Dynamic regulation of plasma matrix metalloproteinases in human diabetic ketoacidosis

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BACKGROUND: Diabetic ketoacidosis (DKA) in children is associated with cerebrovascular-related complications. We recently reported that DKA facilitates leukocyte adherence to the brain microvascular endothelium. Adhered leukocytes can release enzymes that instigate vascular dysfunction. Our aims were to measure plasma levels of leukocyte-derived matrix metalloproteinases (MMPs) from DKA patients and to correlate plasma MMP concentrations with DKA severity.

METHODS: Plasma was obtained from children with type 1 diabetes, either in DKA ($n = 16$) or insulin controlled (CON; $n = 16$). Antibody microarray and gelatin zymography were used to quantify plasma MMPs and their endogenous tissue inhibitors (TIMPs). MMP concentrations were correlated with DKA severity (blood pH). Quantitative PCR of leukocyte mRNA was used to help determine the origin of plasma MMPs.

RESULTS: DKA was associated with altered plasma levels of $\downarrow$ MMP-2 ($P < 0.001$), $\uparrow$ MMP-8 ($P < 0.001$), $\uparrow$ MMP-9 ($P < 0.05$), and $\uparrow$ TIMP-4 ($P < 0.001$), as compared with CON. Elevated MMP-8 and MMP-9 were both positively correlated with DKA severity ($P < 0.05$). DKA was associated with increased leukocyte mRNA for *MMP-8*, *MMP-9*, and *TIMP-4* ($P < 0.005$).

CONCLUSION: MMPs are dynamically regulated during DKA. Plasma MMP-8 and MMP-9 concentrations correlate with DKA severity and are known to degrade brain microvascular endothelial cell tight junctions. Thus, leukocyte-derived MMPs might contribute to DKA-associated cerebrovascular complications.

Type 1 diabetes mellitus (T1DM) is a chronic disease with onset predominantly in childhood and adolescence, which is expected to double over the next decade (1). The most frequent complication of T1DM is diabetic ketoacidosis (DKA), a state of insulin deficiency that leads to metabolic acidosis, hyperglycemia, and ketonemia (2). DKA is associated with intracranial cerebrovascular-related complications such as vasogenic edema (3).

DKA is associated with systemic inflammation (4). We recently reported that DKA elicits significant elevations in the chemokines CXCL1 (GRO α) and CXCL8 (IL-8), resulting in

leukocyte adhesion to human-derived cerebral microvascular endothelium (5). Adhered leukocytes can release substances that mediate endothelial damage and vascular destabilization (e.g., matrix metalloproteinases (MMP)) (6).

MMPs are a family of endogenous proteases that include collagenases (MMP-1,-8,-13), stromelysins (MMP-3,-10,-11), matrilysins (MMP-7,-26), gelatinases (MMP-2,-9), and membrane-type MMPs (MMP-14, -15, -16, -17,-24, -25) (7). Leukocytes are a major source of circulating MMPs during inflammation (8). Alterations of MMPs and their endogenous tissue inhibitors (TIMPs) are observed in inflammation-related pathologies (e.g., sepsis (9)), and MMPs have the potential to compromise cerebrovascular endothelial barrier function (10–12).

We hypothesized that DKA is associated with elevated leukocyte-derived MMPs. Our aims, using blood from pediatric DKA patients, were to measure plasma levels of MMPs/TIMPs, to correlate the MMP/TIMP levels with DKA severity and to determine if MMPs were leukocyte derived.

RESULTS

Study Patients

Plasma samples from DKA patients were drawn 5.7 ± 1.8 h after presentation to our hospital system ($n = 16$). One half of DKA patients were new-onset DKA cases ($n = 8$). Patients with DKA had significantly higher HbA_{1c} values, as compared with age- and sex-matched controls (CON; $P < 0.001$; **Table 1**), indicating elevated blood glucose over the previous 3 mo. DKA patients all had elevated blood glucose (27.4 ± 8.3 mmol/l) and moderate-to-severe metabolic acidosis (pH 7.00 ± 0.03 ; Pco₂ 20.8 ± 2.3 mm Hg; HCO₃⁻ 5.9 ± 0.8 mmol/l; lactate 2.9 ± 0.7 mmol/l). The leukocyte and neutrophil counts for DKA patients were both elevated at $23.0 \pm 2.2 \times 10^9$ /l and $18.4 \pm 2.0 \times 10^9$ /l, respectively.

