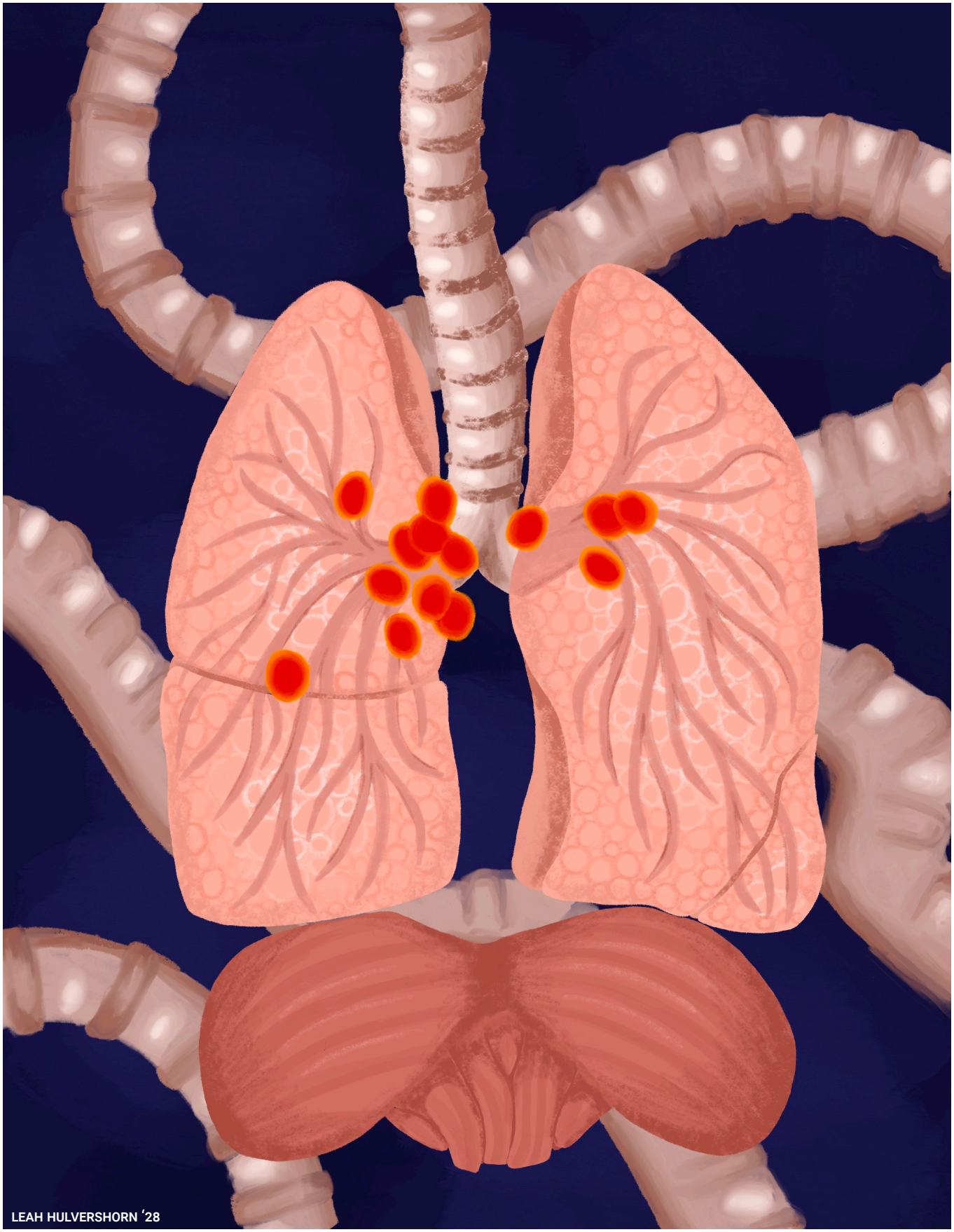

LEAH HULVERSHORN '28

ORIGINAL RESEARCH

Role of S100A8/A9 Proteins in Identifying Tuberculosis Disease States

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Tuberculosis (TB) is an infectious disease spread person to person via coughing and sneezing tiny, aerosolized droplets containing *Mycobacterium tuberculosis*. The disease has two states: active TB, where individuals suffer from all symptoms and are able to spread the disease, and latent TB, where the bacteria is present in the body, but does not grow, preventing the individual from feeling symptoms or infecting others. S100A8/A9 proteins function to regulate inflammatory processes and immune responses both extracellularly and intracellularly. The objective of this study was to determine if S100A8/A9 proteins can be used as a biomarker to differentiate between the two disease states of TB. Levels of S100A8/A9 proteins were tested in human serum samples with varying states of the disease (Active TB, Latent TB, Healthy Donor) using a sandwich ELISA. We found that S100A8/A9 proteins were highest in samples containing Active TB. Latent TB samples also contained a slightly elevated, but not significant, level of S100A8/A9 proteins compared to healthy donor samples. These data suggest that immune responses to the growth of *Mycobacterium tuberculosis* may lead to increased levels of S100A8/A9 proteins in the body, and that these proteins may be a promising candidate biomarker for rapid and reliable TB detection. Future studies are needed to increase statistical power and investigate other alternatives, like different proteins in the S100 family or human lung samples rather than human serum samples.

Known by many names, the "white death" has trailed humanity since earliest antiquity [1]. Today, approximately 1.7 billion individuals have some form of tuberculosis (TB), the disease caused by aerial transmission of *Mycobacterium tuberculosis* [2]. Upon infection, the disease may develop into either latent TB (LTB) or active TB (ATB) in individuals. Latent TB is a state of tuberculosis in which *M. tuberculosis* is present but dormant, causing infected individuals to be asymptomatic. Individuals with latent TB have a 10% chance of the disease developing into active TB [3], where M. tuberculosis is present and growing in the body, allowing the individual to experience symptoms and also transmit the disease to others.

