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Identification and investigation of a novel biomarker signature for active Tuberculosis

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under the supervision of Associate Professor Bernadette Saunders and Dr. Nilesh Bokil

University of Technology Sydney
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Certificate of Original Authorship

I, Jessica Pedersen, declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution. This research is supported by an Australian Government Research Training Program.

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Publications associated with this thesis

Chapter 2

Pedersen, J.L., Bokil, N.J. & Saunders, B.M. 2019, 'Developing new TB biomarkers, are miRNA the answer?', *Tuberculosis (Edinb)*, vol. 118, p. 101860.

Chapter 3

Pedersen, J.L., Barry, S.E., Bokil, N.J., Ellis, M., Yang, Y., Guan, G., Wang, X., Faiz, A., Britton, W.J. & Saunders, B.M. 2021, 'High sensitivity and specificity of a 5-analyte protein and microRNA biosignature for identification of active tuberculosis', *Clin Transl Immunology*, vol. 10, no. 6, p. e1298.

Publications related to this thesis

Hinneburg, H., **Pedersen, J.L.**, Bokil, N.J., Pralow, A., Schirmeister, F., Kawahara, R., Rapp, E., Saunders, B.M. & Thaysen-Andersen, M. 2020, 'High-resolution longitudinal N- and O-glycoprofiling of human monocyte-to-macrophage transition', *Glycobiology*, vol. 30, no. 9, pp. 679-94.

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Chapter 5

Abbreviations

°C	degrees celsius
ACK	ammonium-chloride-potassium
AGO	argonaute
AKT	protein kinase B
ANOVA	one-way analysis of variance
APC	antigen presenting cell
ATP	adenosine triphosphate
AUC	area under the curve
BCG	Bacillus Calmette-Guérin
BMDM	bone-marrow derived macrophages
CBA	cytometric bead array
CFU	colony forming units
COPD	chronic obstructive pulmonary disease
CO_2	carbon dioxide
Ct	threshold cycle
DC	dendritic cell
dL	decilitre
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribose nucleic acid
DOTS	directly observed treatment, short course
EDTA	ethylene-diamine-tetra-acetic acid
ESAT-6	early secreted antigenic target of 6 kDa
FCS	foetal calf serum
fg	femtogram
FZD8	frizzled 8
g	acceleration due to gravity
GM-CSF	granulocyte macrophage colony stimulating factor
H&E	haemotoxylin and eosin
HIV	human immunodeficiency virus
HMDM	human monocyte-derived macrophages
hsa	homo sapiens

IFN-γ	interferon-γ
IGF1R	Insulin-like growth factor receptor 1
IGRA	interferon-γ release assay
IL	interleukin
IP-10	interferon-γ inducible protein 10
KBTBD8	Kelch repeat and BTB domain containing 8
LPS	lipopolysaccharide
LTBI	latent tuberculosis infection
MCP-1	monocyte chemoattractant protein 1
MDR	multi-drug resistant
mg	milligrams
miRNA / miR	microRNA
mL	millilitres
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NF-κB	nuclease factor-kappa B
ng	nanogram
NHAR	Ningxia Hui Autonomous Region
nm	nanometre
NO_2	nitrite
NOX4	NADPH oxidase 4
NPV	negative predictive value
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered sodium
PCR	polymerase chain reaction
PC3	physical containment level 3
pg	picogram
PI3K	phosphoinositol 3 kinase
pM	picomolar

PPD	purified protein derivative
PPV	positive predictive value
PRC	Peoples Republic of China
RANTES	regulated upon activation normal T cell expressed and secreted
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
ROC	receiver operating characteristic
RPMI	Roswell Park Memorial Institute (media)
RT-qPCR	real-time quantitative reverse transcriptase polymerase chain reaction
siRNA	small interfering RNA
sTNFR1	soluble TNF receptor 1
TB	tuberculosis
Th1	type 1 T helper
TLR	toll-like receptors
TNF	tumour necrosis factor
TNFRSF-4	TNF receptor superfamily member 4
TPP	target product profiles
TST	tuberculin skin test
UTR	untranslated region
VEGF	vascular endothelial growth factor
WHO	World Health Organization
WT	Wildtype (C57BL/6 mice)
μF	microfarad
μL	microliter
μm	micrometre
μM	micromolar

Abstract

Tuberculosis (TB) is an infectious respiratory disease caused by the bacteria *Mycobacterium tuberculosis.* Each year 3 million TB cases go undiagnosed and untreated, partly due to limitations in access to care and current diagnostic tests. Eliminating this disease relies on developing new tools that rapidly, and accurately identify those with active TB disease without the need for sputum analysis. This thesis investigated plasma proteins and miRNA as biomarker candidates for TB disease to determine their utility and potential for clinical application.

