

Characterising the immunometabolic profile of HIV/TB co-infection

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Whatever you do, work heartily, as for the Lord and not for men, knowing that from the Lord you will receive the inheritance as your reward. You are serving the Lord Christ.

Colossians 3:23-24

SUMMARY

The synergy between the human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB) during co-infection of a host is well established. While this synergy is driven by immunological deterioration, the metabolic mechanisms that contribute to the associated disease burden experienced during HIV/TB co-infection remain poorly understood. Although antiretroviral therapy (ART) suppresses viral replication, these therapeutics give rise to metabolic disruptions and adaptations beyond that induced by infection. In this study, the serum cytokine and metabolic profiles of 9 untreated HIV/TB co-infected, 12 HIV/TB co-infected on ART, and 22 HIV-negative TB-positive patients, as well as 29 healthy controls, were measured and compared. A cytometric bead array kit was used for multiplexed cytokine measurements, and an untargeted two-dimensional gas chromatography time-of-flight mass spectrometry approach was used for metabolomics analyses. There was no significant difference between the cytokine levels of the diseased groups when compared to each other or the controls, however, the co-infected individuals were characterised by increased interleukin (IL)-6, and interferon-gamma (IFN- γ) when compared to the controls. The concomitant increase of cytokines typically classified as T helper cell type (Th) 1 or Th2, and as pro-inflammatory or immunoregulatory, suggests a failure of immunoregulation, resulting in an increased disease burden. Metabolites indicative of cachexia, damage of the intestinal mucosa and dysbiosis of the microbiome characterised co-infected individuals from the TB-positive population, while co-infected individuals were distinguished from healthy controls by similar alterations with the addition of more extensive amino acid changes. Loss of gut integrity and subsequent microbial translocation results in increased inflammation and immune/cytokine activation, culminating in a reduced appetite and malabsorption, which support the profile of exacerbated wasting in HIV/TB co-infected individuals. Treating HIV in the co-infected group revealed that some altered metabolites returned to values comparable to those of the healthy or the TB-positive population. However, it is unclear whether this represents a return to a more healthy state, as other metabolic alterations were exacerbated upon treatment. These results suggest that HIV augments the HIV-*Mtb* synergy, at least in part, through its detrimental effects on gut health, which in turn, affects energy availability.

Keywords: HIV/AIDS; tuberculosis; HIV/TB co-infection; metabolomics; cytokines; immunometabolism

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LIST OF ABBREVIATIONS

Abbreviation	Meaning	Abbreviation	Meaning
2,4-DHBA	2,4-Dihydroxybutyric acid	LDL	Low-density lipoprotein particles
3,4-DHBA	Dihydroxybutyric acid	LTB	Latent <i>Mtb</i> infection
AIDS	Acquired immunodeficiency syndrome	MCP	Monocyte chemoattractant protein
Ala	Alanine	MDR	Multidrug-resistant
ANOVA	Analysis of variance	MFI	Mean fluorescent intensity
APC	Allophycocyanin	MHC	Major histocompatibility complex
ART	Antiretroviral therapy	MIP	Macrophage inflammatory protein
ARV	Antiretroviral	MPE	Malignant pleural effusion
ASCA	ANOVA simultaneous component analysis	MS/MS	Tandem mass spectrometry
Asp	Aspartate	MSEA	Metabolite set enrichment analysis
ATT	Anti-TB treatment	<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
AUC of ROC	Area under curve of receiver operating characteristic	NA	Nucleoside analogue
BALF	Bronchoalveolar lavage fluid	NMP	National Metabolomics Platform
BCAA	Branched-chain amino acid	NMR	Nuclear magnetic resonance
BH	Benjamini-Hochberg	NNRTI	Non-nucleoside reverse transcriptase inhibitor
BH₄	Tetrahydrobiopterin	NRTI	Nucleoside reverse transcriptase inhibitor
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide	OPLS-DA	Orthogonal projections to latent structure-discriminant analysis
CAP	Community-acquired pneumonia	OS	Oxidative stress
CBA	Cytometric bead array	PAH	Phenylalanine hydroxylase
CD	Cluster of differentiation	PBMC	Peripheral blood mononuclear cell
CP	Catabolic products	PC	Persistent controllers
CPT	Cell Preparation Tube	PCA	Principal component analysis
CSF	Cerebrospinal fluid	PCR	Polymerase chain reaction
CV	Coefficient of variation	PE	Phycoerythrin
CVD	Cardiovascular disease	Phe	Phenylalanine
DL	Dyslipidemic	PI	Protease inhibitor
DM	Diabetes mellitus	PLS-DA	Partial least squares-discriminant analysis
DOTS	Directly observed treatment, short-course	PRR	Pattern recognition receptor
DS	Drug-susceptible	PUFA	Polyunsaturated fatty acid
ELISA	Enzyme-linked immunosorbent assay	QC	Quality control
ELISPOT	Enzyme-linked immunospot	RA	Receptor antagonist
ESI	Electrospray ionization	RFA	Random forest analysis
FAME	fatty acyl methyl ester	ROS	Reactive oxygen species