M. tuberculosis rests among the world's leading causes of death from a single infectious agent. In 2023, tuberculosis infected roughly 10.8 million people worldwide, with an estimated 1.25 million deaths [4]. The disease is curable and preventable, but remarkably resilient, proving very challenging to eliminate fully. After more than a century of use, the BCG vaccine is still the standard of prevention, contributing to a 60-80% decrease in incidence, but with variable efficacy, particularly in adult cases [5]. Current treatments are grueling, including a combination regimen of the antibiotics isoniazid (INH), pyrazinamide (PZA), rifampin (RIF), and ethambutol (EMB) that takes anywhere from 4 to 9 months [6]. And importantly, due to the lack of symptoms within latent TB infected individuals, many are not aware that they have the disease. In fact, more than 80% of people who contract ATB in the United States originally had untreated LTB [3].

TB burden upon patients and healthcare systems is therefore immense, and there is a clear need for superior therapies and diagnostic tools to prevent its continued spread. TB causes inflammation within the body, as *M. tuberculosis* has developed various mechanisms to evade the host immune response, residing primarily in the lungs and surviving even when engulfed by phagocytes. To gain a better understanding of the biology permitting TB's persistence in the body, and to identify novel inflammatory biomarkers reflective of TB burden which could be used in diagnostic settings, we turned to the S100A8/A9 proteins. These Ca/Zn binding cytosolic proteins, part of the S100 family, regulate inflammatory processes and immune responses both intra- and extracellularly [7,8]. Previous lab data (not shown) has implicated these proteins with TB infection and disease. S100A8/A9 proteins have also been found to be a candidate biomarker for many inflammatory diseases, such as islet inflammatory responses, cystic fibrosis, inflammatory arthritis disease, transplantation, and infections [7].