Measurements of Plasma MMPs/TIMPs

In order to generate an MMP/TIMP profile, the levels of MMP and TIMP species were measured in plasma from both DKA and CON groups. Out of all the plasma MMP and TIMP

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Table 1. Clinical data and plasma MMP/TIMP levels in type 1 diabetes patients, either metabolically stabilized (CON) or with diabetic ketoacidosis (DKA) ($n = 16/\text{group}$)

	CON	DKA	<i>P</i> value
Clinical data			
Mean age (years)	11.4 ± 1.0	11.7 ± 0.8	0.830
Male:female ratio	8:8	8:8	1.000
HbA _{1c}	8.3 ± 0.3	11.7 ± 0.5	<0.001
MMP/TIMP microarray			
MMP-1	1.22 ± 0.72	2.24 ± 1.18	0.865
MMP-3	6.04 ± 1.34	9.45 ± 1.72	0.086
MMP-8 ^a	39.96 ± 12.90	684.25 ± 307.59	<0.001
MMP-10	0.28 ± 0.04	0.60 ± 0.16	0.181
MMP-13	0.03 ± 0.01	0.04 ± 0.02	0.201
TIMP-1	5.46 ± 1.75	10.70 ± 2.28	0.318
TIMP-2	4.42 ± 0.37	4.71 ± 0.47	0.925
TIMP-4	1.08 ± 0.12	2.26 ± 0.22	<0.001

To control for repeated measures in MMP/TIMP analyses, a *P* value < 0.005 was considered significant. Boldface indicates significant *P* value.

MMP, matrix metalloproteinase; TIMP, tissue inhibitor.

^aConcentrations of MMP-8 are reported in pg/ml (mean ± SEM). All the rest are all reported in ng/mL (mean ± SEM).

species measured on the array, only MMP-8 and TIMP-4 were found to be significantly increased in DKA (17.1-fold increase and 2.1-fold increase, respectively), as compared with CON (Table 1; $P < 0.001$; $n = 16$ per group). These results demonstrate selective elevation of the collagenase MMP-8 and, to a lesser extent, TIMP-4.

To assess MMP-2 and MMP-9 levels in DKA, we assayed gelatinolytic activity of both CON and DKA plasma via zymography. A representative zymogram of three different CON/DKA sample pairs is shown in Figure 1a. MMP-9 was significantly increased 1.7-fold in DKA plasma compared with CON plasma (Figure 1b; ~100 kDa; $P < 0.05$). Conversely, MMP-2 was significantly decreased 2.8-fold in DKA plasma compared with CON plasma (Figure 1c; ~70 kDa; $P < 0.001$). In addition, DKA plasma had a faint ~135 kDa band on gelatin zymography that may reflect a well-described complex of MMP-9 with neutrophil-gelatinase-associated lipocalin (13). Overall, these results suggest distinct opposing regulation of the MMP gelatinases by DKA in children and a potential neutrophilic source of MMP-9.

The plasma concentration of MMP-9 was estimated from zymography (data not shown) using three age-/sex-matched sample pairs (CON ~670 vs. DKA ~1,509 ng/ml). No significant differences were observed between DKA patients with new-onset T1DM vs. those with existing T1DM in terms of MMP-2 ($P = 0.74$), MMP-8 ($P = 0.96$), MMP-9 ($P = 0.88$), or TIMP-4 ($P = 0.38$) levels.

MMP-8 and MMP-9 Correlated With DKA Severity

In order to determine correlations between altered MMP/TIMP levels in DKA plasma and a clinically relevant parameter, correlation analysis was applied to data points graphed

Figure 1. Gelatin zymography was used to measure plasma MMP-2 and MMP-9. (a) A representative zymogram of three plasma DKA/CON experimental pairs. The right-most lane contains a molecular weight ladder. MMP-9 is represented at ~100 kDa, likely representing the latent form. MMP-2 is represented by the bands at ~70 kDa, also likely representing the latent form. (b) MMP-9 levels in DKA plasma were significantly increased relative to CON plasma. Results are presented as relative optical density (* $P < 0.05$; $n = 16/\text{group}$). (c) MMP-2 levels in DKA plasma were significantly decreased from CON plasma. Results are presented as relative optical density († $P < 0.001$; $n = 16/\text{group}$). CON, control; DKA, diabetic ketoacidosis; MMP, matrix metalloproteinase.

as MMP concentrations vs. blood pH (acidotic blood pH reflects greater DKA severity). Both MMP-8 and MMP-9 were inversely correlated with blood pH (Figure 2a; $r_s = -0.71$; $P < 0.01$ and Figure 2b, $r_s = -0.60$; $P < 0.05$, respectively). In contrast, no correlations could be determined for either MMP-2 or TIMP-4 ($r_s = 0.26$; $P = 0.34$ and $r_s = 0.07$; $P = 0.80$, data not graphically shown). These results suggest that MMP-8 and MMP-9 plasma levels are correlated with DKA severity.

