does not constitute pathology; as indicated, deficiencies in MMP-8 are deleterious to joint health; rather a careful balance is required for maintenance of the anabolic/catabolic balance, and it is dysregulation that brings about the catabolism of articular cartilage in arthritic disease.

Having discussed the role of matrix metalloproteinases in healthy cartilage, it is now pertinent to discuss their overexpression and the relation of this phenomenon to disease states, beginning with osteoarthritis and following with rheumatoid arthritis.

3. The HTRA1-DDR2-MMP-13 Axis

As indicated, several matrix metalloproteinases are upregulated and known to play a role, beneficial or deleterious, in osteoarthritis and as such are candidate biomarkers in osteoarthritis. Nevertheless, we look to one in particular, MMP-13, as the principal effector of cartilage degradation in osteoarthritis and the most obvious and useful candidate for matrix metalloproteinases as a biomarker in the disease.

Common to the pathogenesis of osteoarthritis in known processes culminating in the condition is the activation of the HTRA1-DDR2-MMP-13 axis. High temperature requirement A1 (HTRA1), a serine protease, is strongly expressed in the presence of stressors in murine osteoarthritis models. HTRA1 is responsible for degradation of pericellular matrix components, including fibronectin, matrilin 3, collagen oligometric matrix protein, biglycan, fibromodulin, and type VI collagen [25]. Breakdown of the pericellular matrix exposes the chondrocyte membrane to the type II collagen characteristic of articular cartilage, activating and augmenting the expression of the transmembrane discoidin-containing domain receptor 2 (DDR2) [25]. Heightened expression of DDR2 results in excess binding of the receptor to its ligand [26] in turn stimulating high levels of expression of the MMP-13 gene, culminating in the extracellular matrix and leading to the destruction of articular cartilage [27, 28]. Aside from the wide array of evidence showing HTRA1-DDR2-MMP-13 activity in osteoarthritis of multiple modalities, the convergence of diverse noxious stimuli upon this axis is further supported in the protective effects exerted in DDR2 hypomorphic strains of mice, which, when subject to the DMM procedure, demonstrated a significant decrease in the progression of osteoarthritis compared to wild type littermates [29], comparable to the ablation of MMP-13, which has the effect of protecting cartilage from degradation in osteoarthritis induced through destabilization of the medial meniscal ligament (DMM-induced osteoarthritis) in murine models, though interestingly enough chances to the

chondrocytes themselves and other cells in response to the procedure persisted [17].

4. Molecular Pathways Associated with Transcriptional Regulation of MMP-13 Gene Expression

Gene expression of MMP-13 appears to occur through a number of molecular pathways that work through either inflammation or primary cilia. That is not to say there are not some common themes. Stress-inducible nuclear protein 1 (Nupr1) has been shown to regulate MMP-13 expression in vitro [30]. Yammani and Loeser showed that Nupr1, expressed in cartilage, is required for expression of MMP-13 via IL-1β. This might be a pathway for the catabolic effects of OA to be mediated through inflammation. This is especially interesting in light of the study done by Xu et al., 2015, in which they analyzed differential expression of genes in cartilage involved in OA and RA [31]. While these researchers identified multiple genes associated with the regulation of MMPs, the predominant ones were associated with inflammation. This might give greater credence for the role of early inflammatory signals (i.e., AGEs and IL-1) in the initiation and progression of OA. Meanwhile more obviously, a similar role for inflammation appears to be present in RA. Araki et al., 2016, reported that histone methylation and the binding of signal transducer activator of transcription 3 (STAT3) were associated with RA and OA [32]. They report that histone H3 methylation is associated with elevated expression of MMP-1, MMP-3, MMP-9, and MMP-13. However, STAT3 was shown to increase expression, either spontaneous or IL-6 activated, of MMP-1, MMP-3, and MMP-13 but not MMP-9. As previously indicated, primary cilia appear to also be involved in OA. Sugita et al., 2015, reported that transcription factor hairy and enhancer of split-1 (Hes1) is involved in the upregulation of expression of MMP-13 [33]. Normally Hes1 acts as a transcriptional repressor but under the influence of calcium/calmodulin-dependent protein kinase 2 (CaMK2) it becomes a transcriptional activator, thus upregulating MMP-13 expression [34]. Thus Hes1 acts to increase expression of MMP-13. It is of particular interest to note that Hes1 acts through Notch signaling pathway [35]. Notch has previously been shown to modulate sonic hedgehog signaling and work through primary cilia [36, 37]. In an apparent unrelated mechanism, Niebler et al., 2015, showed that the transcription factor AP-2ϵ is intimately involved in the upregulation of MMP-13 as OA progresses [38].

5. Metabolic Syndrome and Upregulation of Matrix Metalloproteinases

An area of study in the field of rheumatology that presents ample opportunity for research and great promise for therapeutic intervention involves the interaction between articular cartilage and metabolic (insulin resistance) syndrome. The comorbidity between osteoarthritis and metabolic syndrome and its individual manifestations is striking—epidemiological data reveals that 49% of individuals with heart disease, 47% with diabetes, 44% with hypertension, and 31% of obese individuals also have some form of arthritis [39], suggestive of a common or overlapping etiology between osteoarthritis and these conditions. Further incriminating a load-bearing hypothesis of osteoarthritis has been the determination that hand osteoarthritis is more strongly correlated with body mass index than hip osteoarthritis, the latter of which was found to have such a weak relationship as to not being statistically significant [40].

The opportunities for interaction between metabolic syndrome and osteoarthritis are vast and cross a temporal spectrum spanning from an initial hyperinsulinemic state [41] to proper insulin resistance [42] to the downstream effects of metabolic syndrome, including but not limited to type II diabetes mellitus [43], a decrease in circulating HDL particles [44], and high circulating levels of adipokines.

While hyperinsulinemia is implicated in the chondrocyte apoptosis facet of osteoarthritis [41], expression of MMP-13 has not been documented to occur until the point of insulin resistance in the joint capsule. In one study, mice fed a high fat diet to generate the obese/type II diabetes mellitus (ob/t2d) phenotype showed considerably increased levels of tumor necrosis factors (TNFs) in the synovial fluid of the knee joint, and studies comparing ob/t2d with TNF knockout mice demonstrated that TNF species were linked to increased expression of MMP-1, MMP-13, and ADAMTS4—a mouse homologue of matrix metalloproteinases. Supplementation with insulin in these mice inhibited these effects by 50% [42].

Leptin, a peptide hormone involved in maintaining insulin sensitivity and contributing to the sensation of satiety, is expressed at very high levels in obese individuals. It appears to be correlated with osteoarthritis as well, with intervention at the level of MMP-13 expression occurring. Downregulation of leptin mRNA translation via small interference RNA molecules inhibits MMP-13 expression in cultured osteoarthritic chondrocytes [45]. The situation appears to be most exacerbated in cases of extreme obesity; there exists a strong positive correlation between the responsiveness of the MMP-13 gene to leptin and the BMI of osteoarthritic individuals [46]. Its effects are not limited to MMP-13 alone; MMP-1 expression and MMP-3 expression are strongly upregulated in osteoarthritic cartilage, and leptin levels strongly correlated with the presence of these two matrix metalloproteinases in osteoarthritic synovial fluid [47]. Adiponectin also appears to have some ability to stimulate expression of MMP-13. The presence of adiponectin is positively correlated with the presence of membrane-associated prostaglandin E2 synthase (mPGES) and MMP-13 [48]. Furthermore, cultured osteoarthritic chondrocytes treated with adiponectin showed increases in production of nitric oxide via inducible nitric oxide synthase (iNOS), expression of MMP-1, MMP-3, and MMP-13, and levels of collagenase-cleaved type II collagen neoepitope. These effects were attenuated in the presence of AMP-activated protein kinase (AMPK), c-Jun N-terminal kinase, and iNOS inhibitors, implicating these as mediators of adiponectin-induced insult [49].

Hyperglycemia is linked to the presence of high levels of circulating advanced glycation end products, particularly of the S100 family of proteins [50]. This phenomenon is

linked to an array of diabetes-induced complications, including atherosclerosis, and a deficiency in the receptor for advanced glycation end products (RAGE) proves to have protective effects, implicating this receptor in the pathway. Ablation of RAGE in osteoarthritic murine models likewise shows a protective effect; wild-type mice, on the other hand, demonstrate increased expression of proinflammatory markers and catabolic mediators, including MMP-13 [51]. RAGE is known to act through a host of proinflammatory means, including NF-κB, TNF, IL-1, and TGF-β [52], and though it has not been conclusively demonstrated, there is abundant potential for a catabolic role of RAGE in metabolic osteoarthritis.

6. Matrix Metalloproteinases in Response to Mechanical Insult

Activation of matrix metalloproteinases, especially MMP-13, is known to occur in response to mechanical injury to the joint, and factors leading to its expression are far more clear-cut than in metabolic osteoarthritis. In response to injury to the joint, the first response that appears to be elicited from chondrocytes and synoviocytes secretes interleukin-1β. This in turn activates the cell surface receptor IL-1RI, which initiates a cascade of intracellular events involving NF-$\kappa\beta$, MAPK, p38, and JNK. This results in increased expression of TNF-α, which further potentiates inflammatory events already in play [53] and initiates chondrocyte expression of MMP-1 [54], MMP-2 [55] and MMP-13 [56].

Over the course of this process, chondrocytes begin to express the cell proliferant transforming growth factor-β (TGF-β) [57], perhaps in response to its ability to mitigate some of the activities of IL-1β and produce additional chondrocytes to cope with stressors placed on the joint. Overexpression of this factor, however, appears to somehow be involved in initiating the osteoarthritic process [58]. This elevation in TGF-β corresponds to an increase in HTRA-1 [59], perhaps in consequence of the ability of the latter to cleave the former [60], and consequentially DDR2 is activated and MMP-13 is highly expressed. The body of evidence implicates MMP-13 as a major effector of mechanically mediated joint destruction in osteoarthritis.

Consistent with previously discussed studies, DDR2 is shown to be a major effector of MMP-13 expression in DMM models, such that almost complete protection from osteoarthritis is shown in DDR2 hypomorphic strains. It is also telling to note that genetic ablation of the receptor for advanced glycation end products (RAGE) corresponds to a decreased expression of MMP-13 in DMM models, though not as dramatic as DDR2 hypomorphism, and that this decreased expression corresponds with decreased progression and severity of osteoarthritis. This suggests a contributing role of RAGE in the pathogenesis of osteoarthritis and certainly a target for therapeutic intervention. Conversely and for reasons that are not yet completely understood, pharmacological antagonism of the proinflammatory transmembrane protein Toll-like receptor 4 (TLR-4) results in heightened MMP-13 expression and a corresponding dramatic increase in the severity of osteoarthritis [61]. Further research is required to elucidate a rationale for this phenomenon, and this information must be considered in devising therapeutic intervention for acute joint injury with the goal of delaying or preventing osteoarthritis development later in life.

7. Genetic Anomalies Resulting in Matrix Metalloproteinases Overexpression and Osteoarthritis

As stated, a number of disease states involve overexpression of matrix metalloproteases, and there exist diseases in which mutations result in overexpression of MMP-13 and premature development of osteoarthritis. One such abnormality is spondyloepiphyseal dysplasia congenita (SEDC), which serves as an experimental model of osteoarthritis. In SEDC, a mutation occurs in the COL2A1 gene, resulting in the formation of superfluous disulfide bridges in type II collagen, adversely affecting association between individual collagen molecules and thus the triple helix that is characteristic of the typical collagen molecule [62]. It is possible that this failure of collagen monomers to form proper interactions predisposes the individual to premature osteoarthritis rendering the molecules ideal targets for the binding and activity of HTRA1 and DDR2 and downstream to these MMP-13 explaining the dramatically increased levels of each characteristic of the syndrome [4].

Another disease that has been the focus of osteoarthritis research as of late is the ciliopathy Bardet-Biedl syndrome (BBS). BBS may result from mutations in a number of the known genes that are involved in ciliary formation and transport [63], resulting in a number of congenital abnormalities including polydactyly, cognitive impairment, cardiac and renal malformation, obesity hypertension, and type II diabetes mellitus. In addition, mouse models of BBS manifest early onset osteoarthritis; whether this is a direct result of ciliary malformation or secondary to osteoarthritis risk factors such as obesity and type II diabetes mellitus is presently unclear. What is known is that, in BBS osteoarthritic cartilage, TGF-β is downregulated, HTRA1 is upregulated, and MMP-13 is strongly expressed in the absence of DDR2. This finding strongly suggests a role of primary cilia in DDR2 activity and/or signal transduction, and further research is clearly warranted to elucidate the link between cilia and DDR2.

8. Matrix Metalloproteinases and Rheumatoid Arthritis

Rheumatoid arthritis is a form of arthritis in which the host immune system mounts an attack on connective tissue in the joint. MMPs are associated with rheumatoid arthritis [64]. In their study, Ahrens et al. demonstrated that MMP-9 is elevated in the synovial fluid of patients with rheumatoid arthritis. Indeed, they indicated that SF levels of MMP-9 were higher in rheumatoid arthritis patients compared to osteoarthritis. It is of interest to note that they also speculated that elevated MMP-9 was associated with connective tissue turnover in rheumatoid arthritis patients.

These observations led to the intriguing possibility of determining the severity of rheumatoid arthritis by examination of matrix metalloproteinases in blood. Keyszer et al. were the first to show a correlation between blood circulating matrix metalloproteinases and the clinical manifestation of rheumatoid arthritis [65]. They demonstrated that circulating levels of MMP-3 were a better predictor of rheumatoid arthritis severity or activity than cytokine level. These observations led to the thinking that perhaps clinicians could separate the immunological and inflammatory mechanisms associated with RA from the actual erosion of articular surface. Uncoupling these two pathophysiological pathways may aid in new treatment regimens and strategies. Indeed, Cunnane et al. were the first to make this connection [66]. They showed that the degree of articular surface erosion correlated with levels of MMP-1 and MMP-3. They concluded that treatments for rheumatoid arthritis which specifically target MMP-1 may limit the number of new joint erosion foci and thus improve the overall functional outcome for RA patients.

Gender can be a complicating issue for both osteoarthritis and rheumatoid arthritis. In a recent study in which matrix metalloproteinases associated with tuberculosis were examined in relation to gender, Sathyamoorthy et al. found that while plasma MMP-8 concentrations inversely correlated with body mass index, they were significantly higher in males than in females [67]. The authors pointed out that this significant difference in gender expression of MMP-8 was not associated with or due to disease severity. Indeed, they concluded that plasma analysis of MMP-1 and MMP-8 was a better discriminator for tuberculosis in men than in women. In a more recent study, it was shown that plasma MMP-3 was significantly higher in men compared to women in a number of clinical conditions that included both infectious and noninfectious diseases including those rheumatic in nature [68].

9. Conclusion

Expression of MMPs is ubiquitous characteristic of development. Adherently high expression of MMPs is associated with a host of diseases—infectious and noninfectious. They are particularly significant in the progression and severity of osteoarthritis regardless of etiology. This attests to their utility as major biomarkers for osteoarthritis and mediators of joint destruction. It is worthy to note that MMP-13 is most notably associated with osteoarthritis and once its expression is elevated in the joint significant damage is imminent and the progression to joint destruction is rapid. Present medical knowledge does not provide a way to reverse damage to cartilage caused by osteoarthritis regardless of the insulting modality. It is highly likely that pharmacologic intervention of osteoarthritis will target upstream of MMP-13 expression.

The present short-term treatment for osteoarthritis is pain management, typically in the form of opiates and nonsteroidal anti-inflammatory drugs. While effective at improving the quality of life for osteoarthritis sufferers for a time, the long-term health consequences are severe, and in spite of such interventions joint replacement is almost invariably necessary eventually. There is much opportunity for research leading to an understanding of the processes upstream of the expression of MMP-13.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

Expressions of Matrix Metalloproteinases 2, 7, and 9 in Carcinogenesis of Pancreatic Ductal Adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) is a highly fatal disease, usually diagnosed in an advanced stage which gives a slight chance of recovery. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that participate in tissue remodeling and stimulate neovascularization and inflammatory response. The aim of the study was to evaluate the expression of MMP-2, MMP-7, and MMP-9 in normal ducts, tumor pancreatic adenocarcinoma cells, and peritumoral stroma in correlation with clinicohistopathological parameters. The study material was obtained from 29 patients with pancreatic ductal adenocarcinoma. The expressions of MMP-2, MMP-7, and MMP-9 were performed by immunohistochemical technique. Microvessel density (MVD) was visualized by special immunostaining. The expressions of MMP-2, MMP-7, and MMP-9 were mainly observed in tumor cells and peritumoral stroma. MMP-2 expression in cancer cells was correlated with female gender, stronger inflammation, and histopathological type of cancer ($R = 0.460$, $p = 0.013$; $R = 0.690$, $p = 0.0001$; $R = -0.440$, $p = 0.005$, resp.). The expression of MMP-7 in tumor cells was found to positively correlate with the presence of necrosis and negatively correlate with MVD ($R = 0.402$, $p = 0.031$; $R = -0.682$, $p = 0.000$). We also showed that positive MMP-9 expression in tumor cells was associated with MVD ($R = 0.368$, $p = 0.084$); however, it was not statistically significant. Our results demonstrate that MMP-2, MMP-7, and MMP-9 expressions correlate with various morphological features of the PDAC tumor such as inflammation, necrosis, and formation of the new blood vessels.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly fatal disease, usually diagnosed in an advanced stage which gives a slight chance of recovery. Pancreatic cancer is characterized by a rapid course, poor prognosis, and high mortality since most patients are diagnosed with metastases to lymph nodes and distant organs [1]. This increases with age and is closely associated with genetic factors [2].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases [3]. MMPs are produced by connective tissue cells (fibroblasts), leukocytes, macrophages, and endothelial cells [4]. They are involved in degradation of the extracellular matrix and have been implicated in physiological processes. MMPs are involved in supporting tissue remodeling and are required for the skeletal development. They stimulate neovascularization, both in physiological and in pathological conditions, for example, in tumors [3]. MMPs can regulate vascular stability and permeability in response to tissue injury [5]. Metalloproteinases are responsible for proper migration of cells involved in the inflammatory response [6]. They allow the cells to move into the damaged tissue and to release cytokines and their receptors through

their ability to destroy the basement membrane. It has been shown that metalloproteinases destroy interleukin-2 receptors on T cells which mute the immune response against tumor cells. The imbalance between the activity of MMPs and their tissue inhibitors (TIMPs) has been attributed to the ability of cancer cells to migrate [7]. Recent studies have confirmed that the overexpression of MMPs in tumor and stromal cells in various cancers was associated with tumor invasion and progression [8]. Moreover, MMPs released from distant organs, along with tumor cell growth factors, can participate in premetastatic niche formation and metastasis [9, 10].