S100A8/A9 proteins have diverse functions and are known to regulate calcium balance, cell apoptosis, cell proliferation, cell differentiation, energy metabolism, and inflammation [7,8]. They are mainly present in activated immune cells of myeloid origin, such as neutrophils, monocytes, and also macrophages [9]. Intracellular S100A8/A9 protein complexes play a role in cytoskeleton modulation, amino acid metabolism, and protection against pathogens. Extracellular S100A8/A9 proteins stimulate leukocyte recruitment and cytokine secretion, among other functions. S100A8 and S100A9 often bind each other, forming the abundant (representing 30-60% of neutrophil total cytosolic protein) heterodimer calprotectin which has pro-inflammatory and anti-microbial properties [10,11]. While these proteins are often involved in inflammatory responses, they also exhibit anti-inflammatory properties under specific conditions, suggesting that these proteins modulate and restrict excessive inflammation [7].

Although previous studies showed that secretion of S100A8/A9 proteins can be driven by infection-induced inflammation [7,8], whether and how S100A8/A9 protein levels are affected by the onset of various disease states in the context of tuber-

culosis has not been investigated. Thus, we tested the levels of S100A8/A9 proteins in samples of active TB, latent TB, and healthy donor (no TB) through the use of a sandwich ELISA. We present data here suggesting that S100A8/A9 proteins may be used as a biomarker for distinguishing between ATB and healthy sera. More work is needed to clarify the efficacy of S100A8/A9 serum detection in distinguishing between various disease states of TB (ATB vs. LTB).

Materials and Methods

Tuberculosis Samples

Sera from ATB, LTB, and healthy control (uninfected, HC) patients were collected from Instituto Nacional de Enfermedades Respiratorias (INER), Mexico City, Mexico. Diagnosis of patients were found through tuberculin skin testing (TST), interferon gamma release assays (IGRA), and sputum analysis. TSTs are not specific for TB and may have false positive and negative rates, so positive TST tests must be followed by additional IGRA testing specific for TB. This testing cannot confirm that the individual is currently infected with TB, but rather that they have been exposed to it. Latent infections will not expand in culture, so sputum is obtained from TST+/IGRA+ patients and subsequently cultured *in vitro* to determine whether *M. tuberculosis* is growing or not. Active TB patients test positive for all TST/IGRA/sputum tests. Latent TB patients will have positive TST/IGRA tests but negative sputum tests. Healthy donors have negative TST/IGRA/sputum tests.

S100A8/A9 Protein Quantification by ELISA

Plate Preparation. Sandwich Enzyme-Linked Immunosorbent Assays (ELISAs) (R&D Systems Human S100A8/S100A9 Heterodimer DuoSet Elisa) were performed on 3 conditions to quantify S100A8/A9 protein levels: healthy serum (negative control), ATB, and LTB. Assay standards and samples were generally prepared as detailed in the provided R&D ELISA protocol. Reagent diluent was created using 50 mM Tris, 10 mM $CaCl_2$, 0.15 M NaCl, and 0.05% Brij35. HCl was also added to the solution to adjust the pH to 7.42-7.44. Then, anti-S100A8/A9 capture antibody was diluted with 1x PBS to a final concentration of 120 µg/mL. 100 µl of diluted capture antibody was dispensed to each well in the 96-well plate. Border wells can be avoided in order to prevent evaporation/heat distribution changes. The samples were sealed and left at room temperature (20-22°C) overnight. The next morning, wash buffer and block buffer were created with 0.05% Tween 20 in PBS and filtered 1% BSA in PBS, respectively. Samples were washed with wash buffer for a total of 3 washes at 200 µl/well and blotted on paper towels. 100 µl of Block Buffer was added to each well and was left to incubate at room temperature for 1 hour. Serial dilution was then performed on the standards, and the final concentration of dilution was 93.75 µg/mL. Samples were washed with wash buffer and blotted on paper.

Assay Procedure. 100 µl of standards and samples were added to all wells and allowed to incubate at 4°C overnight. The following day, monoclonal 27E10 detection antibody was diluted to a final concentration of 60 ng/mL, added to wells, and incubated for 2 hours. Samples were washed with wash buffer, added by 100 µL/well, and washed 3 more times with 200 µL wash buffer/well. Strep-HRP solution was then created at a working concentration of 1:40 dilution of reagent diluent and 7.5 mL solvent. This solution was then added at 100 µL/well and allowed to incubate in the dark at room temperature for 20 minutes. Samples were washed using wash buffer and blotted on a paper towel in the dark. Then, substrate solution was created using a 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (tetramethylbenzidine). 100 µL of substrate solution were added to each well while simultaneously tapping on the plate in order to ensure thorough mixing. After 20 minutes of incubation in the dark, 1 M H_2SO_4 stop solution was added. Finally, the plate was read at 450 nm to quantify S100A8/A9 protein levels.