FC	Fold change	RPLC-Q-TOFMS	Reversed-phase high-performance liquid chromatography quadrupole time-of-flight mass spectrometry
FCS	Flow cytometry standard	SATVI	South African Tuberculosis Vaccine Initiative (SATVI)
FDC	Fixed-dose combination	SD	Standard deviation
FDR	False discovery rate	SMPDB	Small Molecule Pathway Database
FIA-MS/MS	Flow injection analysis tandem mass spectrometry	SSC	Side scatter
FluoroSPOT	Fluorescence-based variation	TB	Tuberculosis
FSC	Forward scatter	TC	Transient controllers
GCH	GTP cyclohydrolase (GCH)	TCA	Tricarboxylic cycle
GC-MS	Gas chromatography mass spectrometry	TGF	Transforming growth factors
GCxGC-TOFMS	Two-dimensional gas chromatography time-of-flight mass spectrometry	Th	T-helper cell type
Gln	Glutamine	TMCS	Trimethylsilyl chloride
Glu	Glutamate	Tn	Treatment
GM-CSF	Granulocyte-macrophage colony-stimulating factor	TNF	Tumour necrosis factor
HCA	Hierarchical cluster analysis	TPE	Tuberculous pleural effusion
HDL	High-density lipoprotein particles	Tregs	T regulatory cells
His	Histidine	Trp	Tryptophan
HIV	Human immunodeficiency virus	TST	Tuberculin skin test
IAA	3-Indoleacetic acid	Tyr	Tyrosine
ICS	Intracellular cytokine staining	UHPLC-MS/MS	Ultra-high-performance liquid chromatography-tandem mass spectrometry
IDO1	Indoleamine-2,3-dioxygenase 1	UPLC-MS	Ultra-high-performance liquid chromatography-mass spectrometry
IDO2	Indoleamine 2,3-dioxygenase 2	UPLC-qTOF	Ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry
IFN	Interferon	UPLC-TQMS	Ultra-pressure liquid chromatography triple quadrupole mass spectrometry.
IGRA	Interferon-gamma release assay	VEGF	Vascular endothelial growth factor-C
IR	Immunological responders	VL	Viral load
IRIS	Immune reconstitution inflammatory syndrome	VLDL	Very-low-density lipoprotein particles
Kyn	Kynurenine	WHO	World Health Organization
LAM	Lipoarabinomannan	XDR-TB	Extensively drug-resistant tuberculosis
LC-MS	Liquid chromatography mass spectrometry	xMAP	Multi-analyte profiling
LC-MS/MS	Liquid chromatography-tandem mass spectrometry	γ-GC	Gamma-glutamyl cycle

CHAPTER 1: PREFACE

1.1 Background, motivation and problem statement

Although tuberculosis (TB) is an ancient disease (Wang *et al.*, 2016), it still ranked as the top cause of death due to a single pathogen from 2007–2019, ranking above the human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) (Lyles *et al.*, 1999; World Health Organization, 2019). It is expected that coronavirus disease 2019 (COVID-19) will now outrank TB as the leading cause of infection-related death as of 2020 (World Health Organization, 2021). HIV/AIDS and TB are pandemics with high morbidity and mortality rates (Tiberi *et al.*, 2017), with an estimated 1.3 million global TB deaths among HIV-negative individuals and an additional 214 000 deaths amongst HIV-positive individuals in 2020 alone (World Health Organization, 2020). When co-infection with HIV and *Mycobacterium tuberculosis* (*Mtb*; the causative agent of TB) is established, these pathogens act synergistically to increase the disease burden in the host (Kwan & Ernst, 2011). Due to the immunosuppressive effects of HIV on the host, HIV-positive individuals have a 26–31 times greater risk of developing active TB compared to HIV-negative individuals (World Health Organization, 2012).