Therefore, the aim of our study was to evaluate the immunohistochemical expressions of MMP-2, MMP-7, and MMP-9 in the normal pancreas, pancreatic adenocarcinoma tumor cells, and stromal cells in correlation with clinicopathological features.

2. Material and Methods

2.1. Materials. The study material was obtained from 29 patients (23 men, 6 women; 14 patients aged ≤60 and 15 aged >60) with pancreatic ductal adenocarcinoma (PDAC), operated on in the 2nd Department of General Surgery and Gastroenterology, Medical University of Białystok. Patients received neither preoperative cancer therapy nor inflammation therapy.

The study was performed in conformity with the Declaration of Helsinki for Human Experimentation and received approval by the Local Bioethics Committee of the Medical University of Białystok (number R-I-002/167/2013).

2.2. Histopathological Examination. The routine histopathological assessment of the sections (stained with H+E) was conducted with reference to the histological type, malignancy grade (G), clinicopathological pT status, regional lymph node involvement, and the presence of distant metastases. Inflammation was defined as mild when it consisted of <10 immune cells per 10 hpf under 100x magnification; as moderate when it consisted of 10 to 50 immune cells; and as severe when it consisted of > 50 immune cells [11, 12]. Desmoplasia was classified as poor when it occupied <25% of tumor area observed in 10 hpf under 100x magnification, whereas it was predominant for >25%. Hemorrhage was measured under 400x magnification and defined as single (one focus) or numerous (more than one hemorrhagic focus). Necrosis in the central tumor was graded as absent (none), weak/focal (<10% of tumor area), moderate (10–30%), or strong/extensive (>30%) [13].

2.3. Assessment of Vessel Formation. Microvessel density (MVD) was visualized by special immunostaining of collagen type IV. Since protein can be expressed on the mesenchymal components and epithelial basal laminae, to prevent misdiagnosis, we analyzed only vascular structures with distinct (slot-like or tubular) lumens showing positively stained endotheliocyte layers. They were counted as a number of intratumoral microvessels per unit area of the tumor, subjectively selected from the most vascularized areas (5 hpf under 200x magnification) [14]. The PDAC tumors were divided into two groups, with low or high MVD. The cutoff value was the mean MVD. The mean MVD of the tumor tissue was 5.28 vessels (range: 0–21), confirming the histopathological features of hypovascular pancreatic tumors.

2.4. Immunohistochemical Analysis. Formalin-fixed and paraffin-embedded tissue specimens were cut on a microtome into 4 μm sections. The sections were deparaffinized in xylenes (CHEM*115208603; Chempur, Poland) and hydrated in alcohols. To visualize the antigens of MMP-2, MMP-7, and MMP-9, the sections were heated in a microwave oven for 20 min in EDTA buffer (pH = 9.0) (EDTA buffer, Antigen Retriever; E1161; Sigma-Aldrich Co., MO, USA). Then, they were incubated with 3% hydrogen peroxide solution for 20 min in order to block endogenous peroxidase. Next, incubation was performed with anti-human antibodies against monoclonal antibody of matrix metalloproteinase 2 (clone 17B11; Novocastra, UK; dilution 1 : 60), mouse monoclonal antibody of matrix metalloproteinase 7 (clone 111433; R&D Systems, USA; dilution 1 : 75), and mouse monoclonal antibody of matrix metalloproteinase 9 (clone 15W2; Novocastra, UK, dilution 1 : 80) during a period of 1 hour at room temperature. The reaction was carried out using the streptavidin-biotin system (Biotinylated Secondary Antibody, Streptavidin-HRP, Novocastra, UK). A color reaction for peroxidase was developed with chromogen DAB (DAB, Novocastra, UK). The negative control section was incubated instead of the primary antibody. All section slides were counterstained with hematoxylin.

Immunohistochemical staining was evaluated by two independent pathologists who were blinded to the clinical information. A positive reaction was observed in the cytoplasm of the normal pancreas, pancreatic ductal adenocarcinoma, and peritumoral stroma. Due to the small study group, immune cells and fibroblasts were analyzed jointly. The expression of proteins was evaluated and defined as positive (reaction present in >25% of normal ductal cells, tumor cells, or peritumoral stroma components) or negative (lack of reaction or reaction present in <25% of normal ductal cells, tumor cells, or peritumoral stroma components). The percentage of the reaction-positive cells was calculated in 500 neoplastic cells in each study sample at 400x magnification.

2.5. Statistical Analysis. Statistical analysis was conducted using Statistica 10.0 (StatSoft, Kraków, Poland). In order to compare the two groups, the parameters were calculated by the Chi-quadrate test. Correlations between the parameters were calculated by Spearman's correlation coefficient test. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Histopathological Findings. Pancreatic adenocarcinomas were moderately differentiated (G2) in 25/29 cases and poorly differentiated (G3) in 4/29 cases. They were classified as mucinous in 3/29 cases and as nonmucinous in 26/29 cases. We noted lymph node involvement in 12/29 cases and metastases to distant organs (liver, intestine) in 9/29

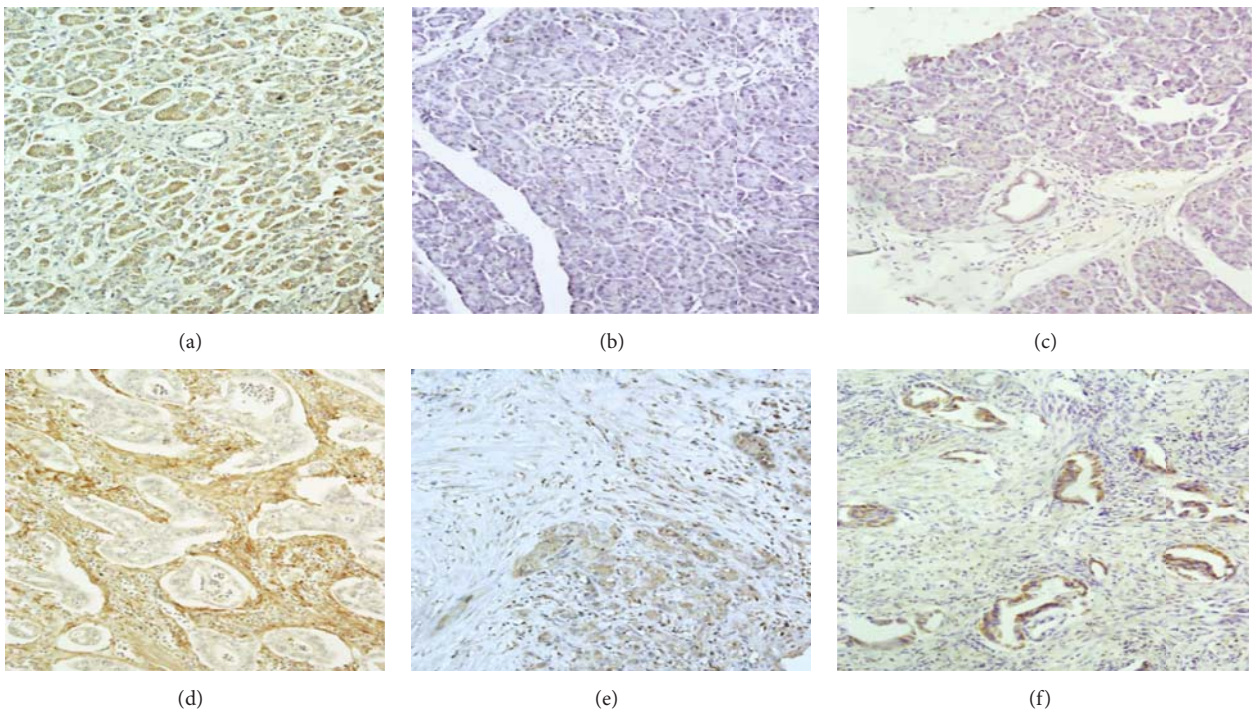

Figure 1: Immunohistochemical expressions of MMP-2, MMP-7, and MMP-9 in normal ducts, tumor cells, and peritumoral stroma of PDAC. The lack of MMP-2 (a), MMP-7 (b), and MMP-9 (c) expressions was found in normal ducts. Positive reaction of MMP-9 (d) was noted in the cytoplasm of pancreatic adenocarcinoma cells. MMP-2 (d) overexpression was observed in the peritumoral stroma in the majority of PDAC cases and color reaction of MMP-7 was observed in the cytoplasm of both cancer cells and the stroma (e). Positive reaction of MMP-9 was noted in the cytoplasm of pancreatic adenocarcinoma cells (f).

cases. In addition, we assessed the degree of inflammation, desmoplasia, necrosis, and foci of hemorrhage. We observed a weak inflammatory response in 9 cases, moderate response in 10 cases, and strong response in 10 cases. Tumors with poor and prominent fibrosis were noted in 12 and 17 cases, respectively. Hemorrhage was numerous in 5 cases and single in 10 cases. Pancreatic adenocarcinomas were associated with weak or moderate necrosis in 13 cases.

3.2. Expression of Matrix Metalloproteinases (MMP-2, MMP-7, and MMP-9) in Normal Ducts, Pancreatic Adenocarcinoma Tumor Cells, and Stromal Cells. The expression of MMP-2 was predominantly present in tumor cells and stroma (55.17% and 79.31%, resp.) in comparison to its absence in the normal pancreas (96.55%). In contrast, the positive expression of MMP-7 was observed mainly in tumor cells (96.55%), less in the stromal cells (55.17%), as compared to the normal pancreas (93.10%). Immunohistochemical assessment of MMP-9 in patients with PDAC showed the protein presence in tumor cells in 44.83% of cases and its absence in normal pancreatic ducts and stroma (100% and 75.87%, resp.) (Figure 1). Furthermore, statistical analysis showed a significant difference between the expressions of MMP-2 and MMP-7 in normal and tumor tissues ($p = 0.0001$). In addition, the expression of MMP-7 in tumor cells differed statistically significantly from that noted in the peritumoral stroma ($p = 0.005$). The differences in MMP expressions (MMP-2, MMP-7, and MMP-9) in normal tissue, tumor cells, and stroma are shown in Table 1.

3.3. The Correlation between the Expression of Matrix Metalloproteinases (MMP-2, MMP-7, and MMP-9) in Tumors of PDAC and Clinicopathological Features. The analysis of the expression of matrix metalloproteinases in a variety of tissues and selected anatomical clinical factors demonstrated a correlation between MMP-2 expression in tumor and female gender ($R = 0.460$, $p = 0.013$). A strong positive correlation was noted between MMP-2 in tumor tissue and the presence of stronger inflammation ($R = 0.690$, $p = 0.0001$). MMP-2 expression in stromal cells was found to negatively correlate with the nonmucinous type of PDAC ($R = -0.440$, $p = 0.005$). Positive expression of MMP-2 was more frequent in patients over 60 years of age, although it was not statistically significant. MMP-7 expression in tumor cells revealed a positive association with more frequent occurrence of necrosis in the main mass of tumor ($R = 0.402$, $p = 0.031$) and a negative correlation with the lower index of MVD ($R = -0.681$, $p = 0.000$). Positive expression of MMP-7 was accompanied by frequent changes indicating the presence of necrosis. The expressions of matrix metalloproteinases were not correlated with malignancy grade, fibrosis degree, the incidence of hemorrhage, or the presence of metastases to lymph nodes and distant organs (Tables 2 and 3).

Table 1: Expressions of MMP-2, MMP-7, and MMP-9 in normal pancreas, cancer cells, and stroma.

	MMP-2		MMP-7		MMP-9	
	Negative	Positive	Negative	Positive	Negative	Positive
Normal tissue	28 (96.55%)	1 (3.45%)	27 (93.10%)	2 (6.90%)	29 (100%)	0 (0%)
Cancer cells	13 (44.83%)	16 (55.17%)*	1 (3.45%)	28 (96.55%)**	16 (55.17%)	13 (44.83%)
Tumor stroma	6 (20.69%)	23 (79.31%)	13 (44.83%)	16 (55.17%)***	22 (75.87%)	7 (24.13%)

*MMP-2 in cancer cells versus MMP-2 in normal tissue, $p = 0.0001$.
**MMP-7 in cancer cells versus MMP-7 in normal tissue, $p = 0.0001$.
***MMP-7 in cancer cells versus MMP-7 in tumor stroma, $p = 0.005$.

Table 2: Correlations between MMP-2, MMP-7, and MMP-9 expressions in tumor cells and clinicopathological parameters.

Variables	Patients, N (%)	MMP-2		MMP-7		MMP-9	
		R	p value	R	p value	R	p value
Gender							
Male	23 (79.3)	0.460	**0.013**	−0.047	0.814	−0.042	0.804
Female	6 (20.7)						
Age							
≤60	14 (48.3)	−0.012	0.949	0.339	0.071	−0.383	**0.046**
>60	15 (51.7)						
Inflammation							
Absent	2 (6.9)						
Weak	9 (31.0)	0.690	<**0.001**	0.053	0.782	0.081	0.666
Moderate	10 (34.5)						
Strong	8 (27.6)						
Desmoplasia							
Poor	12 (41.4)	0.016	0.931	0.215	0.262	0.135	0.491
Prominent	17 (58.6)						
Foci of hemorrhage							
Absent	14 (48.3)						
Single	10 (34.5)	0.088	0.648	0.161	0.403	−0.271	0.162
Numerous	5 (17.2)						
Necrosis							
Absent	16 (55.2)						
Weak	7 (24.1)	−0.007	0.968	0.402	**0.031**	0.084	0.668
Moderate	6 (20.7)						
Strong	0 (0.0)						
MVD							
Low	15 (51.7)	0.072	0.738	**−0.682**	<**0.001**	0.368	0.084
High	14 (48.3)						
Adenocarcinoma type							
Nonmucinous	26 (89.7)	−0.193	0.314	−0.139	0.495	−0.081	0.681
Mucinous	3 (10.3)						
Grade of malignancy							
G2	25 (86.2)	−0.156	0.417	−0.036	0.850	−0.129	0.512
G3	4 (13.8)						
Lymph node involvement							
Absent	17 (58.6)	0.024	0.899	−0.261	0.172	0.033	0.866
Present	12 (41.4)						
Distant metastases							
Absent	20 (69.0)	0.014	0.940	−0.193	0.313	−0.081	0.681
Present	9 (31.0)						

Table 3: Correlations between MMP-2, MMP-7, and MMP-9 expressions in peritumoral stroma cells and clinicopathological parameters.

Variables	Patients, *N* (%)	MMP-2		MMP-7		MMP-9	
		R	*p* value	*R*	*p* value	*R*	*p* value
Gender							
Male	23 (79.3)	−0.051	0.793	−0.603	0.060	−0.237	0.215
Female	6 (20.7)						
Age							
≤60	14 (48.3)	0.199	0.299	0.029	0.879	−0.261	0.170
>60	15 (51.7)						
Inflammation							
Absent	2 (6.9)						
Weak	9 (31.0)	0.263	0.167	0.132	0.494	−0.042	0.826
Moderate	10 (34.5)						
Strong	8 (27.6)						
Desmoplasia							
Poor	12 (41.4)	0.033	0.864	−0.129	0.505	0.189	0.325
Prominent	17 (58.6)						
Foci of hemorrhage							
Absent	14 (48.3)						
Single	10 (34.5)	−0.198	0.302	−0.010	0.958	−0.164	0.395
Numerous	5 (17.2)						
Necrosis							
Absent	16 (55.2)						
Weak	7 (24.1)	−0.196	0.306	−0.050	0.796	0.121	0.529
Moderate	6 (20.7)						
Strong	0 (0.0)						
MVD							
Low	15 (51.7)	−0.220	0.300	−0.022	0.916	−0.046	0.829
High	14 (48.3)						
Adenocarcinoma type							
Nonmucinous	26 (89.7)	−0.440	**0.005**	0.106	0.584	−0.194	0.313
Mucinous	3 (10.3)						
Grade of malignancy							
G2	25 (86.2)	−0.130	0.500	0.125	0.518	−0.021	0.912
G3	4 (13.8)						
Lymph node involvement							
Absent	17 (58.6)	−0.021	0.910	−0.176	0.360	−0.105	0.586
Present	12 (41.4)						
Distant metastases							
Absent	20 (69.0)	0.101	0.602	−0.106	0.582	−0.224	0.241
Present	9 (31.0)						

4. Discussion

PDAC has poor prognosis and patients' survival time is estimated to be several months. The research into mechanisms of the outstanding malignancy and invasiveness of tumor cells is still being conducted [15]. It has been proven that metalloproteinases are involved in different phases of tumor development, such as extracellular matrix degradation, neovascularization, inhibition of inflammatory cell migration, and metastasis formation in the lymph nodes and distant organs [3, 5, 9, 15]. Their role in the above mechanisms has been investigated in various types of the digestive system tumors located in the colorectum, the stomach, and the liver [16–19].

In our study, we noted a positive expression of MMP-2 in 55.17% of tumor cells and in 79.31% of the stroma, which was significantly higher than in the normal pancreas (3.45%). Giannopoulos et al. [17] also showed the presence of MMP-2 in cancer cells in most cases of PDAC. In turn, Gress et al. [18] demonstrated that the overexpression of MMP-2 was significantly higher in tumor cells than in the stroma. We also reported a positive correlation between the

expression of MMP-2 in tumor cells and inflammation. This may suggest that MMP-2 protein is secreted from tumor cells to the stroma where it modifies the immune response cells. In our research, MMP-2 expression was significantly more frequent in the nonmucinous type of PDAC. Histologically, the nonmucinous type of pancreatic cancer is characterized by tubular structures generally located in the rich desmoplastic stroma. Tumor cells of the mucinous type have the ability to produce a large amount of mucus that fills in the tumor microenvironment and may limit the development of connective tissue. The results of our small study suggest that MMP-2 expression correlates with the histopathological type of PDAC and that its expression may be involved in the regulation of the inflammatory response.

