Potential diagnostic biomarkers for pulmonary tuberculosis in humans are not elevated in *Mycobacterium tuberculosis* culture–positive Asian elephants (*Elephas maximus*)

Laura L. Coughlin, BS1*; Carlos R. Sanchez, DVM, MSc2; Margot I. Monti, CVT, VTS2; John A. Griffioen, DVM3; Felicia B. Nutter, DVM, PhD, DACZM1; Gillian L. Beamer, VMD, PhD, DACVP1

1Department of Infectious Disease and Global Health, Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA
2Veterinary Medical Center, Oregon Zoo, Portland, OR
3Fort Worth Zoo, Fort Worth, TX
*Corresponding author: Laura Coughlin (laura.coughlin@tufts.edu)

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OBJECTIVE
To determine (1) if chemokine (C-X-C motif) ligand 1 (CXCL1), matrix metalloproteinase 8 (MMP8), interleukin-10 (IL-10), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) can be detected in serum from Asian elephants, and (2) if their concentrations are significantly elevated in *Mycobacterium tuberculosis* (*M.tbc*) culture–positive elephants compared to –negative elephants. CXCL1, MMP8, IL-10, IFN-γ, and TNF-α were recently identified as potential diagnostic biomarkers for pulmonary tuberculosis in experimental studies in animals and humans. Therefore, we hypothesized that they would be detectable and significantly elevated in *M.tb* culture–positive elephants compared to *M.tb* culture–negative elephants.

SAMPLE
101 Asian elephant serum samples, including 91 samples from 6 *M.tb*–negative elephants and 10 samples from 5 *M.tb*–positive elephants (none of which exhibited clinical signs of disease). *M.tb* status was determined by trunk wash culture.

PROCEDURES
Commercially available ELISA kits were used to determine the concentrations of each biomarker in serum samples.

RESULTS
Biomarker concentrations were below the limit of detection for the assay in 100/101 (99%) samples for CXCL1, 98/101 (97%) samples for MMP8, 85/101 (84%) samples for IL-10, 75/101 (74%) samples for IFN-γ, and 45/101 (45%) samples for TNF-α. Multiple *M.tb* culture–positive elephants did not have detectable levels of any of the 5 biomarkers.

CLINICAL RELEVANCE
CXCL1, MMP8, IL-10, IFN-γ, and TNF-α were not elevated in *M.tb* culture–positive Asian elephants compared to *M.tb* culture–negative Asian elephants. This may be related to disease state (ie, clinically asymptomatic). More sensitive assays are needed to better understand the role of these biomarkers in *M.tb* infection in Asian elephants.
The current gold standard for detecting *M. tb* infection in Asian and African elephants is trunk wash culture. Trunk wash samples are collected by instilling sterile saline into the trunk, elevating the trunk to allow for deeper distribution of the liquid, and having the elephant forcefully blow the liquid into a plastic bag. This technique has several limitations, including the time required to train animals for sample collection, the time required to obtain culture results given that *M. tb* is a slow-growing organism (up to 12 weeks), the fact that elephants shed *M. tb* intermittently and therefore a negative culture cannot rule out infection, and the possibility of bacterial and fungal contamination given the variety of tasks for which elephants use their trunks. Serologic tests to detect serum *M. tb* antibodies are also available, including the Dual Path Platform VetTB Assay (Chembio Diagnostic Systems Inc), which has demonstrated high sensitivity and specificity for detecting *M. tb* infection in Asian and African elephants months to years before diagnosis could be made with trunk wash culture. However, investigation into new or complementary serological testing is warranted, as knowledge gaps remain about elephants’ immune responses to *M. tb* bacilli and infected animals often show no clinical signs until the disease has significantly progressed, making diagnosis more challenging.