The rate of co-infection is highest in sub-Saharan Africa, with South Africa having the second-highest (after Lesotho) co-infection mortality rate in 2019 when approximately 38 500 HIV-positive individuals died of TB (World Health Organization, 2020). With timely diagnosis and treatment, many of these deaths can be prevented as TB is a curable disease, but current diagnostic tests have a limited capacity to detect TB in HIV-positive individuals (Tiberi *et al.*, 2017).

During co-infection, the core of the synergistic actions of HIV and *Mtb* lies in the deterioration of immunological functions (Pawlowski *et al.*, 2012). This has profound effects on the host metabolism, hence in recent years, the field of immunometabolism has emerged in an attempt to better characterise these changes. Immunometabolism refers to the convergence of bioenergetic pathways and the specific functions of immune cells, as defined by Shi *et al.* (2016). It is well-established that HIV infection, TB, and HIV/TB co-infection cause complex alterations to metabolism, a topic of intense research in recent decades, however, these changes have not been fully characterised. Metabolomics provides unique insights into pathogen-specific metabolic changes, as well as the effects of these pathogens on the host, as it can relate to the ongoing disease phenotype, which is closely related to the metabolic state of the host. Though the individual metabolic profiles associated with HIV infection and TB have been studied using various metabolomics approaches, this has not yet been done in an exploratory context for the co-infected disease state.

In light of the close association between the immune and metabolic systems, concurrently measuring immunological (e.g. cytokine) and metabolic alterations may lead to a deeper understanding of the

mechanisms underlying the synergy of these pathogens in the HIV/TB co-infected host. This has been done for HIV infection (Peltenburg *et al.*, 2018) and TB (Weiner *et al.*, 2012). The blood cytokine alterations during untreated HIV/TB co-infection have been investigated in multiple contexts, although most often measurements were made after *in vitro* exposure to *Mtb* antigens (Murray *et al.*, 2018), and not concurrently with metabolic alterations. The existing literature on the inherent cytokine profile in the blood of co-infected patients is contradictory, as the expression of a particular cytokine at any moment in time is highly dependent on a multitude of factors, including other cytokines.

As the metabolic profile of HIV/TB co-infected individuals has hitherto remained largely uncharacterised, untargeted metabolomics, by virtue of its exploratory nature, could contribute to a better understanding of this disease phenotype. The specific metabolic effects of HIV infection and its treatment on TB patients have also not been characterised comprehensively, i.e. has only been done in specific contexts. Furthermore, seeing as metabolic alterations can originate from altered immunological functions, concomitantly measuring cytokine and metabolic levels, could contribute to the better characterisation of the underlying mechanisms of these processes. Ultimately, the findings may lead to the development of improved diagnostics, better prognosis and disease management, and treatment monitoring of this combined disease state, which should be a priority considering the global prevalence of HIV/TB co-infection and the devastation to people's lives and the health care system.

1.2 Aims and objectives

1.2.1 Aims

This investigation aims to use flow cytometry and two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOFMS) to better characterise:

- 1) HIV/TB co-infection by comparing the serum immune and metabolic profiles of untreated comorbid HIV-positive patients with active pulmonary TB (HIV+/TB+/Tn-; n=9) to those of healthy controls (HIV-/TB-/Tn-; n=29).
- 2) The serum immune and metabolic effects of HIV and its treatment on a TB-positive population via the comparison of untreated HIV-negative patients with active pulmonary TB (HIV-/TB+/Tn-; n=29), untreated HIV-positive patients with active pulmonary TB (HIV+/TB+/Tn-; n=9), and HIV-positive patients with active pulmonary TB, on ART (HIV+/TB+/Tn+; n=12).

1.2.2 Objectives

Considering these aims, the objectives are to:

- 1) Determine and validate the repeatability of both the cytokine and metabolomics analytical methods, as well as that of the apparatuses used (flow cytometer and GCxGC-TOFMS respectively);
- 2) Apply these validated methodologies to all collected serum samples;
- 3) Apply various statistical approaches to extract and identify significant cytokine and metabolite markers pertaining to the aims of this investigation;
- 4) Interpret all results based on previously published literature.

1.3 Structure of the dissertation and research outputs

This dissertation is written in article format according to the guidelines of the North-West University (NWU), Potchefstroom Campus, South Africa, for the completion of the degree *Magister Scientiae* (Biochemistry). As such, a complete literature review (Chapter 2) as well as a methods chapter (Chapter 3) are provided. The subsequent chapter (Chapter 4) will have an introduction, materials and methods, results, discussion, conclusion, and reference list. The dissertation will be concluded with a summary of all findings and future prospects in this area of research.