MMP-8, MMP-9, and TIMP-4 levels were not correlated with HbA_{1c} ($r_s = 0.16$; $P = 0.56$; $r_s = -0.08$; $P = 0.76$; $r_s = -0.21$; $P = 0.44$, respectively) or blood glucose ($r_s = 0.32$; $P = 0.23$; $r_s = 0.13$; $P = 0.64$; $r_s = 0.36$; $P = 0.17$, respectively).

Leukocyte mRNA Expression of MMPs and TIMP-4

To determine whether the observed changes in MMP-2, MMP-8, MMP-9, and TIMP-4 were leukocyte derived, we assayed the respective gene's mRNA levels with quantitative real-time PCR. MMP-8, MMP-9, and TIMP-4 gene expression was significantly increased in leukocytes from DKA patients, as compared with CON patients (Figure 3; 45.0-, 6.3-, and 31.8-fold change, respectively; $P < 0.005$). In contrast, MMP-2 showed no significant changes. These results suggest that elevated plasma MMP-8, MMP-9, and TIMP-4 are, at least in part, leukocyte derived.

DISCUSSION

In this study, we report that DKA induced a markedly altered plasma MMP/TIMP profile in pediatric DKA patients and that both MMP-8 and MMP-9 concentrations correlated with DKA severity. DKA-induced MMPs were, at least in part, leukocyte derived. MMP-8 and MMP-9, at concentrations similar to those measured in DKA plasma, have been reported to disrupt brain endothelial cell tight junctions. To our knowledge, this

Figure 2. Plasma MMP concentrations correlated with DKA severity. (a) MMP-8 plasma concentrations were inversely correlated with blood pH in DKA patients. A best-fit line was added to aid in visual interpretation of the graphs ($r_s = -0.714$; $**P < 0.01$, $n = 16$). (b) MMP-9 plasma concentrations were inversely correlated with blood pH in DKA patients. A best-fit line was added to aid in visual interpretation of the graphs ($r_s = -0.6024$; $*P < 0.05$; $n = 16$). CON, control; DKA, diabetic ketoacidosis; MMP, matrix metalloproteinase.

study is the first to employ human DKA tissues in relation to pathological changes in systemic proteases.

Our current data, taken together with our previously reported results (5), show that DKA is an inflammatory state associated with highly elevated levels of systemic cytokines (IL-6 and IFN α 2), chemokines (CXCL1 and CXCL8), and MMPs (i.e., MMP-8 and MMP-9). MMPs have diverse actions that may involve exacerbation of existing inflammatory cascades (14), as well as direct actions on the microvasculature (15). This latter action of MMPs is particularly intriguing given that DKA induces leukocyte adherence to the brain microvasculature (5) and that the DKA-induced MMPs appear to be leukocyte derived.

MMP-8 was significantly elevated in DKA plasma, and plasma MMP-8 concentrations correlated with DKA severity. MMP-8 mRNA was elevated in leukocytes from DKA patients, a finding that is consistent with the almost exclusive production of MMP-8 by neutrophils (16). The primary substrates for MMP-8 are the fibrillary collagens, I, II, III, as well as other extracellular matrix components (8). Vascular leukocyte trafficking and blood-brain barrier disruption are mediated by MMP-8 and may involve cleavage of the endothelial cell tight

Figure 3. Elevated MMPs and TIMP-4 in DKA plasma are leukocyte derived. Gene expression of MMP-8, MMP-9, and TIMP-4 were significantly elevated in DKA, as compared with CON patients. Open bars represent CON gene expression and filled bars represent DKA gene expression. Target gene expression is normalized to β -actin and GAPDH and presented as fold change from CON (N.S., not significant; $**P < 0.01$ (controlled for repeated measures); $n = 5$ –7/group). CON, control; DKA, diabetic ketoacidosis; MMP, matrix metalloproteinase; TIMP, tissue inhibitor.

junction proteins (17,18). Our results raise the possibility that the DKA-induced elevations in MMP-8 may contribute to cerebrovascular endothelial perturbations.