MMPs are involved in the degradation of ECM leading to promotion of cancer cell invasion. The smallest family of MMPs, namely, MMP-7, shows the greatest proteolytic activity [19]. In our study, the positive MMP-7 expression was present in most cases in tumor cells, in more than half of the cases in the stroma, and in only two cases in normal pancreatic ducts. Crawford et al. [20] and Li et al. [21] showed the presence of MMP-7 expression in the cytoplasm of most tumor cells and its absence in normal pancreatic ducts. Our statistical analysis demonstrated a significant correlation between MMP-7 expression in PDAC cells and a positive reaction in stromal cells as well as in normal ducts. These results are consistent with the observations of Jones et al. [22]. In contrast, Yamamoto et al. [23] reported an increase in MMP-7 expression in tumor nests located in the front of PDAC tumors. Tan et al. [24] showed that MMP-7 overexpression in the tumor front was present much less frequently than in the center of the tumor mass. In our study, the positive expression of MMP-7 in PDAC tumors was associated with the presence of necrotic lesions. Other studies have confirmed that MMP-7 shows proteolytic activity, participates in cell dissociation through disruption of tight junction structures, determines tumor dissociation from the primary tumor, and stimulates cancer cell invasion [25, 26]. Moreover, we observed a strong, negative relationship between MMP-7 expression in tumor cells and MVD. MMP-7 can cleave collagen IV which constitutes the skeleton of the basement membranes on blood vessels and the ECM components. In turn, MMP-7 may lead to the degradation of the tumor stroma, decay of current vessels, and the inhibition of neovascularization. As a result of these processes, necrotic lesions were observed in tumor mass and hypovascular features of PDAC tumors were noted. Our findings suggest that MMP-7 expression in PDAC cancer cells may contribute to the degradation of the peritumoral stroma, thus facilitating tumor cell invasion and metastasis.

MMP-9 is considered to be a strong factor stimulating the secretion of proangiogenic factors, such as vascular endothelial growth factor (VEGF) which participates in the new blood vessel formation [26, 27]. The study of mouse model has confirmed that tumor cells in MMP-9+/+ mice produce bigger pancreatic tumors with high index of MVD [24]. Moreover, Huang et al. [28] also demonstrated an increased incidence of cancer and tumor size in MMP-9+/+ mice, which was associated with a high index of MVD and increased macrophage infiltration. We noted positive expression of MMP-9 in the cytoplasm of tumor cells and in the stroma of patients with PDAC. Our observations are consistent with those reported by Giannopoulos et al. [17] and Gress et al. [18]. Statistical analysis confirmed that the positive expression of MMP-9 only in the tumor cells showed a growing tendency in MVD. These observations suggest that MMP-9 has an angiogenic property in comparison with much stronger desmoplastic activity of MMP-2 and proteolytic activity of MMP-7 in the tumor stroma in PDAC tumors.

In conclusion, our findings confirmed that MMP-2, MMP-7, and MMP-9 may take part in various morphological features of PDAC tumor. MMP-2 is mainly responsible for the regulation of inflammatory response. MMP-7 takes part in the degradation of the peritumoral stroma whereas MMP-9 may have an impact on the formation of the new blood vessels. In our opinion, immunohistochemical evaluation of the study metalloproteinases in the tissue of patients with PDAC may help to better understand the morphology and development of PDAC tumors. Our observations need to be confirmed in a larger group of PDAC tumors.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Serum Gelatinases Levels in Multiple Sclerosis Patients during 21 Months of Natalizumab Therapy

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Background. Natalizumab is a highly effective treatment approved for multiple sclerosis (MS). The opening of the blood-brain barrier mediated by matrix metalloproteinases (MMPs) is considered a crucial step in MS pathogenesis. Our goal was to verify the utility of serum levels of active MMP-2 and MMP-9 as biomarkers in twenty MS patients treated with Natalizumab. *Methods*. Serum levels of active MMP-2 and MMP-9 and of specific tissue inhibitors TIMP-1 and TIMP-2 were determined before treatment and for 21 months of therapy. *Results*. Serum levels of active MMP-2 and MMP-9 and of TIMP-1 and TIMP-2 did not differ during the treatment. The ratio between MMP-9 and MMP-2 was increased at the 15th month compared with the 3rd, 6th, and 9th months, greater at the 18th month than at the 3rd and 6th months, and higher at the 21st than at the 3rd and 6th months. *Discussion*. Our data indicate that an imbalance between active MMP-9 and active MMP-2 can occur in MS patients after 15 months of Natalizumab therapy; however, they do not support the use of serum active MMP-2 and active MMP-9 and TIMP-1 and TIMP-2 levels as biomarkers for monitoring therapeutic response to Natalizumab.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) of presumed autoimmune origin that is characterized by demyelination and axonal loss [1]. MS affects young adults, women more frequently than men, and is clinically marked by exacerbations, called relapses, which typically show dissemination in space and time [2]. Brain inflammation is initiated and sustained by lymphocyte migration across the blood-brain barrier (BBB) [3]. In particular, the interaction of $\alpha 4\beta 1$ integrin on the surface of lymphocytes with vascular-cell adhesion molecule 1 (VCAM-1) and on the surface of vascular endothelial cells in brain and spinal cord blood vessels mediates the adhesion and migration of lymphocytes in inflamed CNS sites [4]. Natalizumab (Tysabri, Biogen Idec Inc., Cambridge, Massachusetts, USA) is a humanized anti-$\alpha 4$ integrin monoclonal antibody approved for relapsing-remitting multiple sclerosis (RRMS) [5, 6]. The efficacy of Natalizumab monotherapy was demonstrated in clinical trials by the reduction in relapse rate and the progression of disability [7]. Consequently, Natalizumab is used as a second-line treatment in MS patients who have a suboptimal response to first-line disease-modifying therapies or as a first-line therapy in those with a highly active disease [8]. Notwithstanding the unquestionable benefits, anti-$\alpha 4$ integrin treatment is, however, associated with John Cunningham Virus- (JCV-) mediated progressive multifocal leukoencephalopathy (PML), a severe adverse event [9].

Although MS etiology remains largely unknown, the migration of immunocompetent cells into the CNS is dependent on several factors, but it fundamentally requires the opening of the BBB, a mechanism in which matrix metalloproteinases (MMPs) play a crucial role [10, 11]. These enzymes are a family of zinc-containing and calcium-requiring endopeptidases, which are secreted into extracellular space as a latent inactive proform that becomes activated through a proteolytic cleavage [12]. Specific tissue inhibitors of metalloproteinases (TIMPs) are molecules capable of binding either activated MMPs or their preforms and then finally of regulating MMP activity [13]. Due to their ability to degrade type IV collagen and gelatin, which are the main constituents of basal lamina, MMP-2 (gelatinase A, 72 kDa type IV collagenase) and MMP-9 (gelatinase B, 92 kDa type IV collagenase) are the most extensively studied subfamily of MMPs in the course of MS. In particular, previous studies demonstrated that cerebrospinal fluid (CSF) and serum levels of MMP-9 were higher in RRMS compared to progressive forms [14–16] and that elevated CSF and serum concentrations of MMP-9 were associated with clinical and magnetic resonance imaging (MRI) evidence of disease activity [15, 17–19] and disease evolution [20]. Moreover, CSF levels of MMP-9 were reduced after 12 months of Natalizumab treatment in 7 MS patients and in the same study CSF MMP-9 mean levels were higher in MS patients before Natalizumab treatment than in patients with other neurological diseases [21]. On the other hand, the significance of MMP-2 is more controversial in MS. In fact, while MMP-9 is predominantly upregulated in inflammatory conditions, MMP-2 is constitutively expressed in the brain [22]. Contradictory results have been reported in previous studies where MMP-2 levels in acute and chronic demyelinated lesions [23–25] as well as in CSF [26] in serum [15, 17, 20] and in peripheral blood mononuclear cells (PBMCs) [27–29] were increased, decreased, or represented in equivalent amounts in MS patients and in controls. In two previous studies we investigated the role of the active forms of MMP-9 and MMP-2 in MS and our results showed a reciprocal variation in these enzymes compared to the activity of the disease. In particular, serum and CSF levels and the intrathecal synthesis of active MMP-9 forms were associated with clinical and MRI disease activity [30] whereas CSF levels and intrathecal synthesis of active MMP-2 were more elevated in MS patients without MRI evidence of disease activity [31].

Considering these findings, in this study we, aimed to investigate serum temporal concentrations of active MMP-2 and active MMP-9 in a cohort of RRMS patients during 21 months of Natalizumab therapy.

2. Materials and Methods

2.1. Study Design and Sample Handling. The study design and the sample population were the same as in an earlier study [32]. Briefly, twenty consecutive RRMS [33] patients (17 female and 3 male) in treatment with Natalizumab were included in the study. All the patients were enrolled in the "Fondazione Istituto Neurologico C. Mondino" in Pavia. Serum samples were collected before starting therapy and then every three months for 21 months of treatment. All the samples were withdrawn, stored, and analyzed under the same conditions. At any time point: (a) disease severity was scored using Kurtzke's Expanded Disability Status Scale (EDSS) [34]; (b) presence of relapse was recorded as clinical activity; and (c) anti-JCV antibodies were determined to assess the risk of PML [35]. During the treatment, disability progression was defined as an increase of one point on EDSS score from baseline [5]. Brain MRI scans were performed at entry and at the end of the study and the occurrence of a new lesion on T2-weighted scans and/or a new gadolinium-(Gd-) enhancing lesion on T1-weighted scans was defined as MRI activity [33]. The approval of the Committee for Medical Ethics in Research was obtained for experiments involving human subjects. Written informed consent was obtained from all subjects participating in the study.

2.2. MMP-2 and MMP-9 Activity Assays. Serum levels of active MMP-2 and active MMP-9 were determined using commercially available specific activity assay systems as published before [30, 31] (Activity Assay System, Biotrak, Amersham Biosciences, Little Chalfont, UK; code RPN2631 and code RPN2634, resp.). With these methods, only circulating active forms of MMP-2 and MMP-9 were measured. All the reagents and standards were included in the kits. Briefly, in both activity assays, serum samples were applied in duplicate into 96-microwell microtiter plates precoated with anti-MMP-2 or anti-MMP-9 antibodies. Human pro-MMP-2 and pro-MMP-9, respectively, activated with p-aminophenylmercuric acetate, were used in six serial dilutions, as standard, in each plate. The detection enzyme was the proform of a modified urokinase, an enzyme that can be activated by captured active MMPs in an active detection enzyme. The natural activation sequence in the prodetection enzyme was replaced using protein engineering, with an artificial sequence recognized by specific MMP. Activated urokinases were then measured using a specific chromogenic substrate (S-2444™). The amount of active MMP-2 or active MMP-9 in all samples was determined by interpolation from a standard curve. According to the manufacturer's instructions, for the MMP-2 activity assay, the lower limit of quantification was 0.19 ng/mL, the range of intra-assay coefficient of variations (CV) was 4.4–7.0%, and the range of interassay CV was 16.9–18.5%, while for the MMP-9 activity assay the lower limit of quantification was assumed at 0.125 ng/mL, the range of intra-assay CV was 3.4–4.3%, and the range of interassay CV was 20.2–21.7%.

2.3. TIMP-1 and TIMP-2 Detection Assays. As previously described [30, 31], serum levels of TIMP-1 and TIMP-2 were measured using commercially available "sandwich" ELISA kits (Biotrak, Amersham Biosciences, Little Chalfont, UK; codes RPN2611 and RPN2618, resp.) according to the manufacturer's instructions. All the reagents and standards were included in the kits. The limit of sensitivity in both the assays was 3.13 ng/mL.

2.4. Data Analysis. Statistical analysis was performed with GraphPad Prism®. The normality of each variable was

Table 1: Demographic and clinical characteristics of 20 RRMS patients stratified according to response to therapy before and during treatment with Natalizumab.

	Responders	Nonresponders
Patients (n)	15	5
Sex (male/female)	3/12	0/5
Age at entry, years (mean ± SD)	35.1 ± 10.1	31.6 ± 9.4
EDSS at baseline (mean ± SD)	1.0 ± 1.1	2.3 ± 2.4
EDSS after 21 months of therapy	1.3 ± 1.3	2.8 ± 2.4
Relapses during 21 months of therapy (mean ± SD)	0	1.6 ± 0.9
Patients with new MRI lesions at the end of treatment	4	0

EDSS = Expanded Disability Status Scale; MRI = magnetic resonance imaging; SD = standard deviation.

checked by using the Kolmogorov-Smirnov test. When normality of data distribution was found in all variables, statistical analysis was performed by a parametric approach. Accordingly, ANOVA test was used to compare variables among the various groups, and when significant differences were found, Student's t-test was used for the comparison between two groups. On the other hand, when normality of data distribution was rejected, statistical analysis was performed by a nonparametric approach. Kruskal-Wallis test was used to compare variables among the various groups and if significant differences were found, Mann-Whitney U-test was then used to compare two different groups. In case of multiple comparisons, a Bonferroni post hoc correction was applied. A value of $p < 0.05$ was accepted as significant.

3. Results

Demographic and clinical characteristics of 20 RRMS patients treated with Natalizumab are listed in Table 1. During the therapy five patients experienced relapses (3 patients had 1 relapse between baseline and 3 months, one had 2 relapses between 6 and 9 months and at 12 months, and one had 2 relapses between 9 and 12 months and between 18 and 21 months) and four patients showed new T2 and/or Gd-enhancing lesions on the last MRI examination at the 21st month. No patients showed anti-JCV seroconversion during the 21 months of Natalizumab treatment. The timing of sample collection was not sequential and resulted incomplete for ten patients. However, we decided to analyze all the variables in RRMS patients considered as a whole. Serum levels of active MMP-2, active MMP-9, and TIMP-1 were detected in all samples, while serum levels of TIMP-2 were measured in 145/148 (98%) of samples. As reported in Figure 1, no differences were found for serum levels of active MMP-2 (panel (a), ANOVA: n.s.) and active MMP-9 (panel (b), Kruskal-Wallis: n.s.) and TIMP-2 (panel (c), Kruskal-Wallis: n.s.) and TIMP-1 (panel (d), ANOVA: n.s.) among the various time points. The ratios between MMPs and the specific tissue inhibitors and between active MMP-9 and active MMP-2 were then calculated for all the patients at each time point (Figure 2). No differences were found for the MMP-2/TIMP-2 (panel (a), Kruskal-Wallis: n.s.) and MMP-9/TIMP-1 (panel (b), Kruskal-Wallis: n.s.) ratios while the active MMP-9/active MMP-2 ratio was different at various time points (panel (c), Kruskal-Wallis: $p < 0.001$) and in particular it was higher at the 15th month (Mann-Whitney with Bonferroni correction) than at the 3rd ($p < 0.01$), 6th ($p < 0.01$), and 9th months ($p < 0.05$), more elevated at the 18th month than at the 3rd and 6th ($p < 0.05$), and finally more increased at the 21st month of treatment than at the 3rd and 6th months ($p < 0.05$). Afterwards, we tried to compare patients who were free of relapses during the treatment, considered as "responders," with patients who experienced at least one relapse, "nonresponders." Despite the small number of patients in each group, we compared all the variables: serum concentrations of active MMP-2 and active MMP-9 and TIMP-2 and TIMP-1 and the ratios calculated between MMPs and TIMPs and between active MMP-9 and active MMP-2. No differences were found between the responders and the nonresponders for all the data analyzed (data not shown).

4. Discussion

To the best of our knowledge, this is the first study that longitudinally analyzes serum levels of active MMP-2 and active MMP-9 with sensitive activity assay systems in a cohort of RRMS patients during the treatment with Natalizumab in an attempt to provide further insight into the real significance of gelatinases in MS pathology and their role in monitoring efficacy of treatment. The involvement of MMP-2 and MMP-9 in MS pathogenesis and progression has been widely investigated in the past decades. There is a large agreement, particularly on the proinflammatory role of MMP-9 and on the protective function of TIMP-1. In particular, serum MMP-9 levels were greater in RRMS than in the progressive forms [14–16] in MS patients with MRI evidence of disease activity [15, 17, 19] and in MS subjects with clinically isolated syndromes who developed clinically definite MS [18]. On the other hand, TIMP-1 levels were lower in MS patients than in controls [14, 15, 17] and serum MMP-9/TIMP-1 ratio has been indicated as a potential biomarker of MS disease activity [15, 18]. The study of the active forms of MMP-9 also demonstrated that CSF and serum levels of active MMP-9 could represent a potential biomarker for monitoring MS disease activity and that serum active MMP-9/TIMP-1 ratio seems to be an indicator of ongoing MS inflammation [30]. In addition, beneficial effects of Natalizumab treatment were associated with decreased CSF MMP-9 levels after 12 months of therapy, and for this reason MMP-9 was proposed as a biomarker for clinical trials on new drugs for MS [21]. On the contrary, the role of MMP-2 still remains unclear. Previous studies have reported that MMP-2 was elevated in PBMCs and CD4+ Th1 cells of MS patients and could contribute to the homing of these immune cells inside the brain through the BBB [28, 29]. In addition, while CSF MMP-2 levels appeared to be comparable between MS and controls [14, 15, 22, 25, 26], serum MMP-2 concentrations were similar or lower in MS patients than in controls [15, 20]. The active