Statistical Analysis of Data

The use of a Shapiro-Wilk test of normality was employed to determine whether a non-parametric test should be used or not. All samples were ranked from least concentration to highest concentration. The resulting p-value was found to be statistically significant. Thus, the results of this test showed that the data does not follow a normal distribution, and therefore, a non-parametric test should be used. The Kruskal-Wallis test was then run on the data set. All samples were given a rank from lowest to highest. Then, the sum of these ranks was added, and an H value was calculated. Finally, a p-value was calculated using a Kruskal-Wallis p-value chart. This test showed a p-value of <0.0001, meaning the null hypothesis is rejected and at least one of the sample groups stochastically dominates another. The Dwass, Steel, Critchlow-Fligner (DSCF) method was subsequently used to check for pairwise 2-sided multiple comparison analysis.

Results

Elevated S100A8/A9 Proteins Are Found in Samples With Active Tuberculosis (ATB)

S100A8/A9 protein levels were found to be higher in ATB samples than LTB and healthy controls. ATB samples had a mean value of 1268 pg/mL, while the LTB sample mean was 345 pg/mL and healthy control mean was 196 pg/mL (Figure 1).

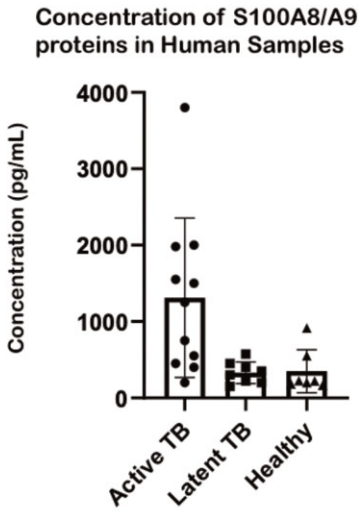

Fig. 1 | Concentration of S100A8/A9 heterodimers in human serum samples with active TB, latent TB, and no TB (healthy).

The concentration of S100A8/A9 proteins in human blood serum samples, quantified by ELISA. Active TB is presented in the first bar with 11 samples, while the latent TB and healthy donor bars contain 10 samples each.

The p-value across the three groups by Kruskal-Wallis was <0.0001. In the following post hoc DSCF pairwise comparisons, the following p-values were reported: ATB vs. LTB: <0.0001; ATB vs. HC: <0.0001; LTB vs. HC: 0.1376. ATB vs. LTB and ATB vs. HC values are considered significant against a standard p-value of significance, 0.05. This suggests that S100A8/A9 is increased by a significant amount in the sera of ATB patients when compared to the sera of either LTB or healthy control. However, the data do not demonstrate that S100A8/A9 serves as an effective biomarker in differentiating and identifying LTB patients from otherwise healthy individuals (HC).

Discussion

Leukocytes such as neutrophils, macrophages, and monocytes play a critical role in the body's immune response and contain the majority of S100A8/A9 proteins in the body. In the presence of persistent *Mycobacterium tuberculosis*, we hypothesized that a sustained level of inflammation mediated by these leukocytes could be detected through upregulated serum S100A8/A9. To investigate if S100A8/A9 proteins could serve as a biomarker for distinguishing between active and latent TB states, we employed a sandwich ELISA, selected for its high sensitivity and specificity in identifying our desired proteins. We predicted that active TB samples would contain higher levels of S100A8/A9 proteins compared to latent TB samples and that latent TB samples would contain higher levels of S100A8/A9 proteins compared to healthy donor samples. Here, we provide evidence suggesting that S100A8/A9 proteins are effective markers in distinguishing between ATB and healthy (no TB) individuals. However, we did not demonstrate that S100A8/A9 is significantly upregulated in LTB vs. healthy individuals.