Chapter 1 (the current chapter) provides the background and motivation for this study, as well as the aims and objectives defined in light of that. It also outlines the structure of the dissertation and all the publications and other research outputs generated from this study.

Chapter 2 presents a literature review of the immune and metabolic characteristics of HIV infection, TB, and HIV/TB co-infection. A part of this chapter has been published as a review paper (see Appendix B):

- Liebenberg, C., Luies, L., and Williams, A.A. 2021. Metabolomics as a tool to investigate HIV/TB co-infection. *Frontiers in Molecular Biosciences*, 8: 1009. (Impact factor: 5.246)

Chapter 3 explains the overall study design, the sample collection, as well as the validation and details of the cytokine and metabolomics methodologies used.

In **Chapter 4** we report on the immune and metabolic changes associated with HIV/TB co-infection as well as the effects of HIV infection and its treatment on a TB-positive population (post-application of the validated methodology and data reported in Chapter 3). This chapter has been submitted for publication in *eLife* (submission no: 06-12-2021-RA-eLife-76156; see Appendix B).

Parts of this chapter have also been presented as posters at two international conferences and as an oral at one South African conference:

- Liebenberg, C., Luies, L., Loots, D.T., Williams, A.A. Characterising the serum metabolic profile of HIV/TB co-infection. Presented as a poster at the 23rd International AIDS Conference: Virtual, 6–10 July 2020.
- Liebenberg, C., Luies, L., Loots, D.T., Williams, A.A. Characterising the serum metabolic profile of HIV/TB co-infection in cohorts treated and untreated for HIV. Presented as a poster at the 16th Annual Conference of the Metabolomics Society: Metabolomics 2020 Online.
- Liebenberg, C., Luies, L., Loots, D. and Williams A.A. (2022). Characterising the immunometabolic profile of HIV/TB co-infection in cohorts untreated and treated for HIV. Presented as an oral at the SASBMB-2022 national conference, Online, 23–26 January 2022.

Chapter 5 summarises all the results and conclusions of this study in relation to the aims and objectives set out in Chapter 1. It also highlights considerations for future studies.

1.4 Author contributions

The contributions of the main author of this dissertation (Ms Chandré Liebenberg), co-authors to publications and other research outputs, and co-workers are outlined in Table 1.1.

The following statement from the main author, supervisor and co-supervisors confirms their roles in the study and gives their permission that the data and conclusions generated from this study may be used as part of this dissertation: I declare that my role in this study, as indicated in Table 1.1, is a representation of my actual contribution, and I hereby give my consent that this work may be published as part of the M.Sc. dissertation of Chandré Liebenberg.

			
Dr. Aurelia Alvina Williams (supervisor)	Dr. Laneke Luies (co-supervisor)	Prof. Du Toit Loots (co-supervisor)	Chandré Liebenberg (M.Sc. candidate)

Table 1.1: Researchers involved in this study.

Co-author	Collaborator	Contribution
Chandré Liebenberg (B.Sc. Hons. Biochemistry)		Conceptualised, planned, executed cytokine experiments, analysed all data, and wrote the dissertation and all associated documents and publications, together with the supervisor and co-supervisor
Dr Aurelia Williams (Ph.D. Biochemistry)		Supervisor: Conceptualised and co-ordinated all aspects of the study, including study design, planning, execution, writing of the dissertation and all associated documents and publications
Dr Laneke Luies (Ph.D. Biochemistry)		Co-supervisor: Conceptualised and co-ordinated all aspects of the study, including study design, planning, execution, writing of the dissertation and all associated documents and publications
Prof Du Toit Loots (Ph.D. Biochemistry)		Co-supervisor: Conceptualised the study design, contributed to conference outputs, data interpretation and reviewed the dissertation
	National Metabolomics Platform (NMP)	Assisted with the GCxGC-TOFMS analysis for the metabolomics section of this study
	The South African Tuberculosis Vaccine Initiative and the Desmond Tutu HIV Centre at the Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa	Collected and provided all serum samples and associated clinical information

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CHAPTER 2: LITERATURE REVIEW

Parts of this chapter have been published:

- Liebenberg, C., Luies, L. & Williams, A.A. 2021. Metabolomics as a tool to investigate HIV/TB co-infection. *Frontiers in Molecular Biosciences*, 8:1009. (Impact factor: 5.246)

This chapter introduces immunometabolism and its measurement and presents a review of the immune and metabolic characteristics of HIV infection, TB, and HIV/TB co-infection. Considering that limited HIV/TB co-infection metabolomics studies have been published to date, this Chapter summarises what is known regarding the HIV/TB co-infection synergism from a conventional and metabolomics perspective. It then explores metabolomics as a tool for the improved characterisation of HIV/TB co-infection in the context of previously published human-related HIV infection and TB investigations, respectively, as well as for addressing the gaps in existing knowledge based on the similarities and deviating trends reported in these HIV infection and TB studies.