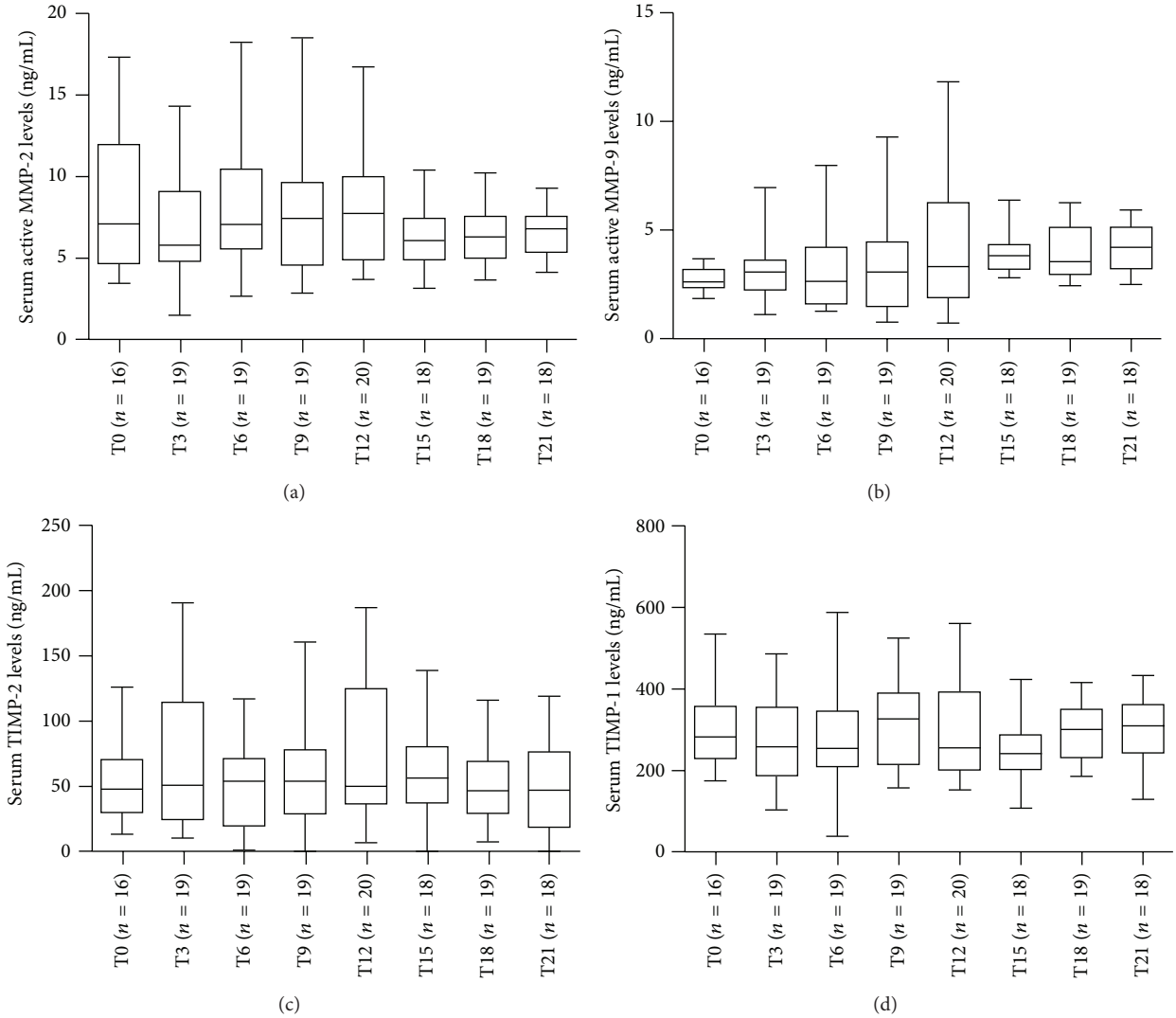

Figure 1: Longitudinal fluctuations of serum active MMP-2 (a) and active MMP-9 and (b) TIMP-2 (c) and TIMP-1 (d) in patients with relapsing-remitting multiple sclerosis (RRMS) treated with Natalizumab for 21 months. MMP = matrix metalloproteinases; TIMP = tissue inhibitors of metalloproteinases; T0 = baseline; T3 = 3rd month; T6 = 6th month; T9 = 9th month; T12 = 12th month; T15 = 15th month; T18 = 18th month; and T21 = 21st month. Horizontal bars indicate medians and error bars correspond to interquartile range. The boundaries of the box represent the 25th–75th quartiles. The line within the box indicates the median. The whiskers above and below the box correspond to the highest and lowest values, excluding outliers.

form of MMP-2 has been described as a potential marker of MS recovery, as detected by MRI, suggesting a beneficial function that sustains the resolution of the inflammatory response and the remission of the disease [31]. In the present study, serum levels of active MMP-2 and active MMP-9 and of the specific tissue inhibitors TIMP-2 and TIMP-1, respectively, were found to be stable during the 21 months of Natalizumab therapy in all patients. Surprisingly, despite the small number of patients included in the study, we did not find differences between patients who experienced relapses during the treatment, considered as "nonresponders," and patients that were free of relapses, considered as "responders," for active MMP-2 and active MMP-9 and TIMP-2 and TIMP-1 serum levels at each time point. Moreover, the ratios between active MMPs and the respective TIMPs did not appear influenced by the Natalizumab treatment and did not differ between responders and nonresponders during the 21 months of observation. On the one hand, this could indicate that Natalizumab treatments maintain stable serum levels of MMPs and TIMPs, but, on the other hand, this excludes the use of these molecules in monitoring the pharmacological response. Previous studies on recombinant interferon beta-1a, one of the most used disease-modifying therapies for MS, showed that low MMP-9 serum levels were associated with a positive outcome, while MMP-2 serum levels were stable during treatment [36] and that serum MMP-9/TIMP-1 ratio may be regarded as a reliable marker and may be predictive of MRI activity in RRMS [37]. Moreover, TIMP-1 has also been suggested as a good indicator of response to therapy [38]. Our principal finding was that an imbalance

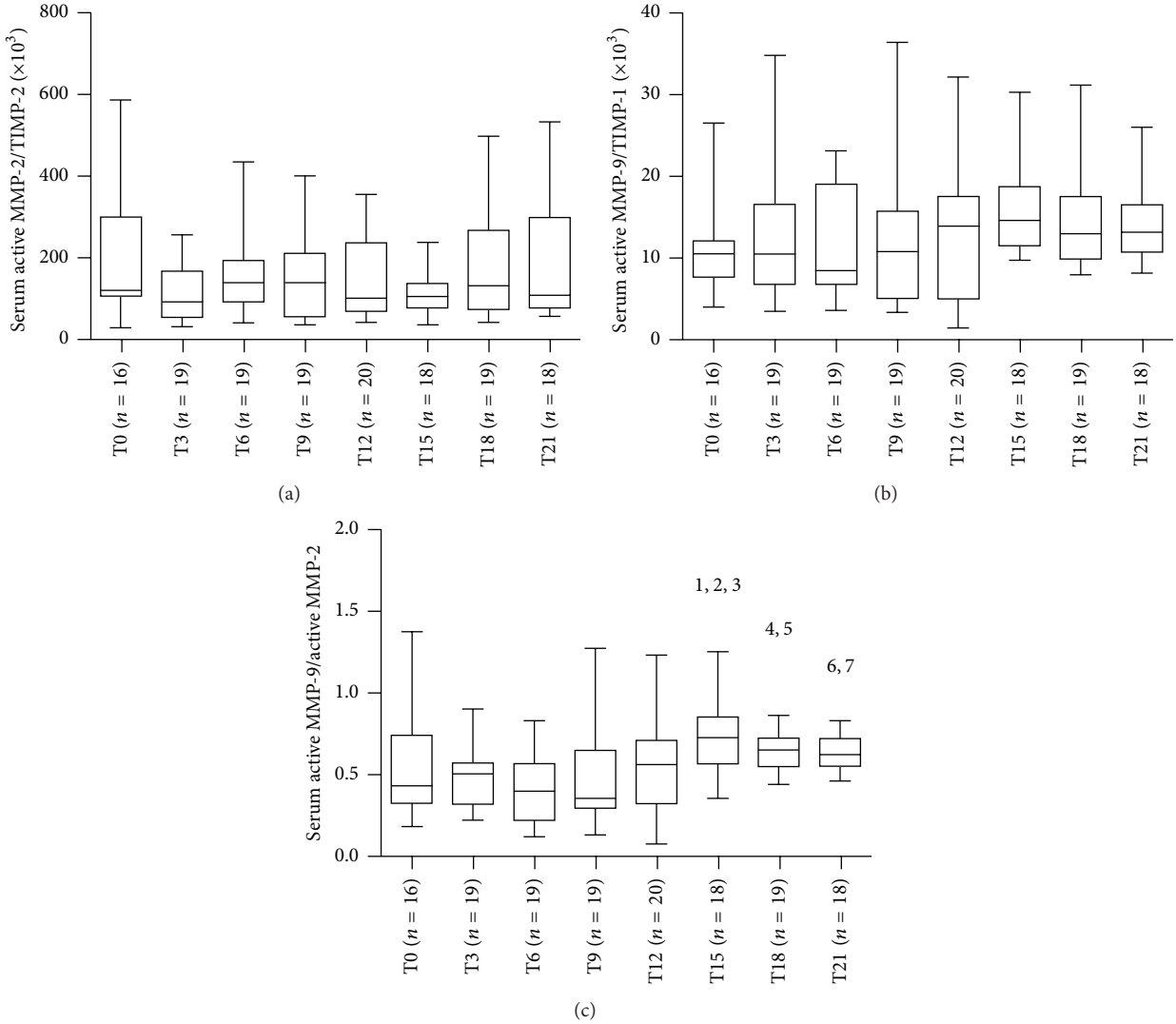

Figure 2: Longitudinal fluctuations of serum active MMP-2/TIMP-2 ratio (a), serum active MMP-9/TIMP-1 ratio (b), and serum active MMP-9/active MMP-2 ratio (c) in relapsing-remitting multiple sclerosis (RRMS) patients during 21 months of Natalizumab treatment. No differences were found for the MMP-2/TIMP-2 (a) and MMP-9/TIMP-1 (b) ratios while the active MMP-9/active MMP-2 ratio was different at various time points ((c), $p < 0.001$); in particular it was higher at the 15th month than at the 3rd ($^1p < 0.01$), 6th ($^2p < 0.01$), and 9th months ($^3p < 0.05$), increased at the 18th month than at the 3rd and 6th ($^{4,5}p < 0.05$), and more elevated at the 21st month of treatment than at the 3rd and 6th months ($^{6,7}p < 0.05$). MMP = matrix metalloproteinases; TIMP = tissue inhibitors of metalloproteinases; T0 = baseline; T3 = 3rd month; T6 = 6th month; T9 = 9th month; T12 = 12th month; T15 = 15th month; T18 = 18th month; and T21 = 21st month. Horizontal bars indicate medians and error bars correspond to interquartile range. The boundaries of the box represent the 25th–75th quartiles. The line within the box indicates the median. The whiskers above and below the box correspond to the highest and lowest values, excluding outliers.

occurred between active MMP-9 and active MMP-2 serum levels after 15 months of Natalizumab therapy without further differences between responder and nonresponder patients. The ratio between MMP-9 and MMP-2 was proposed as a serum marker to monitor the progression of liver disease, and the ratio between MMP-2 and MMP-9 was associated with poor response to chemotherapy in osteosarcoma in two previous studies [39, 40]; however this is the first time that the ratio between the active forms of MMP-9 and MMP-2 was calculated in MS patients, and for this reason further studies are required to investigate the biological significance of the imbalance between serum levels of gelatinases. The main limitations of this study were the small number of enrolled patients and above all the lack of samples collected at the time of relapse. In conclusion, taken together, our data seems to indicate that Natalizumab treatment could either maintain serum levels of active MMP-2 and active MMP-9, as well as of the specific tissue inhibitors TIMP-1 and TIMP-2, stable or make them not affected at all; however, this influence tends to be reduced after 15 months of therapy resulting in an imbalance between serum active MMP-9, considered as a marker of disease activity [30], and serum active MMP-2, described as a marker of disease remission [31]. Moreover, the present study argues against the use of serum levels of

gelatinases for the monitoring of Natalizumab treatments in MS patients. Nevertheless, more extensive research in a larger number of patients is advisable for a better understanding of the correlations between Natalizumab therapy and gelatinases in MS.

Competing Interests

The authors declare that they have no conflict of interests.

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Review Article

Association of Common Variants in MMPs with Periodontitis Risk

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Background. Matrix metalloproteinases (MMPs) are considered to play an important role during tissue remodeling and extracellular matrix degradation. And functional polymorphisms in MMPs genes have been reported to be associated with the increased risk of periodontitis. Recently, many studies have investigated the association between MMPs polymorphisms and periodontitis risk. However, the results remain inconclusive. In order to quantify the influence of MMPs polymorphisms on the susceptibility to periodontitis, we performed a meta-analysis and systematic review. *Results.* Overall, this comprehensive meta-analysis included a total of 17 related studies, including 2399 cases and 2002 healthy control subjects. Our results revealed that although studies of the association between *MMP-8* −799 C/T variant and the susceptibility to periodontitis have not yielded consistent results, *MMP-1* (−1607 1G/2G, −519 A/G, and −422 A/T), *MMP-2* (−1575 G/A, −1306 C/T, −790 T/G, and −735 C/T), *MMP-3* (−1171 5A/6A), *MMP-8* (−381 A/G and +17 C/G), *MMP-9* (−1562 C/T and +279 R/Q), and *MMP-12* (−357 Asn/Ser), as well as *MMP-13* (−77 A/G, 11A/12A) SNPs are not related to periodontitis risk. *Conclusions.* No association of these common MMPs variants with the susceptibility to periodontitis was found; however, further larger-scale and multiethnic genetic studies on this topic are expected to be conducted to validate our results.

1. Introduction

Periodontitis being one of the most common forms of destructive periodontal disease in adults can be defined as bacterial plaque induced inflammation of the attachment apparatus of teeth and supporting structures, which initially manifests as gingivitis and is characterized by extension of inflammation from the gingiva into deeper periodontal tissues that if left untreated results in destruction of periodontium associated with progressive attachment loss and irreversible bone loss [1]. Currently, periodontitis is considered to be multifactorial disease, developing as a result of complex interactions between specific host genes and the environment [2]. Although periodontitis is initiated and sustained by bacterial plaque, host factors determine the pathogenesis and rate of progression of the disease [3].

Matrix metalloproteinases (MMPs) are a large family of metal-dependent extracellular proteinases which are responsible for the tissue remodeling and degradation of the extracellular matrix (ECM), including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycans [4]. To date, at least 26 members of MMPs have been identified [5]. The majority of MMPs proteins are secreted as inactive proMMPs, which are subsequently processed by other proteolytic enzymes (such as serine proteases, furin, and plasmin) to generate the active forms. The proteolytic activities of MMPs are precisely controlled during activation from their precursors and inhibition by endogenous inhibitors, a-macroglobulins, and tissue inhibitors of metalloproteinases (TIMPs) or by nonselective synthetic inhibitors (batimastat, BB-94) [6].

Significant evidence suggests that MMPs comprise the most important pathway in the tissue destruction associated

with periodontal disease [7]. And based on previous studies, dramatically elevated levels of MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9 have been detected in gingival crevicular fluid, peri-implant sulcular fluid, and gingival tissue of periodontitis patients [8]. Likewise, recent studies have also shown that mRNA levels of MMPs are significantly increased in inflamed gingival tissue. MMPs activity may be regulated by interactions with their endogenous inhibitors (TIMPs) and posttranslational modifications, as well as at the levels of gene transcription [9]. Consequently, it can be hypothesized that functional polymorphisms in MMPs genes may affect MMPs expression or activity and, thus, may predispose to periodontal disease conditions.

According to some genotype analyses of single nucleotide polymorphisms (SNPs) in MMPs genes, they have shown increased frequency of several common MMPs SNPs in patients with periodontitis [10–13]. On the contrary, some other studies have demonstrated little or no association of these SNPs in MMPs genes with etiopathogenesis of periodontitis [14–17]. Despite comprehensive studies focusing on the association of gene polymorphisms with the susceptibility and/or severity of periodontitis, there exists a high degree of inconsistency and the results are inconclusive; therefore, for the purpose of deriving a more precise estimation of association between these MMPs SNPs and periodontitis risk, we performed a meta-analysis and systematic review of all eligible studies.

2. Materials and Methods

2.1. Protocols and Eligibility Criteria. The meta-analysis and systematic review reported here are in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) statement (Appendix S1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2016/1545974). The research question for this study was formulated based on the PICO (population, intervention, comparison, and outcomes) criteria. The literature search was limited to original studies performed in humans on the association of matrix metalloproteinases SNPs with periodontitis risk.

2.2. Search Strategy. Studies addressing the correlations of MMPs genetic polymorphisms with the risk of periodontitis were identified by performing an electronic search in PubMed (1966 to May 2015), Medline (1950 to May 2015), and Web of Science databases (1900 to May 2015) by using the following search terms in PubMed: (((((((“Matrix Metalloproteinases” [Mesh]) OR Matrix Metalloproteinases) OR Matrix Metalloproteinase) OR MMPs) OR MMP)) AND (((((“Polymorphism, Genetic” [Mesh]) OR Polymorphism) OR “Genetic Variation” [Mesh]) OR Genetic Variation) OR genetic variant)) AND ((((((((((“Periodontitis” [Mesh]) OR Periodontitis) OR “Chronic Periodontitis” [Mesh]) OR Chronic Periodontitis) OR CP) OR “Aggressive Periodontitis” [Mesh]) OR Aggressive Periodontitis) OR AgP) OR “Periodontal Diseases” [Mesh]) OR Periodontal Diseases) OR PD). Other databases were searched with comparable terms suitable for the specific database. Furthermore, in order to identify any additional studies that may have been missed, a computer-assisted strategy based on manual searching of reference lists from potentially relevant reviews and retrieved articles was performed. Full texts of the relevant articles and studies published in English were retrieved and included to explore the association between MMPs polymorphisms and the susceptibility to periodontitis.

2.3. Selection of Studies. The studies included in the present meta-analysis and systematic review had to meet the following inclusion criteria: (a) studies used validated genotyping methods (such as PCR-RFLP and TaqMan) to measure the association of SNPs in MMP genes with periodontitis risk; (b) studies were in an appropriate analytical design, including case-control, cohort, or nested case-control; (c) studies were published in English; (d) the full text of studies was available, and (e) the data of studies were not duplicated in another manuscript. However, studies were excluded if they did not provide enough information on genotype frequency or did not report sufficient genotype distribution for calculation of odds ratios (ORs) and its variance. Besides, studies investigating the mixed population were excluded if they did not provide the detailed information for each ethnicity. Moreover, studies were also excluded if genotype distributions of control subjects were varied from Hardy-Weinberg equilibrium (HWE).

2.4. Data Extraction. To ensure homogeneity of data collection and to rule out the effect of subjectivity in data gathering, data extraction was performed independently by two investigators (Ying Zhu and Pradeep Singh), using a predefined protocol. Disagreements were resolved by iteration, discussion, and consensus. A series of items were collected for each trial, including first author’s surname, publication year, country, ethnicity (Caucasian, Asian, or mixed (excluding the detailed ethnic results of mixed population in the original study)), type and severity of periodontitis, matching criteria of cases and controls, source of controls, allelic frequency in both cases and controls, genotyping methods, and also the genes and variants genotyped. Furthermore, the evidence of HWE in controls was verified through the application of an online software (http://www.oege.org/software/hwe-mr-calc.shtml). p value less than 0.05 of HWE was considered to be significant.

2.5. Risk of Bias. Methodological quality was independently evaluated by two researchers (Pradeep Singh and Deepal Haresh Ajmera) according to the recently proposed Newcastle-Ottawa Scale (NOS) criteria for the quality assessment of case-control studies. To unravel potential systematic biases, a third investigator (Wenyang Li) performed a concordance study by independently reviewing all eligible studies; complete concordance was reached for all variables assessed. Briefly, the quality of each study was assessed by using the following methodological components: (1) subject selection; (2) comparability of subject; and (3) clinical outcome. Table 2 illustrates the details of each methodological item. NOS scores ranged from 0 to 9, wherein a score of ≥5 was regarded

as high-quality study, while studies with scores <5 were classified as low-quality studies.