MMP-9 was significantly increased in DKA plasma and plasma MMP-9 activity on zymograms correlated with DKA severity. The 100 kDa MMP-9 band suggests that the plasma MMP-9 is primarily a latent form (19), but it is available for immediate activation. Three arguments suggest that neutrophils are a source of MMP-9 in DKA plasma: (i) increased MMP-9 mRNA was found in our leukocyte preparation, (ii) a ~135 kDa band was observed on zymography that likely reflects a well-described complex of MMP-9 with neutrophil-gelatinase-associated lipocalin (13), and (iii) early rises in plasma MMP-9 have been attributed to neutrophil degranulation of stored MMP-9 (20). Delayed sources of plasma MMP-9 may include *de novo* synthesis via monocytes, lymphocytes, dendritic cells, and endothelial cells (21). Megakaryocytes express MMP-9 mRNA that is subsequently transferred into platelets, suggesting that the latter may be a minor source of plasma MMP-9 (22). CXCL8, known to be elevated in DKA, stimulates expression of MMP-9 (23). MMP-9 can proteolytically degrade virtually all components of the extracellular matrix, and MMP-9 has the ability to disrupt components of junctional complexes in the brain microvasculature (24).

Our data suggest that plasma MMP-2 (~70 kDa) is decreased in DKA, possibly due to the hyperglycemic conditions that are characteristic of DKA (25). Decreased MMP-2 facilitates increased extracellular matrix deposition in vascular structures as a primary function of MMP-2 is extracellular matrix turnover (26). Indeed, decreased plasma levels of MMP-2 during DKA may contribute to chronic thickening of basal lamina and pathologic remodeling of the extracellular matrix over multiple DKA episodes.

Plasma TIMP-4 was also mildly elevated in DKA and was at least partially leukocyte derived. Increases in TIMP-4 are likely due to increased monocytic expression, but platelets are

also a potential source of TIMP-4 upon cellular activation (27). While TIMP-4 is considered a broad MMP inhibitor, it also displays some specificity against MMP-2 (28) and has the ability to prevent cell surface activation of MMP-2 (29). Depressed levels of MMP-2, in conjunction with elevated plasma TIMP-4, could facilitate extracellular matrix deposition. TIMP-4 also plays an important role in inflammation-associated vascular diseases, independent of its MMP-inhibitory actions, where its activities can be either detrimental or protective (30).

The actions of elevated plasma MMP-8 and MMP-9 in DKA are largely unknown. The main role attributed to MMPs during inflammation is its proteolytic ability on cellular junctions; however, MMPs are now known to play a role in cytokine processing and generation of biologically active or inactive fragments (14). Processing of the DKA-relevant chemokine CXCL8 (5), by both MMP-8 and MMP-9, has been shown to potentiate its chemotactic effects and biological function (31). MMP-mediated cytokine processing acts to facilitate leukocyte migration, thus facilitating endothelial disruption indirectly (14).

Higher concentrations of plasma MMP-8 and MMP-9 were associated with greater DKA severity (lower pH). Acidic pH directly stimulates the induction and release of cellular MMPs (32,33), and a lower pH is associated with markedly more MMP-8 and MMP-9 catalytic activity (33–36). Thus, DKA patients by virtue of their blood's acidic pH are particularly susceptible to the deleterious actions of MMP-8 and MMP-9. DKA correction with intravenous fluid and exogenous insulin administration normalizes the blood pH. While MMP-8 activity would decrease with increasing pH to normal values (34,36), MMP-9 still maintains much of its catalytic activity (33,35). In contrast, MMP-2 is noted to have decreased activity in acidic pH conditions (37), perhaps resulting in further reduced MMP-2 functional capacity.

MMPs have been shown to increase the permeability of human brain microvascular endothelium (i.e., vasogenic edema) (11,12), likely by degrading tight junction proteins, including occludin, claudin-5, and ZO-1 (10). Active MMP-9 has been most thoroughly investigated and exerts actions at concentrations (100–250 ng/ml) that are compatible with those measured for latent MMP-9 in DKA plasma (1,509 ng/ml) (10,11). The latter would serve as a pool of circulating enzyme ready for immediate pericellular activation. Additional enzymatic action could occur via the sequential addition of MMPs (i.e., MMP-8) that has been demonstrated to boost substrate degradation (38). We have previously demonstrated that reactive oxygen species (ROS) are elevated in human brain microvascular cells *in vitro* after DKA plasma application (5), and ROS are a common trigger for many downstream pathways that directly mediate blood–brain barrier compromise, including MMP activation (39).