2.1 Immunometabolism: What is it and why is it important?

The immune response consists of two different but integrated systems: innate and adaptive immunity. Innate immunity is immediate, non-specific, and consists of two major responses, namely inflammation and the complement system (a network of soluble and membrane proteins) (Dunkelberger & Song, 2010). When a pathogen persists despite the activation of innate defences, adaptive immunity is activated, which produces reactions specific to the particular pathogen through leukocytes. The outcome of the immune response determines whether life continues for the host, the pathogen, or both, at least for some time (Amiel & Perona-Wright, 2020b). As such, a delicate balance is required so that the immune response is rigorous enough to effectively eliminate a pathogen while minimising damage to the host, which may be induced by the very processes meant to protect the host (Amiel & Perona-Wright, 2020a). A major regulator of this balancing act is cellular metabolism. “Metabolism” derives from the Greek word for “change” and refers to all the interconnected biochemical reactions that maintain chemical homeostasis within an organism (Assfalg *et al.*, 2008; Zhang *et al.*, 2018). The attempts of the host to maintain homeostasis are ultimately reflected in the regulation of specific pathways and the resulting changes in the levels of the intermediates of metabolic reactions referred to as metabolites. These changes are mediated largely through cytokines, which serve as messengers of the immune system (Zhang *et al.*, 2018). These small, soluble proteins play a central role in the initiation, modulation, and termination of the immune responses, as well as its interactions with other systems (Holdsworth & Gan, 2015).

Upon infection, the recognition of foreign material initiates an immune response in the host. Significant amounts of energy and biosynthetic precursors are required to mount and sustain an immune response

(Aounallah *et al.*, 2016). Immune cells show a distinct metabolic phenotype in the activated versus the inactivated state; hence the last few years have seen a massive increase in interest in immune cell nutrition and metabolism. This has led to the field of immunometabolism, which is concerned with the metabolic reprogramming that accompanies immune cell activation, proliferation, and effector function. In other words, immunometabolism is the study of the interplay between immune responses and host-pathogen metabolism (O'Neill *et al.*, 2016).

While the host induces various metabolic changes to produce an immune response, the pathogen modulates and/or reprograms host metabolism to suit its own needs, for example, reproduction and immune evasion or persistence (Amiel & Perona-Wright, 2020b). This results in metabolic dysfunction for the host that becomes particularly prominent if the immune response fails to eliminate the pathogen and the infection becomes chronic.

Although the term “immunometabolism” has mainly been used in the context of the metabolism of immune cells, the effects of these alterations extend to systemic metabolism as the organism’s energy and biosynthetic stores are directed towards the immune response. This is most prominent with chronic infections since the immune system is continuously active and the organism must adapt to long periods of higher energy and biosynthetic demand (Palmer *et al.*, 2016). The characterisation of the relationship between immune and metabolic markers in systemic samples such as blood or urine, which are already routinely used for diagnostic and prognostic procedures, may ultimately lead to the development of simple but robust assays for the diagnosis, prognosis, and management of diseases. Cytokines have been extensively studied as potential biomarkers in this regard as they are relatively simple to measure and can be used to develop clinical tests (Monastero & Penttala, 2017).

2.2 Cytokines as indicators of the immune response to infection

Nearly all cell types, except red blood cells (Dinarello, 2007), respond to and secrete cytokines (Kany *et al.*, 2019). Cytokines affect target cells through interaction with specific receptors on the surface of these cells. Although typically secreted in very small quantities, cytokines trigger signalling cascades that have far-reaching consequences for the organism (Dinarello, 2007). The term “cytokine” is a general term encompassing various families of these proteins, which include, but are not limited to, the interleukins (IL), interferons (IFN), chemokines, tumour necrosis factors (TNF), and transforming growth factors (TGF) (Kidd, 2003).