2.6. Heterogeneity. A test for heterogeneity (true variance of effect size across studies) was performed using a Q test (to assess whether observed variance exceeds expected variance) to establish inconsistency in the study results. However, because the test is susceptible to the number of trials included in the meta-analysis, we also calculated I^2. I^2, directly calculated from the Q statistic, indicates the percentage of variability in effect estimates because of true heterogeneity, rather than sampling error. I^2 ranges from 0% to 100%, with 0% indicating the absence of any heterogeneity. Although absolute numbers for I^2 are not available, values <50% are considered low heterogeneity, and the effect is thought to be fixed. Conversely, when I^2 exceeds 50%, then heterogeneity is thought to exist and the effect is random.

2.7. Statistical Analysis. The STATA version 11.0 (Stata Corp, College Station, TX, USA) software was used for meta-analysis. The strength of the association between MMPs SNPs and periodontitis risk was evaluated by ORs with their 95% confidence intervals (CIs) under different genetic models: the allele model (mutant allele versus wild allele), the codominant model (homozygous rare/heterozygous versus homozygous frequent and homozygous rare versus heterozygous), the dominant model (heterozygous + homozygous rare versus homozygous frequent), and the recessive model (homozygous rare versus heterozygous + homozygous frequent), as well as the additive model (heterozygous versus homozygous frequent + homozygous rare). In addition, subgroup analyses were stratified, when feasible, according to the type of disease, racial descent, severity of chronic periodontitis, and smoking habit, respectively. The Z-test was used to estimate the statistical significance of pooled ORs, and the Bonferroni correction was used to account for multiple testing in association analyses. When all genetic models were tested for each SNP, a corrected p value < 0.01 was considered statistically significant.

To estimate the pooled ORs, a fixed effects model (the Mantel-Haenszel method) was used initially, whereas the random effects model (DerSimonian and Laird method) was applied when evidence of significant heterogeneity was found across trials ($p < 0.1$ and $I^2 > 50\%$). In order to evaluate the potential source of heterogeneity, a sensitivity analysis was performed through sequential removal of each included study. Publication bias was investigated using funnel plots, wherein the standard error of log(OR) was plotted against log(OR) for each study. Besides, funnel plot asymmetry was assessed with the Begg rank correlation test (Begg test) and the Egger linear regression approach (Egger test). p values of less than 0.05 from the Egger's test were considered statistically significant. In addition, the results of the trials which could not be pooled through the meta-analysis were assessed using descriptive statistics.

3. Results

The flowchart for the process of including/excluding articles is shown in Figure 1. After abstracts were screened for relevance, 25 full-text studies, comprising chronic periodontitis (CP) and/or aggressive periodontitis (AgP), were comprehensively assessed against the inclusion criteria. Three studies were excluded because they were not in accordance with HWE [10, 18, 19]. Another four studies were excluded because they reported the results of mixed population but did not provide the detailed information for each ethnicity [15, 17, 20, 21]. Besides, one more study was excluded due to insufficient data availability for calculating ORs and their variance [7]. Finally, 17 case-control studies, investigating the association of *MMP-1* (−1607 1G/2G, −519 A/G, and −422 A/T), *MMP-2* (−1575 G/A, −1306 C/T, −790 T/G, and −735 C/T), *MMP-3* (−1171 5A/6A), *MMP-8* (−799 C/T, −381 A/G, and +17 C/G), *MMP-9* (−1562 C/T and +279 R/Q), *MMP-12* (−357 Asn/Ser), and *MMP-13* (−77 A/G and 11A/12A) with periodontitis risk, were included in this meta-analysis [8, 11–14, 16, 22–32]. And the characteristics and quality assessment of all included studies are summarized in Tables 1 and 2.

3.1. MMP-1. Table 3 and Figure 2 show the meta-analysis results of two SNPs in the *MMP-1* gene, namely, −1607 1G/2G and −519 A/G, under various genetic models. In Caucasians, we failed to identify any significant association of these two SNPs with the susceptibility to CP under all comparison models (Table 3; Figure 2). Besides, in Asian population, our results also demonstrated that there was no statistically significant association between *MMP-1* −1607 1G/2G polymorphism and the risk of both CP and AgP (Table 3; Figure 2). Furthermore, analyses of individual polymorphism revealed no differences in distribution of *MMP-1* −422 A/T variant between CP and control groups in Caucasians [14].

Considering the influence of disease severity on polymorphism, we also performed stratified analysis by severity of CP. Pooled ORs revealed that no significant association existed between *MMP-1* −1607 1G/2G polymorphism and the risk of mild to moderate or severe CP in both Caucasians and Asians under all comparison models (Table 3). Besides, a study by Pirhan et al. [26] reported that the −519 G allele carrying genotypes of *MMP-1* gene was not suggested to be related with severe CP in Caucasian population (adjusted OR = 1.25, $p = 0.83$). With smoking being one of the major contributing factors in the susceptibility of periodontitis, we also performed subgroup analysis according to the smoking habit of subjects. In Caucasians, our results revealed that when only nonsmoking or smoking subjects were included, the difference between *MMP-1* −1607 1G/2G polymorphism in CP patients and control population was not significant under all comparison models (Table 3). Likewise, apparent association could not be related with the stratified analysis by individual smoking habit in the allelic and genotype frequencies of MMP-1 −519 A/G polymorphism between CP and control groups in Caucasian population [14]. On the contrary, the results by Holla et al. [14] also suggested that there were significant differences in the distribution of *MMP-1* −422 A/T variant between a subgroup of smoking CP patients versus smoking controls in Caucasians ($p = 0.017$).

Table 1: Main Characteristics of included studies.

Author, year	Country	Ethnicity	Sample size (case/control)	Type of periodontitis	Matching criteria	Genotype method	Gene (polymorphism) & HWE in controls
de Souza et al., 2003 [31]	Brazil	Caucasian	50/37	CP (moderate or severe)	Smoker ratios	PCR-RFLP	MMP-1 (−1607 1G/2G) 0.87
Holla et al., 2004 [14]	Czech Republic	Caucasian	133/196	CP (mild to moderate to severe)	Age, gender	PCR-RFLP	MMP-1 (−1607 1G/2G) 0.52 MMP-1 (−519 A/G) 0.11 MMP-1 (−422 A/T) 0.47
Itagaki et al., 2004 [22]	Japan	Asian	205/142 37/142	CP (mild or moderate or severe) AgP	Age, gender, smoker ratios	TaqMan	MMP-1 (−1607 1G/2G) 0.48 MMP-3 (−1171 5A/6A) 0.87
Holla et al., 2005 [23]	Czech Republic	Caucasian	149/127	CP (mild to moderate to severe)	Age, smoker ratios	PCR-RFLP	MMP-2 (−1575 G/A) 0.40 MMP-2 (−1306 C/T) 0.19 MMP-2 (−790 T/G) 0.67 MMP-2 (−735 C/T) 0.42
Cao et al., 2005 [32]	China	Asian	40/52	AgP	—	PCR-RFLP	MMP-1 (−1607 1G/2G) 0.78
Cao et al., 2006 [13]	China	Asian	60/50	CP (moderate or severe)	—	PCR-RFLP	MMP-1 (−1607 1G/2G) 0.99
Keles et al., 2006 [12]	Turkey	Caucasian	70/70	CP (severe)	Age, gender	PCR-RFLP	MMP-9 (−1562 C/T) 0.82
Holla et al., 2006 [24]	Czech Republic	Caucasian	169/135	CP (moderate or severe)	Age, gender, smoker ratios	PCR-RFLP	MMP-9 (−1562 C/T) 0.59 MMP-9 (+279 R/Q) 0.25
Chen et al., 2007 [16]	China	Asian	79/128	AgP	Age, gender	DHPLC PCR-RFLP	MMP-2 (−1306 C/T) 1.00 MMP-9 (−1562 C/T) 0.63
Gürkan et al., 2007 [8]	Turkey	Caucasian	92/157	AgP	Gender	PCR-RFLP	MMP-2 (−735 C/T) 0.45 MMP-9 (−1562 C/T) 0.35 MMP-12 (357 Asn/Ser) 0.06
Gürkan et al., 2008 [25]	Turkey	Caucasian	87/107	CP (severe)	—	PCR-RFLP	MMP-2 (−735 C/T) 0.43 MMP-12 (357 Asn/Ser) 0.47
Pirhan et al., 2008 [26]	Turkey	Caucasian	102/98	CP (severe)	—	PCR-RFLP	MMP-1 (−519 A/G) 0.79
Ustun et al., 2008 [27]	Turkey	Caucasian	126/54	CP (moderate or severe)	Age	PCR-RFLP	MMP-1 (−1607 1G/2G) 0.75
Pirhan et al., 2009 [28]	Turkey	Caucasian	102/98	CP (severe)	—	PCR-RFLP	MMP-13 (−77 A/G) 0.89 MMP-13 (11A/12A) 0.92
Chou et al., 2011 [11]	China	Asian	361/106 96/106	CP (moderate to severe) AgP	Gender, smoker ratios	PCR-RFLP	MMP-8 (−799 C/T) 0.22
Holla et al., 2012 [29]	Czech Republic	Caucasian	341/278	CP (mild to moderate to severe)	Age, gender, smoker ratios	PCR-RFLP	MMP-8 (+17 C/G) 0.14 MMP-8 (−799 C/T) 0.63
Emingil et al., 2014 [30]	Turkey	Caucasian	100/167	AgP	Gender	PCR-RFLP	MMP-8 (+17 C/G) 0.29 MMP-8 (−799 C/T) 0.09 MMP-8 (−381 A/G) 0.87

CP: chronic periodontitis; AgP: aggressive periodontitis; HWE: Hardy-Weinberg equilibrium. Mild chronic periodontitis: patients with teeth exhibiting < 3 mm attachment loss; moderate chronic periodontitis: patients with teeth exhibiting ≥ 3 mm and <7 mm attachment loss; severe chronic periodontitis: patients with teeth exhibiting ≥ 7 mm attachment loss. A p value less than 0.05 of HWE was considered significant.

Table 2: Assessing the quality of included studies.

Author, year	Selection				Comparability		Exposure			Score
de Souza et al., 2003 [31]	☆			☆	☆		☆	☆		5
Holla et al., 2004 [14]	☆		☆	☆	☆	☆	☆	☆		7
Itagaki et al., 2004 [22]	☆			☆	☆	☆	☆	☆		6
Holla et al., 2005 [23]	☆		☆	☆	☆	☆	☆	☆		7
Cao et al., 2005 [32]	☆			☆	☆		☆	☆		5
Cao et al., 2006 [13]	☆			☆	☆		☆	☆		5
Keles et al., 2006 [12]	☆	☆	☆	☆	☆		☆	☆		7
Holla et al., 2006 [24]	☆	☆	☆	☆	☆	☆	☆☆	☆		9
Chen et al., 2007 [16]	☆		☆	☆	☆	☆	☆☆	☆		8
Gürkan et al., 2007 [8]	☆	☆		☆	☆		☆☆	☆		7
Gürkan et al., 2008 [25]	☆	☆		☆	☆		☆☆	☆		7
Pirhan et al., 2008 [26]	☆	☆		☆	☆	☆	☆	☆	☆	8
Ustun et al., 2008 [27]	☆			☆	☆		☆	☆		5
Pirhan et al., 2009 [28]	☆	☆		☆	☆		☆☆	☆		7
Chou et al., 2011 [11]	☆	☆		☆	☆	☆	☆	☆		7
Holla et al., 2012 [29]	☆	☆	☆	☆	☆	☆	☆	☆		8
Emingil et al., 2014 [30]	☆	☆		☆	☆	☆	☆	☆		7

Selection	(1) Is the case definition adequate? (a) Yes, with independent validation ☆ (b) Yes, for example, record linkage or based on self-reports (c) No description (2) Representativeness of the cases (a) Consecutive or obviously representative series of cases ☆ (b) Potential for selection biases or not stated (3) Selection of controls (a) Community controls ☆ (b) Hospital controls (c) No description (4) Definition of controls (a) No history of disease (endpoint) ☆ (b) No description of source
Comparability	(1) Comparability of cases and controls on the basis of the design or analysis (a) Study controls for the most important factor (HWE in control group) ☆ (b) Study controls for any additional factor (e.g., age, gender, and smoker ratios) ☆
Exposure	(1) Ascertainment of exposure (a) Secure record ☆ (b) Structured interview where blind to case/control status ☆ (c) Interview not blinded to case/control status (d) Written self-report or medical record only (e) No description (2) Same method of ascertainment for cases and controls (a) Yes ☆ (b) No (3) Nonresponse rate (a) Same rate for both groups ☆ (b) Nonrespondents described (c) Rate different and no designation

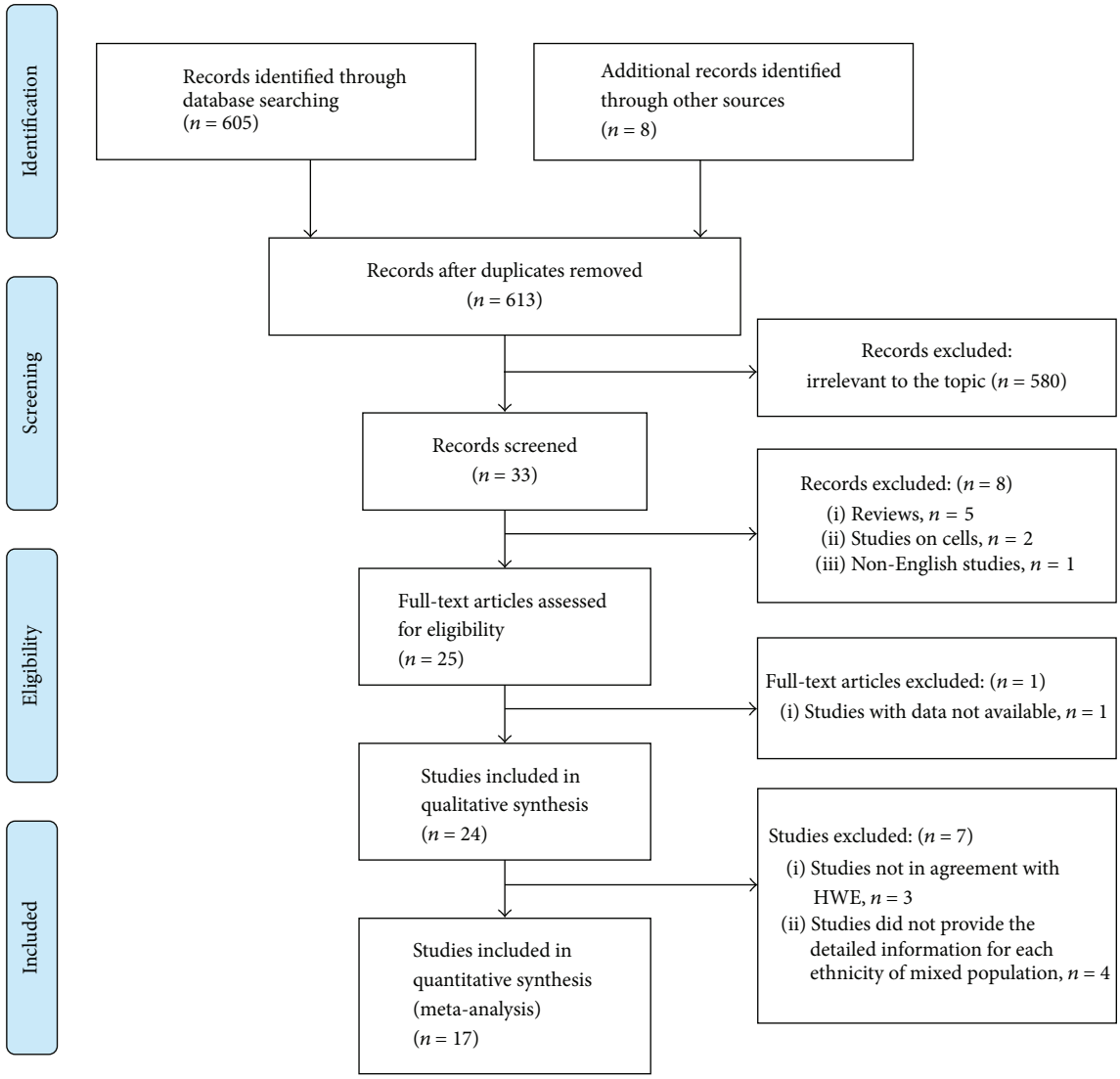


Figure 1: Flow of study identification, inclusion, and exclusion.

3.2. MMP-2. In the present meta-analysis, we failed to associate *MMP-2* −735 C/T polymorphism with CP risk in Caucasian population under all comparison models (Table 3; Figure 2). Besides, a study by Gürkan et al. [8] revealed that this SNP was also not related to AgP risk in Caucasians. Similarly, no significant association of *MMP-2* −1575 G/A, −1306 C/T, and −790 T/G SNPs with the susceptibility to periodontitis was observed in Caucasian and Asian populations [16, 23].

As far as the severity of CP was considered, the allelic and genotype distributions of *MMP-2* −735 C/T variant were similar in severe CP and healthy subjects in Caucasians [25]. When stratified by smoking habit, we found that this polymorphism was not linked with the risk of CP in nonsmoking Caucasian patients and controls without smoking history under all comparison models (Table 3). Likewise, the results of subgroup analysis by Gürkan et al. [8] showed that there was no significant difference regarding the distribution of this SNP between nonsmoking AgP and nonsmoking healthy subjects in Caucasian population. Besides, a similar distribution of other three *MMP-2* variants was also observed between CP patients and controls in subgroup analysis according to smoking status in Caucasians [23].

3.3. MMP-9. Our meta-analysis results revealed that *MMP-9* −1562 C/T SNP might not contribute to CP risk in Caucasians under all comparison models (Table 3; Figure 2). Likewise, the results by Chen et al. [16] and Gürkan et al. [8] failed to find a significant association of this variant with the risk of AgP in Asian and Caucasian populations, respectively. Besides, any significant association of *MMP-9* +279 R/Q polymorphism with the susceptibility to CP was also absent in Caucasians [24].