My initial biomarker study assessed the expression of nine plasma proteins (IP-10, MCP-1, TNF, sTNFR1, VEGF, RANTES, Eotaxin, IL-6 and IL-10) to determine their biomarker potential, alone, and in conjunction with ten miRNA previously detected in a cohort of Chinese TB patients and controls. Six of these nine plasma proteins were significantly elevated in TB patients at diagnosis. Two, IP-10 and IL-6, fell significantly within the first month of TB treatment, while the other four (MCP-1, sTNFR1, RANTES and VEGF) remained significantly upregulated in TB patients throughout the entire 6 months of treatment, compared to controls. Their diagnostic biomarker potential was assessed finding that the nine protein biosignature could distinguish TB patients from controls with an area under the curve (AUC) of 0.908 and a sensitivity of 80% and specificity of 88%. While a promising result, we examined whether the addition of previously detected miRNA candidates could improve this accuracy. Together all 19 analytes demonstrated an AUC of 1.000 to identify the TB patients, though 19 analytes is unrealistic for a biomarker test. A novel 5-analyte biomarker signature (IP-10, miR-29a, -99b, -146a and -221) distinguished newly diagnosed TB cases from control subjects with a high sensitivity and specificity of 96% and 97% respectively. These

results were very encouraging though raised the question as to if we can use this biomarker signature to identify TB patients in other environments, especially asymptomatic incipient TB patients, who are often undetected by current diagnostic tests.

Chapter 4 addressed this question, testing the protein biomarker profile of individuals with incipient TB disease and latent TB infection (LTBI). We validated the protein candidates in a Vietnamese population of predominantly asymptomatic TB patients and control subjects, where samples were collected as part of an active case finding study. Our results demonstrated that only IP-10 and IL-6 were significantly elevated in TB patients compared to controls. Examining the biomarker potential of all nine proteins identified IP-10 to be the most accurate single analyte, able to distinguish TB patients from both control cohorts with an AUC of 0.708 and a specificity 95.5%, but a sensitivity of only 32.9%. The protein expression profile of the healthy controls and individuals with LTBI were not different, indicating that these proteins were upregulated during active TB disease rather than infection. miRNA analysis was also undertaken on a test cohort of 24 individuals. Unexpectedly the miRNA expression in the Vietnamese cohort was quite different to the initial Chinese study, with only one miR (miR-652) out of the ten, expressed in all the samples analysed. Of the four miRNA in the 5-analyte biosignature, miR-99b and miR-221 were expressed in less than 18% of samples while miR-29a and miR-146a were expressed in 50% and 77% of samples respectively. As the test study in the Vietnam cohort did not support the miRNA identified in the Chinese cohort, validation of the biosignature was not progressed further at this stage. The results from these two studies highlight that our biomarker candidates are strongly expressed in advanced TB disease and this may reflect the extensive inflammation in this cohort. Our biosignature requires modification and further study if it is to have utility to identify early stages of disease. One opportunity that these studies do suggest is the potential use of IP-10 and IL-6 as a triage tool for TB. Applying cut-off levels of 1000 pg/mL for IP-10 and 3000 fg/mL for IL-6, positively identifies 97% of the Chinese TB patients and 88% of Vietnamese TB patients, though further work is required to finesse these thresholds.

The other question that was identified in our initial biomarker study was, why are these miRNA dysregulated by TB disease, as their role in TB pathogenesis is not well established. A critical review of all then published miRNA biomarker studies for TB identified 894 miRNA reported as dysregulated in TB patients compared to control subjects. Analysis of validation studies showed only 8 had been reported by two or more publications, suggesting that the optimal miRNA signature for active TB disease has yet to be elucidated.

The last project in this thesis investigated the function of one of these miRs, miR-99b. This was undertaken using miR-99b knockout mice infected with *M. tuberculosis*. miR-99b$^{-/-}$ mice showed a similar response to WT mice to infection, as they controlled bacterial growth for the first 8 weeks as effectively as WT mice, with similar differentiation and recruitment of CD4 T cells, neutrophils and monocytes. At 12 weeks post infection the miR-99b$^{-/-}$ mice did show a small but significant reduction (0.5 log) in bacterial growth in the lung though interestingly this correlated with a small increase (0.2 log) in dissemination of bacteria to the spleen. The biological relevance of this result and function of miR-99b in regulating control to TB during chronic infection needs to be further investigated. miR-99b$^{-/-}$ mice did display increased inflammation in the lungs, early during infection, indicating that miR-99b may have a role as an anti-inflammatory mediator but overall this did not affect control of *M. tuberculosis* growth. Additional work in primary macrophages indicated a potentially contradictory role for miR-99b in *M. tuberculosis* control between humans and mice. Inhibition of miR-99b in human macrophages impeded control of infection however miR-99b$^{-/-}$ murine macrophages displayed enhanced control of *M. tuberculosis*. Future studies are ongoing to identify the gene(s) miR-99b targets during TB, including examining the potential gene target mammalian target of rapamycin (mTOR). mTOR is a validated target for miR-99b in other models, predominantly cancer, however its effect in TB has not yet been examined.

The research presented in this thesis provides valuable data for the development of biomarker candidates for new diagnostics for TB disease and demonstrated that while proteins like IP-10 and IL-6 are valuable options, alone they are not sufficient. miRNA are a promising addition though more studies are required to identify a signature that would be effective across multiple populations, and for both early and advanced TB disease. Increasing our knowledge of miRNA function is essential to understand their role in infection and their utility as biomarkers. My data examining miR-99b$^{-/-}$ mice shows that this miR is not essential to control *M. tuberculosis* infection, though the contradictory role for miR-99b between human and murine macrophages during infection requires investigation. Advancing miRNA and protein biomarkers to a clinical setting requires further research to identify confounding factors that impact the universal application of a new non-sputum biomarker test for active TB disease.