Identification of serum biomarkers of *M. tb* infection in elephants may improve testing capabilities, allowing for earlier detection, isolation, and/or treatment of *M. tb*-positive elephants, which would reduce risk of *M. tb* transmission. Given that Asian elephants develop similar TB pathology to humans and that human TB has been studied much more extensively than elephant TB, emerging diagnostic biomarkers for human pulmonary TB may have potential application in Asian elephants. The biomarkers chemokine (C-X-C motif) ligand 1 (CXCL1), matrix metalloproteinase 8 (MMP8), interleukin-10 (IL-10), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) have been shown to be significantly elevated in the serum of Diversity Outbred mice infected with *M. tb* compared to noninfected mice, and this research recently showed translational relevance to humans. In this study, we sought to determine (1) if these 5 biomarkers could be detected in serum from Asian elephants, and (2) if the serum concentrations of these 5 biomarkers are significantly elevated in *M. tb* culture-positive elephants compared to *M. tb* culture-negative elephants. We predicted that these biomarkers would be both detectable and significantly elevated in serum from *M. tb* culture-positive elephants compared to *M. tb* culture-negative elephants.

**Materials and Methods**

**Elephant serum samples**

Banked Asian elephant serum samples were obtained from Oregon Zoo and Fort Worth Zoo. Samples from *M. tb*-positive elephants included 10 samples from 5 elephants (2 samples per elephant) that had tested positive for *M. tb* infection via trunk wash culture. Elephants positive on trunk wash culture were subsequently tested using the Dual Path Platform VetTB Assay (Chembio Diagnostic Systems Inc); results of these assays were positive, corroborating culture results. Four of these elephants were subspecies *Elephas maximus indicus* and 1 was proposed subspecies *Elephas maximus borneensis*. Nine of the 10 samples were obtained prior to treatment with antitubercular medications, and the remaining 1 sample was obtained after treatment had begun but not during active treatment (ie, treatment had been stopped for other medical reasons). None of the *M. tb* culture-positive elephants had clinical signs of TB. Samples from *M. tb*-negative elephants included 91 samples from 6 elephants (range of 8 to 39 samples per elephant) that tested negative for *M. tb* infection via trunk wash culture collected for routine surveillance. One elephant was subspecies *Elephas maximus indicus*, another was born under human care to *Elephas maximus indicus* elephants. Samples were shipped frozen on dry ice or in coolers with ice packs from collaborating zoos and stored at −80 °C upon receipt. The study was reviewed and approved by research oversight committees at each collaborating zoo.

**Biomarker immunoassays**

Commercially available sandwich ELISA kits were used to determine the concentrations of CXCL1, MMP8, IL-10, IFN-γ, and TNF-α in elephant serum samples. Anti-equine reagents were used to detect TNF-α, IFN-γ, and IL-10 because previous research has validated the use of these reagents for cytokine enzyme immunoassays in elephants. Anti-human reagents were used to detect CXCL1 and MMP8 because anti-human reagents were commercially available for these biomarkers while anti-equine reagents were not, and previous research has validated the use of anti-human reagents to identify acute phase proteins in elephants. Concentrations of CXCL1 were measured using a human CXCL1 enzyme immunoassay (DuoSet DY275; R&D Systems Inc) performed according to modified manufacturer’s instructions. Capture antibody was diluted in reagent diluent (1% BSA in PBS) to a concentration of 4.0 μg/mL, and 100 μL was added to each well of a 96-well microtiter plate. After incubation overnight at room temperature, capture antibody was removed from plates and 200 μL reagent diluent was added to each well to block plates against nonspecific binding. After incubation for 1 hour at room temperature, reagent diluent was removed from plates and 100 μL sample or standard were added to each well. Recombinant human CXCL1 standards were serially diluted in reagent diluent to provide a standard range of 2.0 to 2,000 pg/mL and analyzed in duplicate. Serum samples were run neat and analyzed in triplicate. Plates incubated overnight at 4 °C, following which 90 μL of each sample was transferred to corresponding wells of 96-well microtiter plates precoated with MMP8 capture antibody and pre-blocked with reagent diluent. Remaining sample
and standards were removed from CXCL1 plates and plates were washed 3 times with wash buffer (0.05% Tween-20 in PBS). Following this, 100 µL detection antibody (diluted to 40.0 ng/mL in reagent diluent) was added to each well and allowed to incubate for 2 hours at room temperature. Detection antibody was then removed from plates, plates were washed 3 times with wash buffer, and 100 µL streptavidin-horseradish peroxidase (diluted 1:40 in reagent diluent) was added to each well. After incubation in the dark at room temperature for 45 minutes, contents were removed from plates, plates were washed 3 times with wash buffer, and 100 µL substrate solution was added to each well. After incubation in the dark at room temperature for 20 minutes, 100 µL stop solution (0.25 M HCl) was added to each well and absorbance was measured at 450 nm.