When stratified by the severity of CP, pooled ORs also did not reveal any significant association between *MMP-9* −1562 C/T variant and severe CP risk in Caucasians under all comparison models (Table 3). Similarly, it was reported by

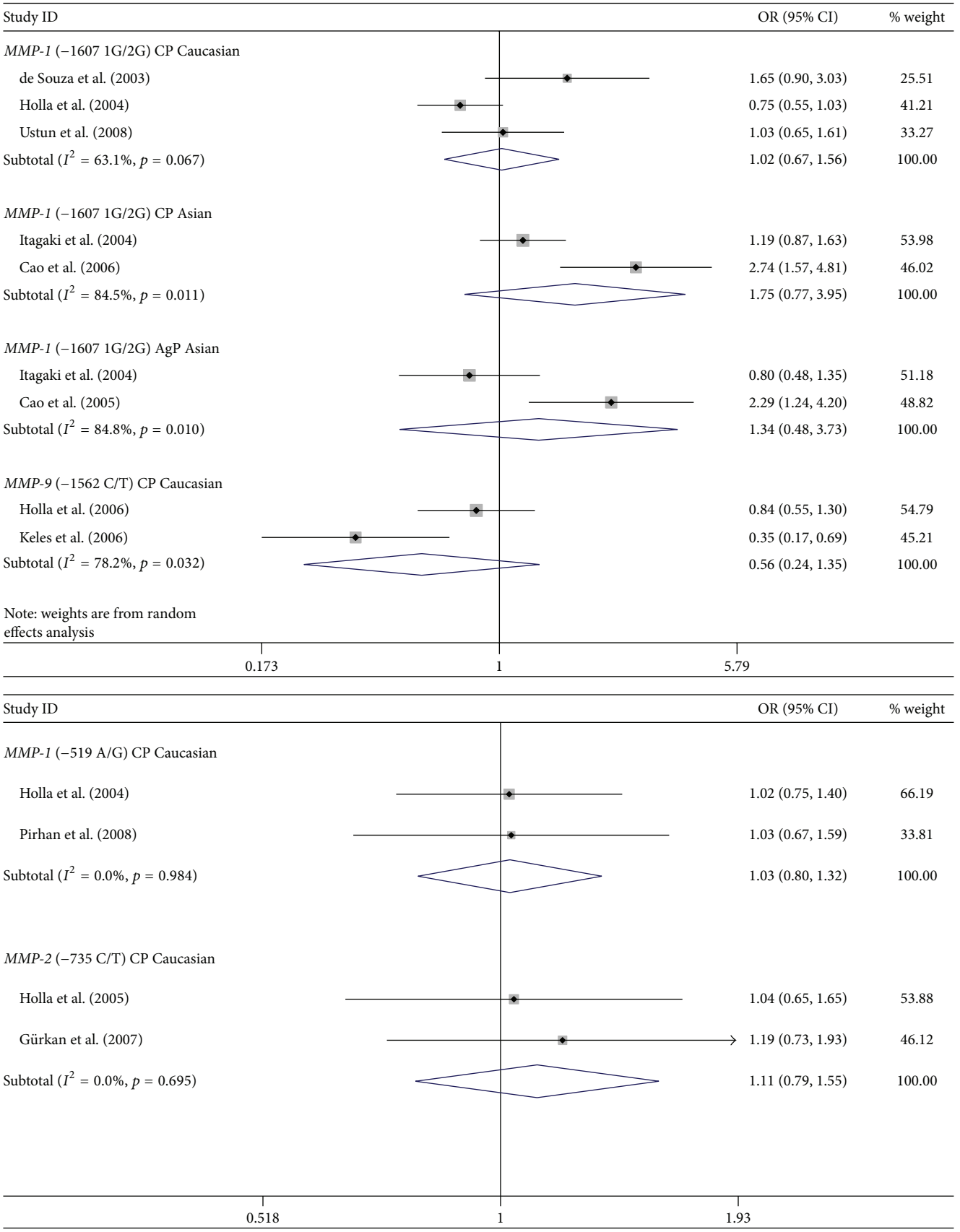
Table 3: Meta-analysis results of the polymorphisms in MMPs gene on periodontitis risk.

		MMP-1						
−1607 1G/2G	Studies (cases/controls)	2G versus 1G OR (95% CI) I^2 (%), p_h, p_c	2G/2G versus 1G/1G OR (95% CI) I^2 (%), p_h, p_c	1G/2G versus 1G/1G OR (95% CI) I^2 (%), p_h, p_c	2G/2G versus 1G/2G OR (95% CI) I^2 (%), p_h, p_c	1G/2G + 2G/2G versus 1G/1G OR (95% CI) I^2 (%), p_h, p_c	2G/2G versus 1G/1G + 1G/2G OR (95% CI) I^2 (%), p_h, p_c	1G/2G versus 1G/1G + 2G/2G OR (95% CI) I^2 (%), p_h, p_c
		Type of disease						
CP								
Caucasian	3 (309/287)	1.02 (0.67–1.56) 63.1, 0.067, **1.000**	1.05 (0.44–2.51) 63.0, 0.067, **1.000**	0.95 (0.64–1.42) 12.0, 0.321, **1.000**	0.90 (0.59–1.37) 0.0, 0.490, **1.000**	0.91 (0.62–1.32) 49.9, 0.136, **1.000**	0.89 (0.60–1.33) 38.2, 0.198, **1.000**	1.00 (0.72–1.40) 0.0, 0.969, **1.000**
Asian	2 (302/192)	1.75 (0.77–3.95) 84.5, 0.011, **1.000**	2.79 (0.56–13.98) 81.5, 0.020, **1.000**	1.31 (0.74–2.32) 27.0, 0.242, **1.000**	1.75 (0.77–3.94) 64.6, 0.093, **1.000**	1.96 (0.63–6.09) 69.4, 0.071, **1.000**	2.01 (0.72–5.61) 79.7, 0.027, **1.000**	0.79 (0.55–1.16) 0.0, 0.467, **1.000**
AgP								
Asian	2 (77/194)	1.34 (0.48–3.73) 84.8, 0.010, **1.000**	1.54 (0.28–8.55) 78.4, 0.031, **1.000**	0.91 (0.42–1.96) 0.0, 0.763, **1.000**	1.67 (0.38–7.31) 81.8, 0.019, **1.000**	1.14 (0.56–2.32) 39.0, 0.200, **1.000**	1.64 (0.35–7.70) 85.7, 0.008, **1.000**	0.75 (0.43–1.30) 59.5, 0.116, **1.000**
		Severe CP						
Caucasian								
Mild to moderate	2 (66/91)	0.99 (0.63–1.55) 0.0, 0.613, **1.000**	0.99 (0.39–2.50) 0.0, 0.613, **1.000**	1.16 (0.52–2.60) 0.0, 0.667, **1.000**	0.85 (0.39–1.84) 0.0, 0.874, **1.000**	1.10 (0.51–2.38) 0.0, 0.613, **1.000**	0.89 (0.43–1.85) 0.0, 0.751, **1.000**	1.17 (0.62–2.21) 0.0, 0.876, **1.000**
Severe	2 (110/91)	1.53 (0.72–3.24) 65.7, 0.088, **1.000**	2.44 (0.47–12.59) 63.4, 0.098, **1.000**	1.52 (0.70–3.30) 15.8, 0.276, **1.000**	1.31 (0.68–2.51) 0.0, 0.369, **1.000**	1.68 (0.81–3.50) 51.1, 0.153, **1.000**	1.47 (0.80–2.73) 42.3, 0.188, **1.000**	0.98 (0.56–1.73) 0.0, 0.845, **1.000**
Asian								
Mild to moderate	2 (168/192)	1.26 (0.93–1.72) 60.1, 0.113, **0.987**	1.62 (0.84–3.12) 62.7, 0.101, **1.000**	1.40 (0.72–2.69) 43.8, 0.182, **1.000**	1.19 (0.75–1.87) 0.0, 0.534, **1.000**	1.51 (0.82–2.80) 56.0, 0.132, **1.000**	1.27 (0.83–1.95) 21.0, 0.261, **1.000**	0.97 (0.63–1.48) 0.0, 0.784, **1.000**
Severe	2 (97/192)	1.99 (0.92–4.26) 73.5, 0.052, **0.546**	2.93 (0.71–12.03) 67.0, 0.082, **0.952**	1.16 (0.53–2.56) 0.0, 0.468, **1.000**	2.19 (1.26–3.79) 48.9, 0.162, **0.035**	1.78 (0.86–3.67) 38.1, 0.204, **0.854**	2.55 (0.95–6.85) 69.7, 0.069, **0.441**	0.58 (0.35–0.98) 0.0, 0.517, **0.287**
		Smoking habit in CP						
Caucasian								
Nonsmoking	3 (200/213)	0.92 (0.55–1.55) 66.1, 0.053, **1.000**	0.90 (0.33–2.43) 63.1, 0.067, **1.000**	0.87 (0.54–1.40) 34.9, 0.215, **1.000**	0.85 (0.52–1.41) 0.0, 0.438, **1.000**	0.96 (0.45–2.05) 56.9, 0.098, **1.000**	0.82 (0.52–1.30) 40.1, 0.118, **1.000**	0.98 (0.66–1.46) 0.0, 0.575, **1.000**
Smoking	2 (109/74)	1.10 (0.71–1.72) 19.0, 0.267, **1.000**	1.14 (0.43–3.02) 32.9, 0.222, **1.000**	1.12 (0.54–2.34) 0.0, 0.976, **1.000**	1.15 (0.50–2.64) 52.2, 0.148, **1.000**	1.12 (0.55–2.29) 0.0, 0.682, **1.000**	1.19 (0.53–2.65) 54.0, 0.140, **1.000**	0.98 (0.53–1.84) 0.0, 0.319, **1.000**
		MMP-1						
−519 A/G	Studies (cases/controls)	G versus A OR (95% CI) I^2 (%), p_h, p_c	GG versus AA OR (95% CI) I^2 (%), p_h, p_c	AG versus AA OR (95% CI) I^2 (%), p_h, p_c	GG versus AG OR (95% CI) I^2 (%), p_h, p_c	AG + GG versus AA OR (95% CI) I^2 (%), p_h, p_c	GG versus AA + AG OR (95% CI) I^2 (%), p_h, p_c	AG versus AA + GG OR (95% CI) I^2 (%), p_h, p_c
		Type of disease						
CP								
Caucasian	2 (235/293)	1.03 (0.80–1.32) 0.0%, 0.984, **1.000**	1.08 (0.64–1.82) 0.0%, 0.806, **1.000**	1.00 (0.68–1.46) 0.0%, 0.811, **1.000**	1.06 (0.64–1.75) 0.0%, 0.691, **1.000**	1.02 (0.71–1.45) 0.0%, 0.884, **1.000**	1.06 (0.67–1.69) 0.0%, 0.736, **1.000**	0.98 (0.69–1.39) 0.0, 0.778, **1.000**
		MMP-2						
−735 C/T	Studies (cases/controls)	T versus C OR (95% CI) I^2 (%), p_h, p_c	TT versus CC OR (95% CI) I^2 (%), p_h, p_c	CT versus CC OR (95% CI) I^2 (%), p_h, p_c	TT versus CT OR (95% CI) I^2 (%), p_h, p_c	CT + TT versus CC OR (95% CI) I^2 (%), p_h, p_c	TT versus CC + CT OR (95% CI) I^2 (%), p_h, p_c	CT versus CC + TT OR (95% CI) I^2 (%), p_h, p_c
		Type of disease						
CP								
Caucasian	2 (236/234)	1.11 (0.79–1.55) 0.0, 0.695, **1.000**	1.19 (0.43–3.37) 0.0, 0.511, **1.000**	1.12 (0.74–1.68) 0.0, 0.940, **1.000**	1.08 (0.37–3.13) 0.0, 0.541, **1.000**	1.00 (0.67–1.49) 0.0, 0.699, **1.000**	1.16 (0.41–3.24) 0.0, 0.522, **1.000**	1.10 (0.74–1.65) 0.0, 0.989, **1.000**
		Smoking habit in CP						
Caucasian								
Nonsmoking	2 (133/198)	1.13 (0.74–1.72) 0.0, 0.337, **1.000**	1.15 (0.32–4.09) 45.2, 0.177, **1.000**	1.17 (0.70–1.94) 0.0, 0.784, **1.000**	0.99 (0.27–3.66) 32.4, 0.224, **1.000**	1.16 (0.71–1.88) 0.0, 0.533, **1.000**	1.10 (0.31–3.89) 42.4, 0.188, **1.000**	1.15 (0.69–1.90) 0.0, 0.917, **1.000**

Table 3: Continued.

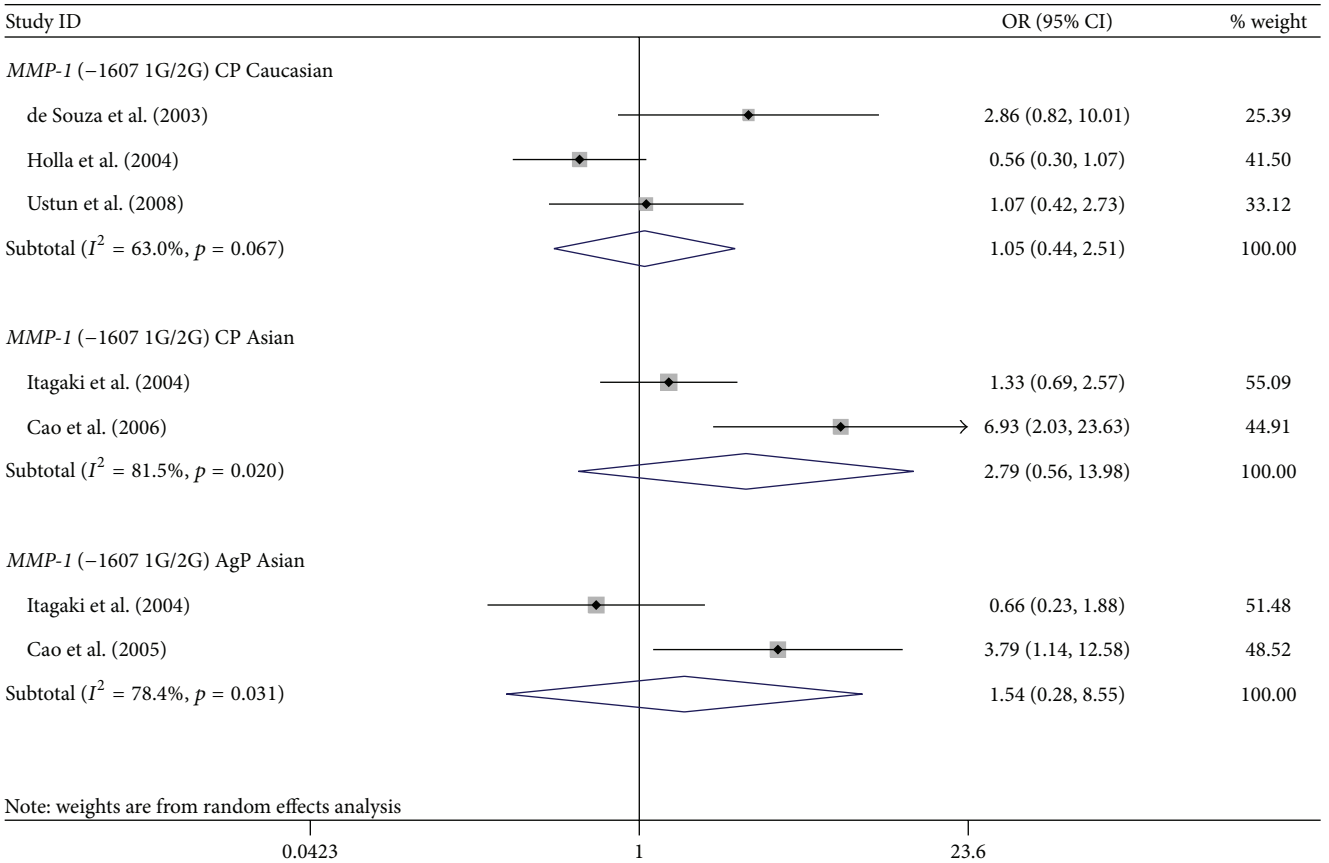
−1562 C/T	Studies (cases/controls)	T versus C OR (95% CI) I^2 (%), p_h, p_c	TT versus CC OR (95% CI) I^2 (%), p_h, p_c	CT versus CC OR (95% CI) I^2 (%), p_h, p_c	TT versus CT OR (95% CI) I^2 (%), p_h, p_c	CT + TT versus CC OR (95% CI) I^2 (%), p_h, p_c	TT versus CC + CT OR (95% CI) I^2 (%), p_h, p_c	CT versus CC + TT OR (95% CI) I^2 (%), p_h, p_c
MMP-9								
Type of disease								
CP								
Caucasian	2 (239/205)	0.56 (0.24–1.35) 78.2, 0.032, **1.000**	0.36 (0.11–1.12) 35.9, 0.212, **0.553**	0.63 (0.29–1.36) 64.0, 0.096, **1.000**	0.51 (0.15–1.72) 0.0, 0.459, **1.000**	0.57 (0.23–1.38) 73.9, 0.050, **1.000**	0.39 (0.12–1.24) 20.4, 0.262, **0.777**	0.72 (0.47–1.10) 57.1, 0.127, **0.924**
Severe CP								
Caucasian								
Severe	2 (163/205)	0.63 (0.20–1.97) 86.1, 0.007, **1.000**	0.44 (0.13–1.49) 54.4, 0.139, **1.000**	0.71 (0.24–2.09) 79.3, 0.028, **1.000**	0.53 (0.14–1.95) 0.0, 0.428, **1.000**	0.65 (0.19–2.15) 84.2, 0.012, **1.000**	0.46 (0.14–1.57) 41.0, 0.193, **1.000**	0.75 (0.28–2.01) 76.0, 0.041, **1.000**

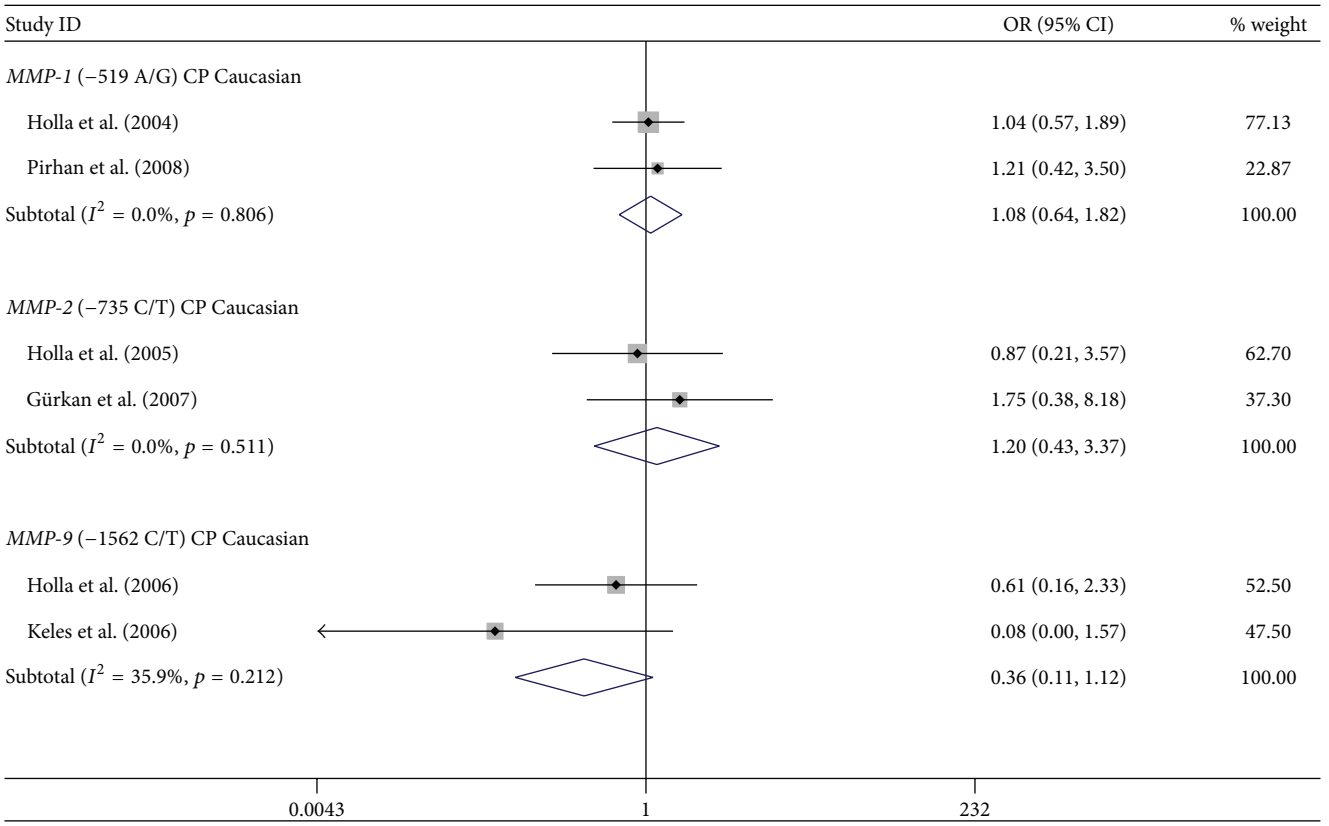
MMP-1: matrix metalloproteinase-1; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; CP: chronic periodontitis; AgP: aggressive generalized periodontitis.
p_h: the p value of heterogeneity; p_c: the p value corrected by Bonferroni correction; OR: odds ratio; CI: confidence interval.
When p_h is <0.1 and I^2 exceeds 50%, the random effects model is used. Conversely, the fixed effects model is used.
$p_c < 0.01$ is considered statistically significant.