The function of S100A8/A9 proteins is well characterized. Previous studies have reported that S100A8/A9 proteins are elevated in patients with systemic inflammatory response syndrome and other inflammatory diseases, and could thus serve as a representative marker to diagnose or detect the progress of these diseases. In a similar manner, *M. tuberculosis* causes symptoms in individuals that in turn activate immune responses. As speculated, our data demonstrate that S100A8/A9's presence in serum is indeed positively associated with TB burden. We therefore suggest that S100A8/A9 proteins may play a plausible and valuable role in the detection and diagnosis of tuberculosis.

This work was limited by a lack of statistical power and small sample sizes. Future work is needed to validate these claims and clarify the difference between S100A8/A9 levels in LTB vs. healthy individuals. This will allow us to conclude whether S100A8/A9 can serve as a reliable indicator of a patient's current disease status (i.e. ATB vs. LTB vs. healthy). Future directions may also look into testing other proteins within the S100 family, which also play valuable roles in regulating immune responses and inflammatory processes. Finally, future projects may look into human lung samples instead of serum and use fluorescence testing or other immunologic techniques.

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General Assay Protocol

"Human S100A8/S100A9 Heterodimer DuoSet Elisa." www.rndsystems.com, https://www.rndsystems.com/products/human-s100a8-s100a9-heterodimer-duoset-elisa_dy8226-05#assay-procedure.

References

[1] Frith, J. (2023). History of tuberculosis. Part 1 - phthisis, consumption and the white plague. JMVH, 22(2). https://doi-ds.org/doilink/11.2021-59178487/JMVH

[2] Houben, R. M. G. J. & Dodd, P. J. (2016). The global burden of latent tuberculosis infection: a re-estimation using mathematical modelling. PLOS Medicine, 13(10): e1002152. https://doi.org/10.1371/journal.pmed.1002152

[3] Centers for Disease Control and Prevention. (n.d.). Preventing tuberculosis. https://www.cdc.gov/tb/prevention/index.html

[4] World Health Organization. (n.d.). Tuberculosis. https://www.who.int/news-room/fact-sheets/detail/tuberculosis

[5] Kuan, R., Muskat, K., Peters, B., & Lindestam Arlehamn, C. S. (2020). Is mapping the BCG vaccine-induced immune responses the key to improving the efficacy against tuberculosis? Journal of Internal Medicine, 288(6), 651–660. https://doi.org/10.1111/joim.13191

[6] Centers for Disease Control and Prevention. (2025). Treatment for TB disease. https://www.cdc.gov/tb/topic/treatment/tbdisease.htm.

[7] Wang, S., Song, R., Wang, Z., Jing, Z., Wang, S., & Ma, J. (2018). S100A8/A9 in inflammation. Frontiers in Immunology, 9. https://doi.org/10.3389/fimmu.2018.01298

[8] Xia, C., Braunstein, Z., Toomey, A. C., Zhong, J., & Rao, X. (2018). S100 proteins as an important regulator of macrophage inflammation. Frontiers in Immunology, 8. https://doi.org/10.3389/fimmu.2017.01908

[9] Averill, M. M., Kerkhoff, C., & Bornfeldt, K. E. (2012). S100A8 and S100A9 in cardiovascular biology and disease. Arteriosclerosis, Thrombosis, and Vascular Biology, 32(2), 223–229. https://doi.org/10.1161/atvbaha.111.236927

[10] Nakatani, Y., Yamazaki, M., Chazin, W. J., & Yui, S. (2005). Regulation of S100A8/A9 (calprotectin) binding to tumor cells by zinc ion and its implication for apoptosis-inducing activity. Mediators of Inflammation, 280–292. https://doi.org/10.1155/mi.2005.280

[11] Stríz, I. & Trebichavský, I. (2004). Calprotectin - a pleiotropic molecule in acute and chronic inflammation. Physiological Research, 53(3), 245–53. http://dx.doi.org/10.33549/physiolres.930448