Concentrations of MMP8 were measured using a human MMP8 enzyme immunoassay (DuoSet DY908; R&D Systems Inc). Steps were performed as described for the CXCL1 enzyme immunoassay except that 90 µL of each component (capture, standard/sample, detection, etc) was added to each well instead of 100 µL, and plates were blocked with 180 µL reagent diluent rather than 200 µL. Assay volumes were reduced with each subsequent enzyme immunoassay to conserve sample given limited sample quantity. A capture antibody concentration of 2.0 µg/mL, standard range of 3.9 to 4,000 pg/mL, detection antibody concentration of 75.0 ng/mL, and 1:200 dilution of streptavidin-horseradish peroxidase were used for MMP8 immunoassays. Concentrations of IL-10 were measured using an equine IL-10 enzyme immunoassay (DuoSet DY1605; R&D Systems Inc). Steps were performed as described previously for the CXCL1 enzyme immunoassay except that 80 µL of each component was added to wells and plates were blocked with 160 µL reagent diluent. A capture antibody concentration of 800 ng/mL, standard range of 0.020–20 ng/mL, detection antibody concentration of 100 ng/mL, and 1:200 dilution of streptavidin-horseradish peroxidase were used for IL-10 immunoassays. Concentrations of IFN-γ were measured using an equine IFN-γ enzyme immunoassay (DuoSet DY1586; R&D Systems Inc). Steps were performed as described previously for the CXCL1 enzyme immunoassay except that 70 µL of each component was added to wells and plates were blocked with 140 µL reagent diluent. A capture antibody concentration of 400 ng/mL, standard range of 12.2 to 12,500 pg/mL, detection antibody concentration of 400 ng/mL, and 1:200 dilution of streptavidin-horseradish peroxidase were used for IFN-γ immunoassays. Finally, concentrations of TNF-α were measured using an equine TNF-α enzyme immunoassay (Innitrogen ESS0017; Thermo Fisher Scientific). Steps were performed as described previously for the CXCL1 enzyme immunoassay except that capture antibody was diluted in carbonate-bicarbonate buffer rather than reagent diluent, 60 µL of each component was added to wells, plates were blocked with 120 µL reagent diluent, and 4% BSA in PBS was used as the reagent diluent per kit instructions. A 1:100 dilution of capture antibody, standard range of 1.0 to 1,000 pg/mL, 1:100 dilution of detection antibody, and 1:200 dilution of streptavidin-horseradish peroxidase were used for TNF-α immunoassays.

Prior to testing all samples, ELISAs were optimized for laboratory conditions. A pilot study was performed on sera from select M.tb culture–positive elephants and M.tb culture–negative elephants to determine the optimal dilution series that would allow us to calculate the concentration of each biomarker on standard curves. This revealed that samples did not contain a high enough concentration of each biomarker to warrant dilution prior to testing, hence the decision to run all samples neat. During subsequent analyses of all samples, 11 samples that were not included in the pilot study registered TNF-α concentrations above what could be interpolated from the standard curve. As a result, a follow-up assay was performed with 1:5, 1:25, and 1:125 dilutions of each of the eleven samples in reagent diluent to measure their TNF-α concentrations.