(a) Mutant allele versus wild allele

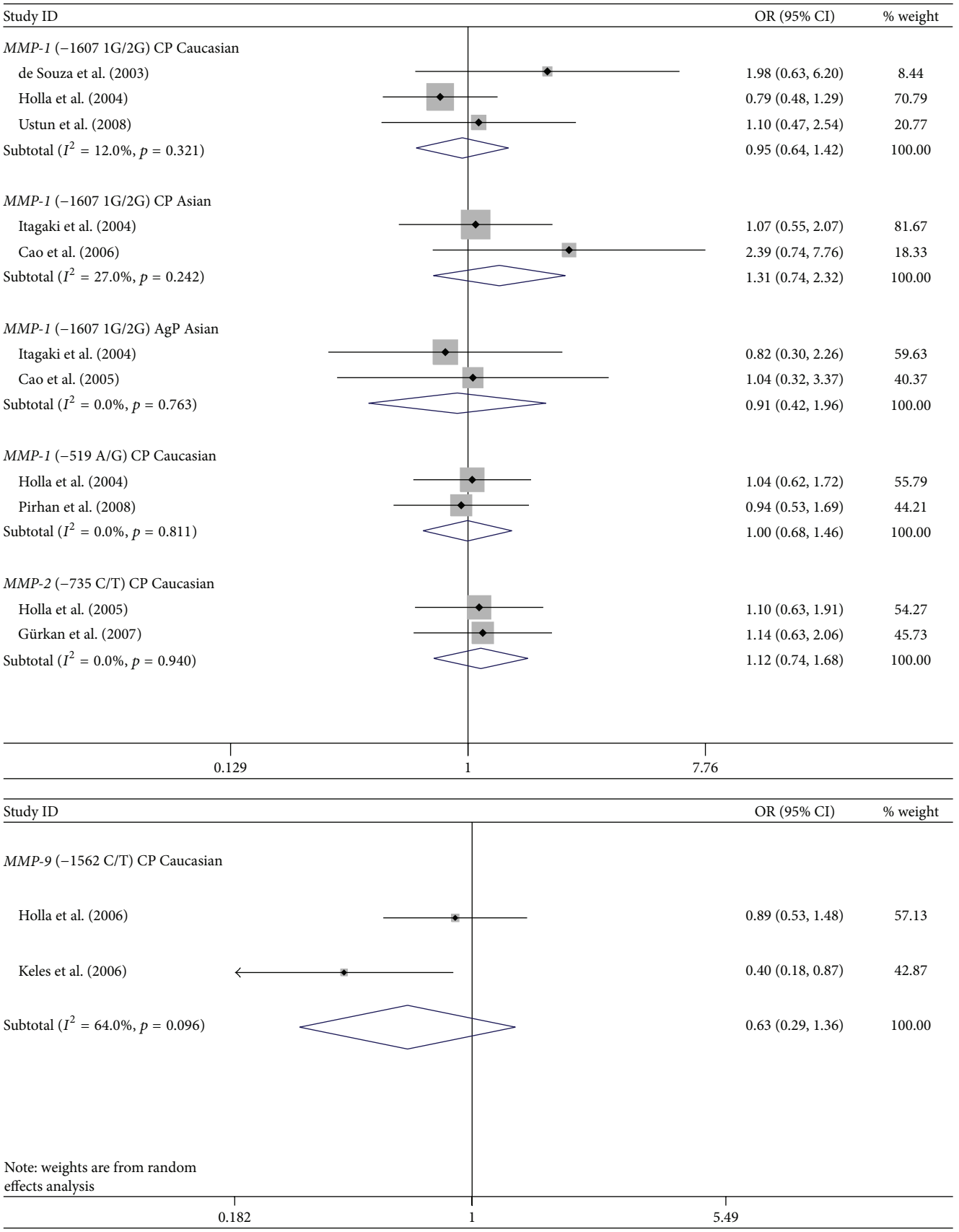
Figure 2: Continued.





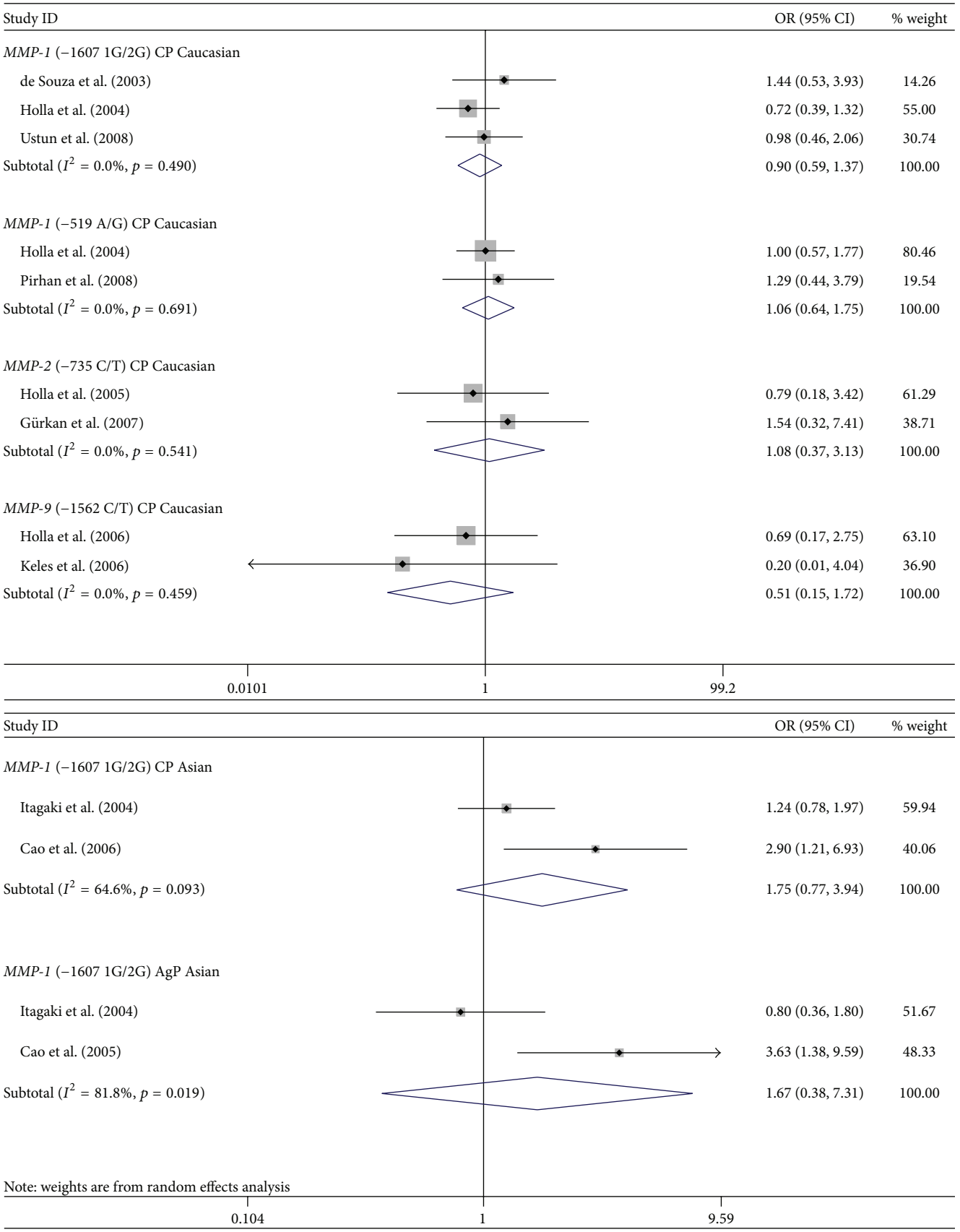
(b) Homozygous rare versus homozygous frequent

Figure 2: Continued.



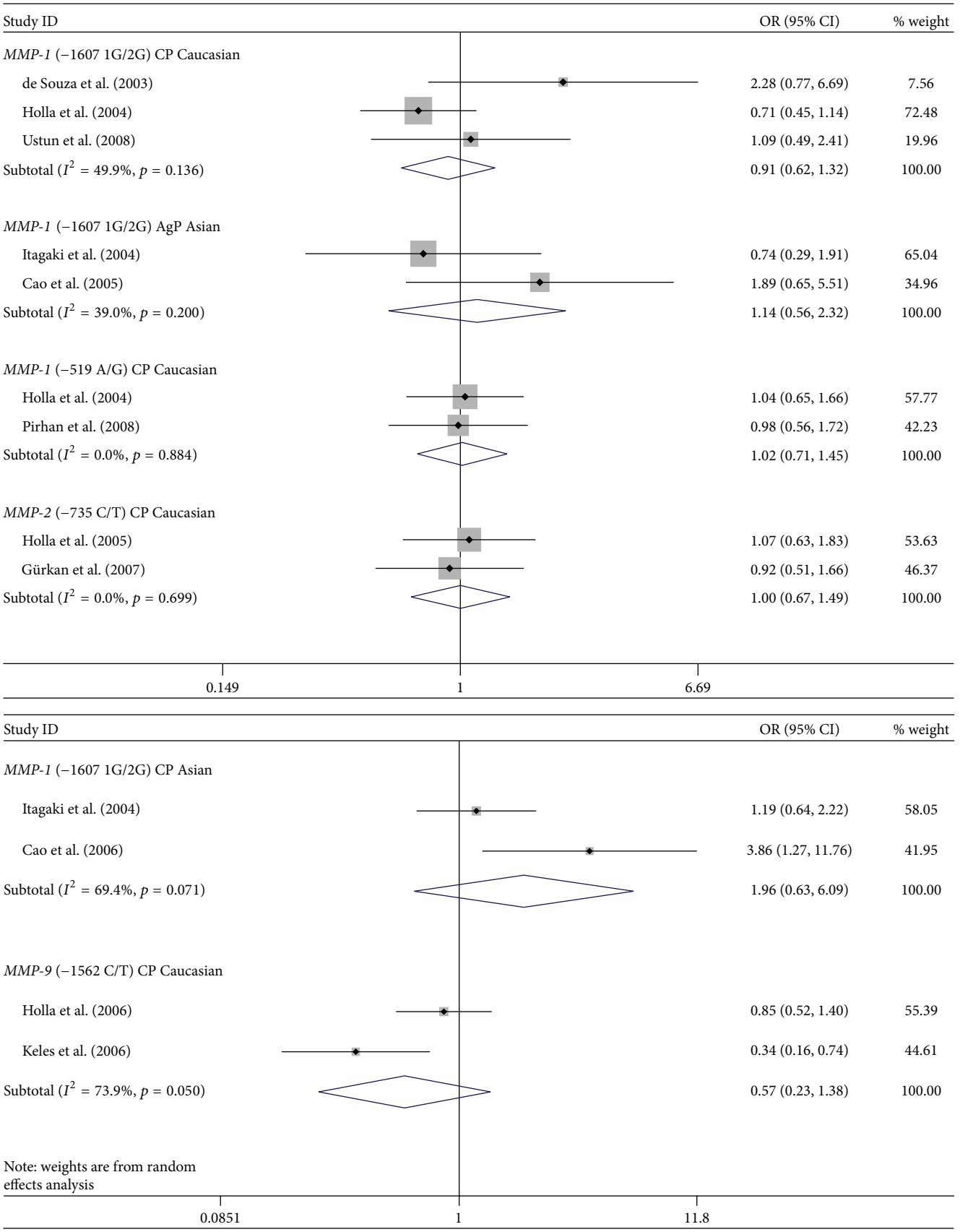
(c) Heterozygous versus homozygous frequent

Figure 2: Continued.



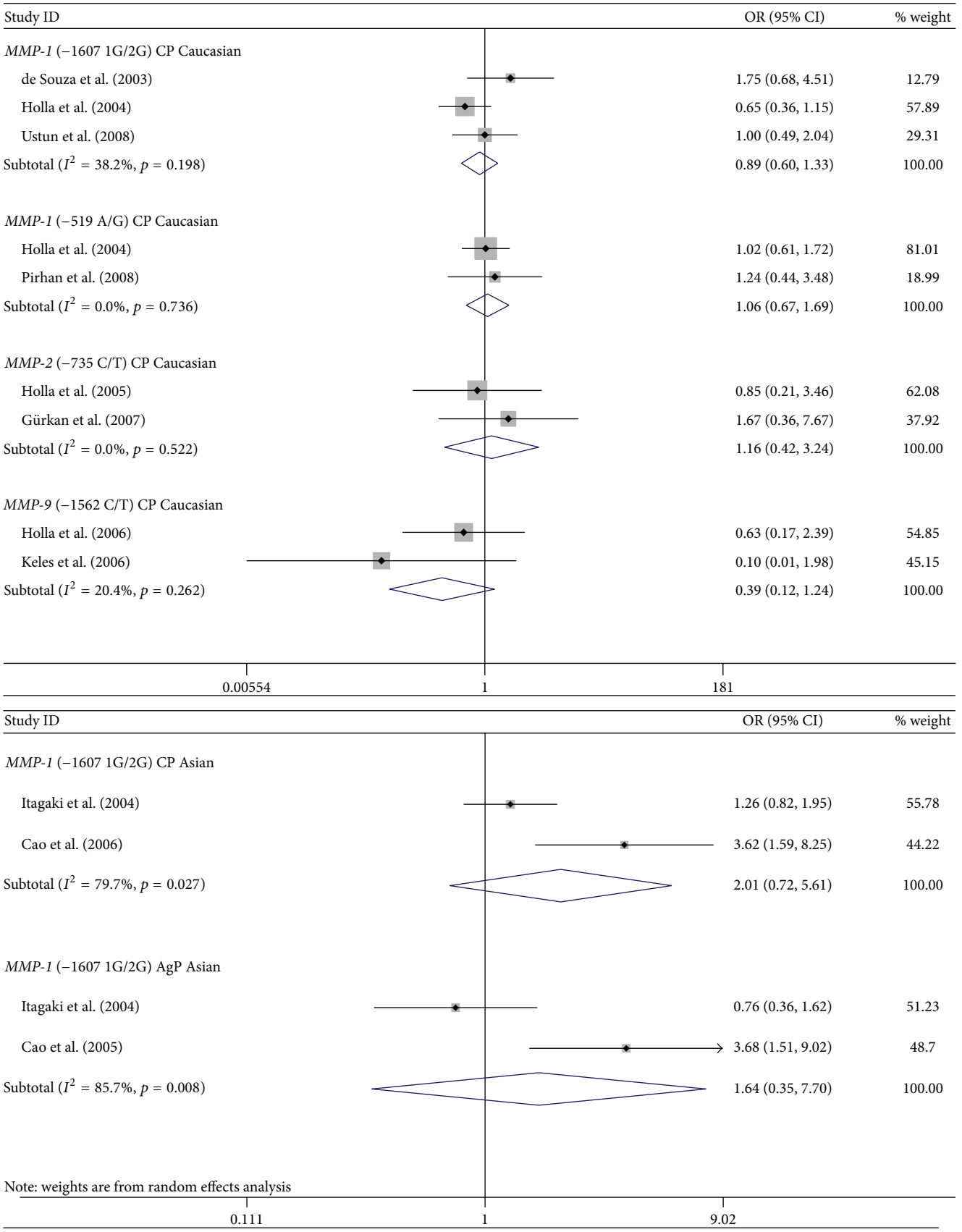
(d) Homozygous rare versus heterozygous

Figure 2: Continued.