Our findings reinforce that DKA-associated inflammation is a factor that should be considered in clinical management, alongside acidosis, hyperglycemia, and dehydration. Specifically, our data suggest a potential mechanism for adhered leukocytes to facilitate human blood–brain barrier disruption

in DKA (5). Clinician awareness is imperative as overaggressive fluid resuscitation will potentially exacerbate subclinical vasogenic edema via the compromised blood–brain barrier. In addition, therapeutic targeting of inflammation-associated molecules may be a potential future intervention.

Our study has several limitations worthy of discussion. First, our data showed a leukocyte origin of the MMP-8 and MMP-9, but we cannot rule out other cellular contributions (i.e., platelets, endothelial cells). Future studies aimed at isolating leukocyte subtypes for analyses from fresh blood samples would be valuable. Second, the measured concentrations of MMP-8 and MMP-9 might not represent the exact concentrations encountered at the brain endothelial cell layer. The MMP concentrations adjacent to leukocytes adhered to the brain microvascular endothelium must be greater, but it is not possible to measure concentrations in such localized domains. Third, chronic hyperglycemia could also play a role in the MMP-associated inflammation; however, several key observations implicate ketoacidosis as the main instigator of the MMP changes in this study: (i) the concentrations of MMPs correlate with DKA severity and (ii) the MMP changes do not correlate with either glucose concentrations or HbA_{1c}. Nonetheless, it is likely that the inflammation lies on a spectrum beginning with hyperglycemia and then greatly exacerbated by ketoacidosis.

In summary, our data show for the first time that DKA is a disease state associated with dynamic regulation of plasma MMPs. In particular, leukocyte-derived MMPs (e.g., MMP-8 and MMP-9) correlated with disease severity, and they have the potential to degrade key components of the cerebrovascular tight junctions (40), perhaps explaining the susceptibility of children with DKA to intracranial complications. Our data provide a plausible mechanism for DKA-induced cerebrovascular perturbations and represent a possible link between DKA-mediated inflammation and DKA cerebrovascular crises.

METHODS

This study was approved by the Health Sciences Research Ethics Board at Western University. Patients were recruited at our regional tertiary care centre, the Children's Hospital, London Health Sciences Centre (London, Ontario, Canada).

Human Subjects

Consent was obtained from the legal guardians of all pediatric patients admitted with DKA, and both legal guardian consent and patient assent were obtained for insulin-controlled T1DM patients. Biochemical diagnostic criteria for DKA included hyperglycemia >11 mmol/L, bicarbonate <15 mmol/L, and ketonuria. DKA is classified according to severity of acidosis as mild DKA (venous pH < 7.3), moderate DKA (pH < 7.2), or severe DKA (pH < 7.1). The majority of DKA cases had severe DKA. Clinic patients with stable T1DM (HbA_{1c} < 10% and no DKA for ≥ 3 mo) served as controls (CON).

Blood Collection and Processing

Blood for research purposes was obtained on hospital presentation at the time of clinically indicated blood draws. Blood was drawn into citrate-containing tubes (Vacutainers; BD Biosciences, Mississauga, Canada) by certified nursing personnel, placed on ice, and immediately transferred to the Translational Research Centre facility for processing by standard operating procedures (www.translationalresearch.ca; London, Canada) (41,42). Briefly, blood was centrifuged at 1,500g for 15 min (4 °C), and the upper plasma layer collected into 250 µl aliquots. Next, the buffy coat was removed and also aliquoted.

Both plasma and buffy coat aliquots were immediately frozen at -80°C until usage. When needed for experiments, plasma was thawed and maintained briefly on ice. Freeze-thaw cycles were avoided.

MMP/TIMP Antibody Microarray

The plasma concentrations of MMP-1, MMP-3, MMP-8, MMP-10, MMP-13, TIMP-1, TIMP-2, and TIMP-4 were measured using a multiplex enzyme-linked immunosorbent assay-based array, the Quantibody Human MMP array (RayBioTech, Norcross, GA) which utilizes a biotin-conjugated detection antibody and a streptavidin-labeled Cy3 equivalent dye that produces fluorescent signal intensity proportional to concentration. Undiluted plasma was loaded into each well and assayed according to the manufacturer's instructions. The completed arrays were then shipped back to the manufacturer for data extraction and analysis.