**Analysis**

We used Gen5 Software (Version 3.08; BioTek Instruments Inc), Excel (Version 2008; Microsoft Corp), and GraphPad Prism (Version 9.2.0; GraphPad Software LLC) to analyze and graph the results of the assays. We calculated the limit of detection for each assay by adding 2 standard deviations to the average optical density of the blank (ie, controls with no sample or standard added) and interpolating the concentration at this value from the standard curve. Visual inspection of the results after graphing was sufficient to conclude a lack of a statistically significant difference in samples from M.tb culture–positive elephants compared to M.tb culture–negative elephants.

**Results**

**CXCL1**

The limit of detection of the assay for CXCL1 was calculated to be 6.15 pg/mL. Of the 101 samples tested, the CXCL1 concentration was above the limit of detection in 1 sample, which was from a M.tb culture–positive elephant (M.tb-positive elephant 1; Figure 1). The CXCL1 concentration in this sample was measured to be 7.90 pg/mL. A second serum sample from the same M.tb culture–positive elephant collected 70 days after the first sample had a CXCL1 concentration below the limit of detection for the assay (Figure 2).

**MMP8**

The limit of detection of the assay for MMP8 was calculated to be 5.95 pg/mL. Of the 101 samples tested, the MMP8 concentration was above the limit of detection in 3 samples, which were consecutive samples from the same M.tb-negative elephant (M.tb-negative elephant 1; Figure 1). The first of these samples contained 7.01 pg/mL MMP8. The...
second sample, which was collected 228 days later, contained 11.8 pg/mL MMP8, and the third sample, which was collected 42 days after the previous sample, contained 13.6 pg/mL MMP8. Subsequent samples from this elephant (collected 63 days after the previous sample and later) contained MMP8 concentrations that were below the limit of detection for the assay (Figure 2).
Figure 2—Concentrations of CXCL1 (A), MMP8 (B), IL-10 (C), IFN-γ (D), and TNF-α (E) in samples from *M. tb* culture-positive and *M. tb* culture-negative elephants, shown as a function of time from the date of the first sample received from each elephant (day 0). A total of 101 samples (10 samples from 5 *M. tb* culture-positive elephants and 91 samples from 6 *M. tb* culture-negative elephants) were tested for each biomarker. Symbols represent the average concentration of the specified biomarker in 1 sample, as calculated by averaging the measured concentrations in 3 replicates. The calculated LOD for each biomarker assay is shown as a gray line.
IL-10
The limit of detection of the assay for IL-10 was calculated to be 7.73 pg/mL. The concentration of IL-10 was above the limit of detection in 16 of the 101 samples tested. These included 2 samples from 1 M.tb culture–positive elephant (M.tb-positive elephant 5), 13 samples from 1 M.tb culture–negative elephant (M.tb-negative elephant 1), and 1 sample from a second M.tb culture–negative elephant (M.tb-negative elephant 6; Figure 1). The 2 samples from M.tb culture–positive elephant 5 were collected 34 days apart and contained 274.0 pg/mL and 216.0 pg/mL IL-10, respectively (Figure 2). The 13 samples from M.tb culture–negative elephant 1 were collected over approximately 5 years (1,848 days); during this time, IL-10 concentrations ranged from 17.0 to 109.3 pg/mL. The single sample from M.tb culture–negative elephant 6 with an IL-10 concentration above the limit of detection contained 47.0 pg/mL IL-10.

IFN-γ
The limit of detection of the assay for IFN-γ was calculated to be 67.8 pg/mL. Of the 101 samples tested, the concentration of IFN-γ was above the limit of detection in 26 samples, all of which were from M.tb culture–negative elephants. These included 13 samples from M.tb-negative elephant 1 (all samples received from this elephant), 9 samples from M.tb-negative elephant 2 (all samples received from this elephant), 2 samples from M.tb-negative elephant 3, and 2 samples from M.tb-negative elephant 6 (Figure 1). The 13 samples from M.tb-negative elephant 1 were collected over approximately 5 years (1,848 days), during which time the concentration of IFN-γ ranged from 542.7 to 1,136.3 pg/mL (Figure 2). The 9 samples from M.tb-negative elephant 2 were collected over 492 days, during which time the concentration of IFN-γ ranged from 164.4 to 339.1 pg/mL. The 2 samples from M.tb-negative elephant 3 were collected 1 day apart and contained 373.8 and 340.9 pg/mL IFN-γ, respectively. Finally, the 2 samples from M.tb-negative elephant 6 were collected approximately 3 months (91 days) apart and contained 313.1 and 93.7 pg/mL IFN-γ, respectively.