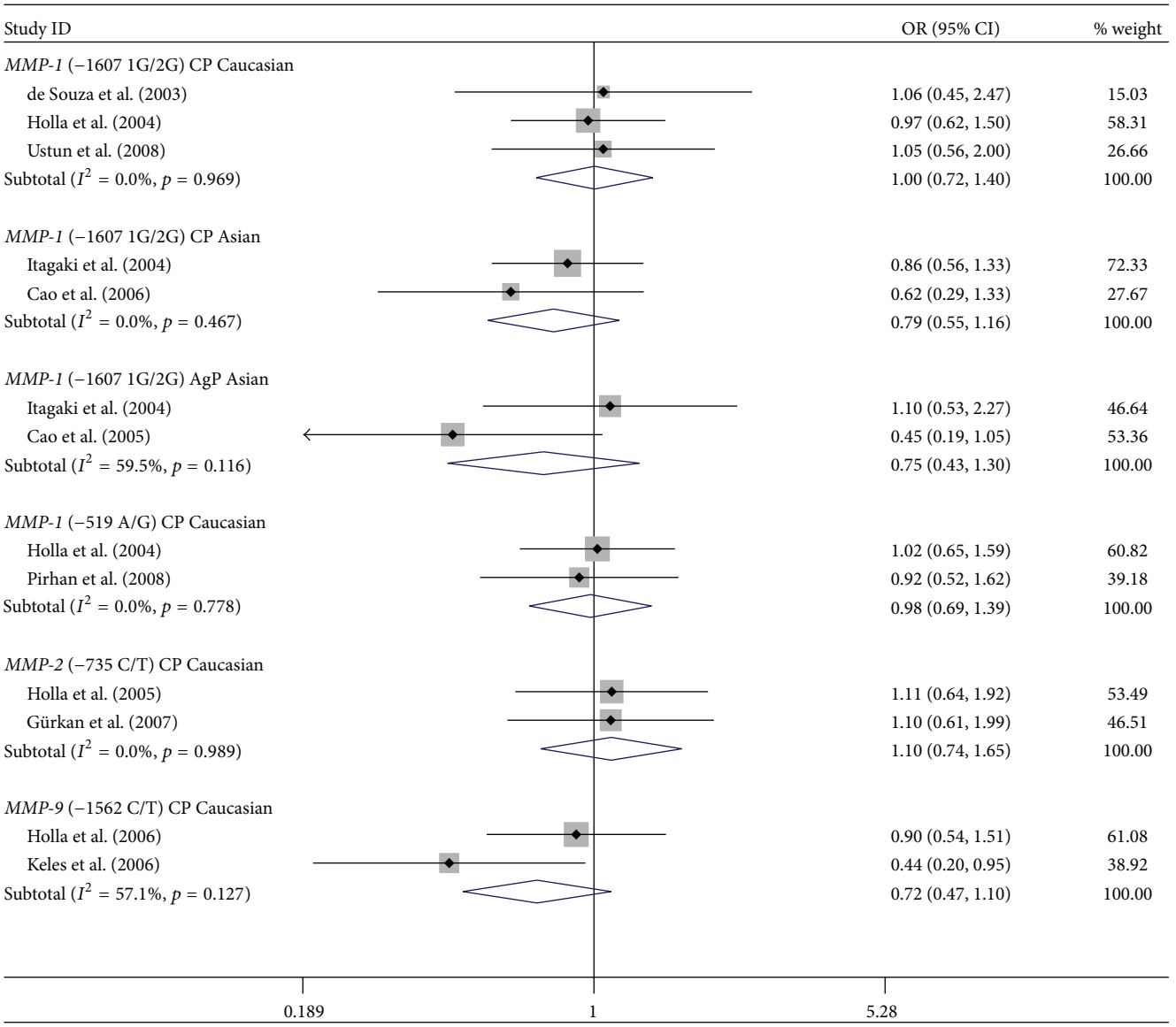
(e) Heterozygous + homozygous rare versus homozygous frequent

Figure 2: Continued.



(f) Homozygous rare versus heterozygous + homozygous frequent

Figure 2: Continued.



(g) Heterozygous versus homozygous frequent + homozygous rare

Figure 2: Forest plot of periodontitis risk associated with MMPs polymorphisms under all comparison models.

Holla et al. [24] that there was no difference in the distribution of *MMP-9* +279 R/Q SNP between the Caucasian CP patients with mild to moderate disease and those with severe disease. Concerning the smoking habit of subjects, the results by Holla et al. [24] suggested no significant difference in the allele and genotype frequencies of *MMP-9* −1562 C/T polymorphism between smoking or nonsmoking CP patients and controls with or without smoking history in Caucasians. Moreover, when the smokers were excluded, the distribution of this SNP in the nonsmoking Caucasian subjects with AgP was similar to that in the healthy group [8].

3.4. Other MMPs. One SNP, −1171 5A/6A (in the promoter region of *MMP-3* gene), has been investigated. In the study by Itagaki et al. [22], they failed to support the influence of this polymorphism on susceptibility to both CP (p = 0.935) and AgP (p = 0.057) in Asians. Moreover, as far as the severity of CP was concerned, the results by Itagaki et al. [22] also revealed that in Asian population, there were no statistically significant differences in the distribution of this variant among three CP phenotypes (severe, moderate, and slight) (p = 0.240, 0.188, and 0.114, resp.).

Variation in *MMP-8* gene, particularly of −799 C/T, −381 A/G, and +17 C/G SNPs, has been investigated in association with periodontitis. As for *MMP-8* −799 C/T polymorphism, analysis of genotypes in periodontitis and healthy control groups in the study by Chou et al. [11] showed that the −799 T allele was associated with increased risk of both AgP (adjusted OR = 1.99, p = 0.04) and CP (adjusted OR = 1.93, p = 0.007) in Asians. Likewise, Emingil et al. [30] has

also found analogous results for the association between this variant and AgP risk ($p < 0.005$) in Caucasians. On the contrary, the results by Holla et al. [29] suggested no differences in the allelic and genotype frequencies of this SNP between Caucasian CP patients and controls ($p > 0.05$). Besides, as for *MMP-8* −381 A/G and +17 C/G polymorphisms, studies conducted by Holla et al. [29] and Emingil et al. [30] revealed that there was no significant association of these two SNPs with the susceptibility to periodontitis in Caucasians. When stratified by smoking habit, a significant difference in T allele carriers of *MMP-8* −799 C/T polymorphism in both AgP (adjusted OR = 2.33, $p < 0.05$) and CP (adjusted OR = 1.84, $p < 0.05$) groups versus control group was found in nonsmokers subgroup analysis in Asian population [11], while studies by Holla et al. [29] and Emingil et al. [30] showed no association of all these three *MMP-8* variants with the risk of CP and AgP in Caucasians when the group of subjects was divided according to smoking status.

A few articles have reported the relation of *MMP-12* −357 Asn/Ser as well as *MMP-13* −77 A/G and 11A/12A SNPs to periodontitis risk in Caucasian population. In the studies by Gürkan et al. [8, 25], they could not succeed in establishing the relationship of *MMP-12* −357 Asn/Ser variant with the susceptibility either to AgP (OR = 1.29, 95% CI = 0.64–2.61; $p = 0.47$) or to severe CP (OR = 0.80, 95% CI = 0.31–2.03; $p = 0.56$). Similarly, a study conducted by Pirhan et al. [28] also failed to reveal any significant influence regarding the distribution of *MMP-13* −77 A/G (OR = 0.11, 95% CI = 0.01–1.59; $p = 0.11$) and 11A/12A (data not shown, $p > 0.05$) polymorphisms on severe CP risk. Furthermore, in the nonsmoker subgroup analysis, the allelic and genotype frequencies of *MMP-12* −357 Asn/Ser variant in the nonsmoking subjects with AgP or CP was similar to those in the healthy group according to studies by Gürkan et al. [8, 25].

3.5. Publication Bias and Sensitivity Analysis. The results of these two analyses are shown in Appendices S2 and S3.

4. Discussion

MMP-1 −1607 1G/2G, located on 11q22-q23 chromosome, is one of the most studied SNPs in periodontitis. Evidence from previous studies revealed that individuals carrying 2G/2G genotype appeared to be at greater risk for developing periodontitis than individuals who had 1G/1G and 1G/2G genotypes [26, 32]. Although the exact mechanism behind these findings is not known, it has reported that the presence of 2G allele together with an adjacent adenosine creates a core binding site (5′-GGA-3′), which is the consensus sequence for the Ets family of transcription factors immediately adjacent to an AP-1 site [33]. Moreover, carriage of 2G allele is also shown to augment transcriptional activity by 37-fold and may potentially increase the levels of protein expression [34]. This mechanism provides the molecular bases for a more intense degradation of periodontal extracellular matrix, leading to increased risk of periodontitis.

However, in our study we could not only find any significant association between *MMP-1* −1607 1G/2G polymorphism and periodontitis risk, but also failed to associate *MMP-1* −519 A/G and −422 A/T SNPs with the susceptibility to periodontitis. Several reasons may contribute to our results. First, an overview of clinical outcomes revealed that, even with the same genotype, the presence of a high variation in MMP-1 expression among periodontitis individuals could be due to additional influence of specific periodontopathogens and cytokine stimulation [35]. Based on the results of these studies, a stronger signaling because of intense and sustained stimulation of host cells by periodontopathogens and by the inflammatory mediators (such as IL-1b and TNF-a) characteristically induced by them may overcome the genetic predisposition, and high levels of *MMP-1* are transcribed irrespective of these SNPs [36].

Also, it is believed that the combination of several significant gene variants in certain individuals synergistically elevate the susceptibility to disease [37]. Since role of TIMPs in MMPs function cannot be denied, it can also be postulated that mutation of the position 2 (Thr in TIMP-1) greatly affects the affinity of TIMPs for MMPs and substitution to glycine essentially inactivates TIMP-1 for MMPs inhibition [38], thus potentiating MMPs activity. Besides, results of the linkage disequilibrium and the haplotype frequencies of *MMP-1* and *MMP-3* variants, both of which are located in 11q22.3 chromosome near to each other, indicated that the risk 2G allele in *MMP-1* was more frequently linked to the nonrisk 6A allele in *MMP-3*, suggesting that the risk and nonrisk linkage combination might lead to the functional compensation of MMP function, to put it in another way, protective function of host homeostasis [22].

Furthermore, previous studies have hypothesized that covariates like severity of the disease and smoking may contribute towards regulation of MMP-1 expression in diseased periodontium [39]. So, we also performed subgroup analyses according to severity of CP and smoking habit of subjects. Similarly, lack of association between *MMP-1* gene variants in terms of CP severity as well as smoking status and periodontitis risk was observed in the present meta-analysis and systematic review. All these above results may lead to the conclusion that an increase in mRNA transcription caused by these *MMP-1* promoter SNPs may not necessarily lead to an increased effect of MMP-1 on the extracellular matrix of periodontal tissues, and many other factors such as bacterial metabolites, cytokines, and other gene variants are supposed to be involved in the regulation of MMP-1 expression and functionality.

The *MMP-2* −735 C/T polymorphism is a synonymous mutation, resulting in the same amino acid (threonine) at codon 460 regardless of the allele present. It has been shown that variation of this SNP at synonymous sites could lead to allele-specific structural differences in mRNA that could affect mRNA structure dependent mechanisms [40], which could have functional consequences of increased MMP-2 expression. In oral cancer, previous studies have verified that patients with *MMP-2* −735 CC genotype present increased risk for developing oral squamous cell carcinoma when compared to those with CT or TT genotype [41]. These findings were consistent with other studies that have linked this genotype with an increased risk of development of lung cancer [42], gastric cardia adenocarcinoma [43],

and abdominal aortic aneurysm [44], suggesting that this polymorphism is identified as a promising candidate for neoplasms.

On the contrary, several studies failed to show association between this variant and the susceptibility to periodontitis [8, 23, 25]. Likewise, our results also found no association of this SNP with the risk of both CP and AgP, so did *MMP-2* −1575 G/A and −1306 C/T, as well as −790 T/G SNPs. A possible explanation would be that the rare allele of these variants could disrupt a Sp-1 binding site within the promoter region of *MMP-2* gene, thus leading to lower *MMP-2* promoter activity [45], which might also contribute towards negative association of these *MMP-2* polymorphisms with periodontitis risk. Besides, when stratified by the severity of CP and smoking, a similar distribution of all these *MMP-2* variants was observed between periodontitis patients and controls. So, we can make a conclusion that genetically determined mechanisms may not be important in tuning the effect of MMP-2 on periodontal tissues.

MMP-9 −1562 C/T SNP, located on 20q11.2-q13.1 chromosome, has been under investigation for its association with an increased risk for the development of cancer and emphysema as well as many other diseases [46]. Based on the evidence of previous studies, the suggested mechanism behind a positive association of this polymorphism with disease risk might be that the MMP-9 expression is primarily controlled at the transcriptional level, where the promoter of *MMP-9* gene responds to stimuli of various cytokines and growth factors [47]. Furthermore, the T allele of this variant can abolish a binding site for a transcription repressor and, thus, change the promoter activity of *MMP-9*, leading to increased MMP-9 expression. Besides, an exchange of C-to-T at position −1562 can also alter the binding of a nuclear protein to this region, resulting in increased transcriptional activity in macrophages [48].

However, the present study failed to find any association of both *MMP-9* −1562 C/T and +279 R/Q SNPs with periodontitis risk. A possible explanation for this discrepancy may be that not only the variant, but several binding sites and also their length-dependent interaction with nuclear proteins may influence the transcriptional activity of the gene due to its close localization to the transcriptional start site [49]. In addition, recent evidence indicates that, in periodontitis, changes in MMP/TIMP balance occur as a result of physiological ageing and that gender might be a significant factor modifying this balance [50]. Although multiple genetic factors, including SNPs, are involved in pro- and anti-inflammatory situations, effect of other factors like oxidant-antioxidant imbalance and tissue remodeling cannot be denied and should be simultaneously considered to understand the entire picture of periodontitis risk.

The −1171 5A/6A variant, a well-characterized insertion/deletion polymorphism in the promoter region of *MMP-3* gene, is considered to be functionally involved in the process of periodontitis. The 5A allele of this polymorphism has been shown to result in higher MMP-3 expression and enzyme activity, thereby increasing extracellular matrix breakdown because of disruption of a binding site for a nuclear factor kappa B, which acts as a transcriptional repressor [51]. Moreover, some studies reported positive association of this SNP with periodontitis and concluded that individuals with the 5A/5A genotype were 2-3 times more likely to develop periodontitis [15, 19]. Conversely, several other studies showed a nonsignificant trend for association of this variant with periodontitis, suggesting a likely attempt of the host environment to contain and perhaps specifically outbalance the increased MMP-3 levels to minimize tissue damage [7, 22].

Recently, several studies have investigated *MMP-8* −799 C/T, −381 A/G, and +17 C/G variants in different periodontal diseases. However, a significant correlation with periodontitis risk was only found in *MMP-8* −799 C/T polymorphism, and it has been reported that T allele carriers have more MMP-8 production in the periodontal environment with bacterial challenge compared to non-T allele carriers [30]. The exact mechanism behind this association is still unknown, but T allele of this variant has been proved to have about 1.8-fold higher promoter activity than the C allele [52]. Besides, MMP-8 activity has also been found to be modified in various organs and body fluids in smokers [53, 54], and tobacco-induced degranulation events in neutrophils and increase in proinflammatory mediator burden can influence the expression level of MMP-8 in smokers' periodontal environment [55]. However, none of the previous studies have succeed in associating these *MMP-8* variants with smoking and periodontitis risk, indicating smoking status may not exert an effect on the association of these SNPs with periodontitis susceptibility.

MMP-12 −357 Asn/Ser as well as *MMP-13* −77 A/G and 11A/12A SNPs, located on 11q22.2-q22.3 chromosomes, has been evaluated with the periodontitis risk in a limited number of studies. And it is suggested that all these polymorphisms do not appear to have a significant influence on the susceptibility to periodontitis and are also not associated with the clinical severity of periodontitis as well as outcome of periodontal therapy and gingival crevicular fluid MMP-12/-13 levels [28]. Moreover, recent studies have also revealed that *MMP-2*, *MMP-3*, *MMP-7*, *MMP-8*, *MMP-11*, or *MMP-12* single gene knockout mice failed to show any apparent disorders, suggesting that a single SNP of MMP might not contribute enough in the susceptibility or progression of a disease. A likely explanation for this behavior would be the sharing of common extracellular matrix substrates by some MMP members which might even compensate these functions for each other [56]. Furthermore, lack of association between these variants and periodontitis may also suggest that an increase in the MMP-12 or MMP-13 transcriptions may not necessarily lead to an increase in the destructive effect of these enzymes on the periodontal tissues.

When compared with previous similar meta-analysis and systematic reviews [57, 58], the present study has several strengths. First, almost all of these prior studies pooled ORs by using the data of trials investigating the mixed population; however, a meta-analysis of mixed ethnicities is meaningless for a genetic association study, owing to high population heterogeneity. As a result, we excluded the trials if they did not provide the detailed information for each ethnicity of a mixed population; moreover, in order to get more reliable results, all

meta-analyses and subgroup analyses in our study were performed according to the racial descent. Besides, some of the previous meta-analyses even included studies in which genotype distributions of control subjects were varied from HWE; however, the allele-frequency comparison test is valid only if HWE conditions prevail. Therefore, in the current study, we also took into consideration this factor that might bias the results, suggesting that evidence from our meta-analysis should be considered to be convincing. Nevertheless, this study still has several potential limitations. One potential limitation is that our restriction on searching studies published in indexed journals and also studies published only in English could introduce an inherent bias for this analysis. Moreover, lack of information for the adjustments of major confounders including age, gender, and environmental factors might cause confounding bias so a more precise analysis would have been performed if all individual raw data had been available. Finally, there were only two ethnicity groups (Caucasian and Asian) included in the present study. Thus, it is doubtful whether the obtained conclusions were generalizable to other populations. Further studies on this topic in different ethnicities are expected to be conducted to strengthen our results.

In conclusion, the present meta-analysis and systematic review suggested that although studies of the association between *MMP-8* −799 C/T variant and the susceptibility to periodontitis have not yielded consistent results, *MMP-1* (−1607 1G/2G, −519 A/G, and −422 A/T), *MMP-2* (−1575 G/A, −1306 C/T, −790 T/G, and −735 C/T), *MMP-3* (−1171 5A/6A), *MMP-8* (−381 A/G and +17 C/G), *MMP-9* (−1562 C/T and +279 R/Q), and *MMP-12* (−357 Asn/Ser), as well as *MMP-13* (−77 A/G and 11A/12A) SNPs are not related to periodontitis risk. However, further well-designed studies with larger sample size and more ethnic groups are required to validate the negative association identified in our study. Besides, we expect that in the future, analyses using polymorphisms will not only identify individual variations within disease comparisons but also help in identification of human response to various therapies. Consequently, even though significant insights have been gained into the role of MMPs and their function, a lot of work needs to be done before the roles of MMPs in development of periodontitis are fully elucidated.

Disclosure

The authors Ying Zhu and Pradeep Singh should be regarded as first joint authors.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

The authors Wenyang Li, Ying Zhu, and Pradeep Singh contributed equally to this study.

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