MMP Gelatin Zymography

MMP-2 and MMP-9 were assayed using gelatin zymography, as we reported previously (9). Plasma samples were diluted 1:1 in phosphate-buffered saline. Protein in the diluted plasma samples was then quantified using the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein (75 μg) were mixed with nonreducing sample buffer and loaded in each well. Samples were run on a 10% sodium dodecyl sulfate polyacrylamide electrophoretic gel impregnated with 0.1% gelatin (m/v) under nonreducing conditions at 140 V until the dye front left the gel. Enzymes were re-natured in 50 ml of 2.7% Triton X-100 (v/v) in distilled water with gentle shaking for 1 h at room temperature. After washing gels for 5 min in 50 ml of distilled water on a rotary mixer, the gels were developed in 50 ml of developing buffer (50 mmol/l Tris-base, pH 7.4, 200 mmol/l NaCl, 5 mmol/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) containing 1 Complete EDTA-free Protease Inhibitor Cocktail Tablet (Roche Applied Science, Indianapolis, IN) for 20 h at 37°C (5% CO_2). The gels were then stained with 0.5% Coomassie Brilliant Blue R-250 (Roche Diagnostics, Mississauga, Canada) for 1 h and then de-stained with a solution consisting of 30% MeOH (v/v) and 10% acetic acid (v/v) until bands of proteolysis were clear. Gels were scanned using a GS-690 densitometer (Bio-Rad Laboratories, Hercules, CA). Proteolytic bands were quantified as a ratio of the optical density of the experimental band to a known amount of human recombinant active MMP-2 standard (EMD Millipore, Etobicoke, Canada) run on the same gel. Quantification of bands was performed using GelQuant Pro v. 11.4 image quantification software (FroggaBio, Toronto, Canada). Negative control zymograms were incubated in the presence of 20 mmol/l EDTA (Bioshop, Burlington, Canada).

Since the zymography technique measures protein as gelatinolytic activity as opposed to concentration, it was necessary to estimate the concentration of MMP-9 from proteolytic bands in a separate experiment. In order to quantify the MMP-9 present in a zymogram, identical volumes (1.0 μl) of three representative age- and sex-matched sample pairs were loaded and run on a zymogram as previously described. A dose-response curve of a known amount of recombinant human active MMP-9 standard (EMD Millipore) was loaded and run on the same gel. The optical density of the proteolytic bands were measured as previously described, and the standard dilutions were used to generate a standard curve to quantify the mass of MMP-9 present in a given proteolytic band. The plasma concentration (ng/ml) of MMP-9 in each experimental condition was estimated by adjusting the average mass of MMP-9 in the zymogram for the volume of plasma initially loaded in the gel and the dilution factor.

Quantitative Real-Time PCR

Quantitative real-time PCR was used to quantify the expression of the genes found to be significantly altered in zymography and the enzyme-linked immunosorbent assay array. RNA was isolated using TRIzol LS Reagent (Life Technologies, Grand Island, NY) from buffy coat extracted from CON and DKA patients then subjected to Turbo DNA-free (Life Technologies) digest according to the manufacturer's suggested protocol. The RNA integrity number of the samples was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, Canada) at the London Regional Genomics Center; samples had a RNA integrity number ≥ 6.7 . RNA from each sample (2 μg) was then

reverse transcribed using iScript Advanced Reverse Transcriptase (Bio-Rad Laboratories). TaqMan Gene Expression Assays (Life Technologies) were used with the SensiFAST Probe No-ROX reagent (Bioline, London, UK) for quantitative real-time PCR. The assay IDs were MMP-2 (Hs01548727_m1), MMP-8 (Hs01029058_m1), MMP-9 (Hs00957562_m1), and TIMP-4 (Hs00162784_m1). Target genes were normalized to β -actin (Hs01060665_g1) and GAPDH (Hs02758991_g1); the M value was determined to be <0.5 indicating they are good normalization genes. Representative samples were used to predetermine the actual amplification efficiency of each probe set in this system which was then used to correct calculations in the BioRad CFX Manager software (v3.1) where efficiency fell outside of the 95–105% range. The reaction was carried out with a CFX96 Real-Time PCR Detection System-IVD (Bio-Rad Laboratories). The PCR was carried out as follows: 2-min initial denaturation at 95°C , 40 cycles of 10-s denaturation at 95°C (up to 70 cycles for undetectable genes), 30-s annealing and extension at 60°C .

Statistical Analysis

All calculations were done using GraphPad Prism software (v. 4.03). Data were statistically analyzed using the Mann-Whitney U -test for nonparametric data with Bonferroni's correction for multiple comparisons when applicable. Correlation analysis utilized Spearman's rank correlation coefficient. All data are presented as mean $\pm$ SEM. Statistical significance utilized a P value of <0.05 .

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