TNF-α
The limit of detection of the assay for TNF-α was calculated to be 23.1 pg/mL. The concentration of TNF-α was above the limit of detection in 56 of the 101 samples tested. These included 2 samples from M.tb-positive elephant 1, 2 samples from M.tb-positive elephant 3, 1 sample from M.tb-positive elephant 5, 12 samples from M.tb-negative elephant 1, 9 samples from M.tb-negative elephant 2, 9 samples from M.tb-negative elephant 3, 8 samples from M.tb-negative elephant 4, 9 samples from M.tb-negative elephant 5, and 4 samples from M.tb-negative elephant 6 (Figure 1).

The 2 samples from M.tb-positive elephant 1 were collected 70 days apart and contained 121.9 and 73.9 pg/mL TNF-α, respectively (Figure 2). The 2 samples from M.tb-positive elephant 3 were collected 82 days apart and contained 91.7 pg/mL and 115.9 pg/mL TNF-α, respectively. The 1 sample from M.tb-positive elephant 5 contained 79.1 pg/mL TNF-α. A subsequent sample collected from this elephant 34 days later contained a TNF-α concentration below the limit of detection for the assay. The 12 samples from M.tb-negative elephant 1 comprised all but 1 sample received from this elephant, collected over approximately 5 years (1,848 days). During this time, measurable TNF-α concentrations ranged from 52.6 to 175.4 pg/mL. The 9 samples from M.tb-negative elephant 2 comprised all samples received from this elephant, collected over a period of 492 days. TNF-α concentrations in these samples ranged from 638.5 to 2,528.4 pg/mL. The 9 samples from M.tb-negative elephant 3 comprised all but 4 samples received from this elephant, collected over approximately 6.5 years (2,475 days). Measurable TNF-α concentrations in these samples ranged from 25.6 to 79.6 pg/mL. The 6 samples from M.tb-negative elephant 4 comprised all samples received from this elephant, collected over a period of 380 days. TNF-α concentrations in these samples ranged from 193.3 to 996.1 pg/mL. The 9 samples from M.tb-negative elephant 5 comprised all samples received from this elephant, collected over approximately 3 years (986 days). TNF-α concentrations in these samples ranged from 120.6 to 89,302.1 pg/mL. Finally, the 4 samples from M.tb-negative elephant 6 comprised 4 consecutive samples of the 39 samples received from this elephant. The first of these samples contained 441.8 pg/mL TNF-α. The TNF-α concentration decreased steadily in subsequent samples, reaching a concentration of 48.2 pg/mL in the fourth sample collected 70 days later.

Discussion
Concentrations of CXCL1, MMP8, IL-10, IFN-γ, and TNF-α were below the limit of detection for our assays in most samples. Except for TNF-α, most samples were below the limits of detection irrespective of M.tb status. However, a concentration above the limit of detection was present in at least 1 sample for each of the 5 biomarkers, confirming that these biomarkers can be detected in the serum of Asian elephants. Two of the 5 M.tb culture–positive elephants (M.tb-positive elephants 2 and 4) did not have detectable levels of any of the 5 biomarkers. These findings suggest that these biomarkers are not substantially elevated in M.tb culture–positive elephants compared to M.tb culture–negative elephants. As such, this work demonstrates that while the biomarkers CXCL1, MMP8, IL-10, IFN-γ, and TNF-α can be detected in Asian elephant serum, they are unlikely to share the same utility in elephant TB that they possess in human TB given that they are not elevated in clinically healthy M.tb culture–positive elephants. This finding may differ with disease state, as experimental studies using human sera have shown significantly higher concentrations of CXCL1 and MMP8 in patients with active pulmonary TB (ie, exhibiting clinical signs) compared to latent M.tb infection (ie, not clinically ill). Given their lack of
clinical signs, *M. tb* culture–positive elephants in this study were likely asymptptomatically infected, analogous to humans with latent *M. tb* infection. However, as most elephants do not exhibit clinical signs until their TB has significantly progressed, the utility of these biomarkers in elephant TB may still be limited. As biomarker concentrations were below the limit of detection for the assay in most samples, more sensitive assays beyond the commercially available ELISA kits used in this study are needed to truly understand the presence of these biomarkers, and concentrations if present, in Asian elephant serum and fully evaluate their utility in detecting *M. tb* infection in Asian elephants.

CXCL1 is a chemokine involved in neutrophil recruitment and activation. In humans, levels of CXCL1 are negligible during health, but they increase significantly during active infection and have been found to correlate with disease severity and extent in human TB patients. Despite being positive for *M. tb* on trunk wash culture, the *M. tb*-positive elephants in this study were clinically asymptomatic, which may explain why concentrations of CXCL1 in most samples from *M. tb* culture–positive elephants were below the limit of detection for the assay, similar to samples from *M. tb* culture–negative elephants. In the 1 sample with a detectable level of CXCL1, the measured concentration was only slightly above the limit of detection (7.90 pg/mL compared to a limit of detection of 6.15 pg/mL).

MMP8 is a collagenase that drives tissue pathology in TB by degrading tissue matrix and contributing to the formation of pulmonary cavities, the hallmark of TB. To the best of our knowledge, levels of MMP8 in health and disease have not previously been studied in elephants. In this study, MMP8 was above the limit of detection in a single elephant (*M. tb*-negative elephant 1), as measured in 3 consecutive serum samples collected from this elephant over 270 days. MMP8 and other matrix metalloproteinases are involved in inflammatory responses in a wide range of diseases, including cancer and arthritis. Therefore, it is likely that *M. tb*-negative elephant 1 was experiencing another inflammatory condition at the time of collection of those 3 samples, which later resolved. Although no clinical signs were observed at the time of sample collection, this animal had lenticular changes or early cataracts consistent with aging lenses, which may have contributed to MMP8 elevation as MMPs have been documented as mediators of primary and secondary cataracts in humans. As none of the *M. tb* culture–positive elephants showed clinical signs of disease, a possible explanation for finding MMP8 levels below the limit of detection in these elephants is that their disease had not significantly progressed at the time of sample collection.

IL-10 is an inhibitory cytokine, and previous research in humans has illustrated that *M. tb* induces IL-10 production to suppress the host immune response and promote survival of mycobacteria. Measurable IL-10 concentrations detected in samples from this study ranged from 47.0 pg/mL (*M. tb*-negative elephant) to 256.5 pg/mL (*M. tb*-positive elephant), all of which were below the value range reported by Edwards et al for serum IL-10 concentrations in Asian elephants (310 to 18,000 pg/mL). Given that 4 of the 5 *M. tb* culture–positive elephants did not have detectable concentrations of IL-10 and that IL-10 is involved in many infectious and inflammatory diseases, it is possible that the elevations in IL-10 seen here are the result of other inflammatory processes rather than a response to *M. tb* infection.

IFN-γ and TNF-α are inflammatory cytokines that, in human TB, have been found to play a role in macrophage activation and granuloma formation. Previous research identified IFN-γ and TNF-α in peripheral blood mononuclear cell cultures from *M. tb*-positive Asian elephants, suggesting that these cytokines are also involved in TB immunopathogenesis in elephants. In our study, IFN-γ was below the limit of detection for the assay in all samples from *M. tb* culture–positive elephants. Two *M. tb* culture–negative elephants had consistently measurable IFN-γ concentrations, and 2 additional *M. tb* culture–negative elephants had measurable IFN-γ concentrations in a subset of samples. Concentrations of IFN-γ in *M. tb* culture–negative elephants ranged from 93.7 to 1136.3 pg/mL, which are within the value range reported by Edwards et al for IFN-γ concentrations in Asian elephants (62.50 to 13,317.40 pg/mL). All *M. tb* culture–negative elephants and 3 of the 5 *M. tb* culture–positive elephants had a measurable TNF-α concentration in at least 1 of their samples, with higher concentrations observed in *M. tb* culture–negative elephants compared to *M. tb* culture–positive elephants. Most measured concentrations were within the value range reported by Edwards et al for TNF-α concentrations in Asian elephants (15.60–1,355.83 pg/mL). However, multiple samples from 2 *M. tb* culture–negative elephants had TNF-α concentrations above this range, with a maximum measured concentration of 89,302.1 pg/mL. Similar to the previously discussed biomarkers, the elevations in IFN-γ and TNF-α in *M. tb*-negative elephants likely indicate other disease processes affecting these animals. The fact that these biomarkers were below the limit of detection for many samples from *M. tb* culture–positive elephants in our study, despite previous evidence that they are involved in Asian elephant immune responses to *M. tb*, may be the result of differences in technique (i.e., sandwich ELISA vs peripheral blood mononuclear cell culture). This further suggests that more sensitive assays are needed to accurately evaluate relative concentrations of these biomarkers in *M. tb*-positive and *M. tb*-negative Asian elephants.

This study is subject to several limitations. First, the study included samples from only 5 *M. tb* culture–positive and 6 *M. tb* culture–negative elephants, meaning that differences in biomarker concentrations may have been missed due to small sample size. Additionally, neither *M. tb* culture–positive nor *M. tb* culture–negative elephants were free from other disease, with medical records describing lenticular changes, arthritis, pododermatitis, and other
conditions, which may have contributed to elevations in biomarker concentrations. Finally, \textit{M. tb} culture-positive and \textit{M. tb} culture-negative elephant samples were received from different zoos (ie, 1 zoo provided all \textit{M. tb} culture-positive samples and a second zoo provided all \textit{M. tb} culture-negative samples), meaning that differing environments could also have contributed to differences observed in biomarker concentrations between the 2 groups. For example, annual seasonality has been documented to influence cytokine production in humans and animals, with expression of Th1 cytokines such as TNF-\(\alpha\) and IFN-\(\gamma\) higher during summer months than winter months.\(^{26,29}\) Therefore, differences in the seasonal profile of regions in which zoos were located may have resulted in differences in biomarker concentrations across the 2 groups. The strengths of this study include the use of serial samples from each elephant, allowing for biomarker concentrations to be assessed over time. Additionally, all serum samples used for this study were banked samples collected during routine procedures, and therefore, no additional procedures or stress to elephants were necessary to conduct this study.

In conclusion, this study demonstrates that the biomarkers CXCL1, MMP9, IL-10, IFN-\(\gamma\), and TNF-\(\alpha\) can be detected in serum from Asian elephants. We found that these biomarkers were not significantly elevated in \textit{M. tb} culture-positive elephants compared to \textit{M. tb} culture-negative elephants. However, this may differ in \textit{M. tb} culture-positive elephants in later stages of disease that are demonstrating active clinical signs. Limiting entry criteria to exclude elephants with other disease conditions would allow for stronger comparison of \textit{M. tb}-positive and \textit{M. tb}-negative elephants. However, elephants under human care commonly experience health problems such as diseases of the musculoskeletal system, and therefore, this may be difficult to achieve. A larger sample size of both \textit{M. tb} culture-positive and \textit{M. tb} culture-negative elephants would also strengthen the study and may help to reduce confounding from other inflammatory disease conditions experienced by study elephants. Finally, using a more sensitive technique such as single molecule array (Simoa), which has high sensitivity given its larger sample size of both culture–positive \textit{M. tb} and therefore, this may be difficult to achieve. A

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