Historically, the classification and naming of these proteins have been controversial, mainly due to the multiplicity of biological properties. Cytokines are pleiotropic, meaning that a single cytokine has different effects depending on the target cell. Cytokine function is also redundant and even synergistic when different cytokines have the same effect. Different cell populations can also produce the same cytokine (Dinarello, 2007). Cytokines are typically divided into superfamilies based on structure and

function, and not on a common gene. As such, each cytokine is similar in function to other members of its superfamily but is a separate gene product (Dinarello, 2007). Additionally, cytokines can be divided into functional classes: lymphocyte growth factors, those that polarise immune function to antigens (adaptive immunity), as well as pro- and anti-inflammatory (Turner *et al.*, 2014).

Cytokines induce protective local inflammation as well as systemic acute-phase responses to coordinate the innate immune response and have roles in initiating, mediating, and regulating adaptive immune responses. When a pathogen breaks through physical barriers such as the mucosal membranes of the host, innate immune cells recognise pathogen-associated molecular patterns as well as danger-associated molecular patterns present on the pathogen through pattern recognition receptors (PRRs). The activation of PRRs result either in immediate phagocytosis of the pathogen (Cheng *et al.*, 2014), or trigger various signal transduction pathways that, in turn, activate various transcription factors. In the case of the latter, cellular responses are initiated and activate the release of soluble mediators such as cytokines to recruit more immune cells, orchestrate further immune cell activation, and recruit humoral defence proteins. These events constitute inflammation (Holdsworth & Gan, 2015; Kany *et al.*, 2019). Additionally, the acute response syndrome is initiated via a set of cytokines to prepare a systemic antipathogenic reaction, and adaptive immunity is initiated through antigen presentation. The initiation and inhibition of the latter are also highly dependent on regulation by cytokines (Holdsworth & Gan, 2015).

Once foreign material has been processed by immune cells, it is presented on the surface of the cell and recognised by T cell receptors. In addition to antigen recognition, co-stimulatory molecules [such as the cluster of differentiation (CD) receptors] expressed on the surface of antigen-presenting cells bind to receptors on T cells for subsequent activation. The specific subset of T cell differentiation depends on the innate cytokines released upon antigen presentation (Holdsworth & Gan, 2015) (see Figure 2.1).

Initially, two subsets of T helper cells (expressing the CD4 glycoprotein) (Spellberg & Edwards, 2001), T helper type 1 and 2 (Th1 and Th2, respectively), were recognised by Mosmann *et al.* (1986). This led to the Th1/Th2 balance hypothesis, which postulated that the homeostasis between Th1 and Th2 cell activity orchestrates all other T cell responses (Kidd, 2003). Th1 responses were also reported to inhibit the induction of Th2 responses and vice versa (Spellberg & Edwards, 2001). It is now accepted that these T cell subsets form part of a larger integrated system rather than being at the centre of its orchestration (Kidd, 2003). Th1 cells have roles in cellular immunity and are prominently involved in stimulating antigen presentation and defence against intracellular pathogens, while Th2 cells are involved in humoral immunity (stimulating high antibody titers) and are prominent in the defence against extracellular pathogens (Zhou *et al.*, 2009). However, these roles are not clear cut as Th1 cells are involved in some aspects of what is typically considered Th2 responses, and vice versa (Spellberg & Edwards, 2001). In 2005, another distinct subset of T helper cells was recognised, Th17 cells, so-called due to the predominant production of IL-17 (Langrish *et al.*, 2005). Th17 cells are important in the defence against bacterial, fungal

and viral infections at epithelial and mucosal surfaces, including the gut mucosa (Khader *et al.*, 2009). Thus, the Th1, Th2, and Th17 subsets have prominent roles in responses to infection with various pathogens and measurement of their levels and functions stand to provide insight into mechanisms of pathology in the corresponding diseases. Although not directly pertinent to this study, several other T helper cell subsets also play important roles in the immune response or have immune regulatory effects that influence the function of other T helper cell subsets. For example, T regulatory cells (Tregs) expressing the forkhead box P3 transcription factor are dedicated to the regulation of immune responses to pathogens (Josefowicz & Rudensky, 2009). These cells are induced by TGF- β and various interleukins. They also produce TGF- β in addition to several other immunosuppressive cytokines, such as IL-10 (Read *et al.*, 2000) and members of the IL-12 family (Collison *et al.*, 2007). These cells have also been implicated in the orchestration of Th1, Th2, and Th17 functions and are important to the regulation of immune cell responses as a whole.

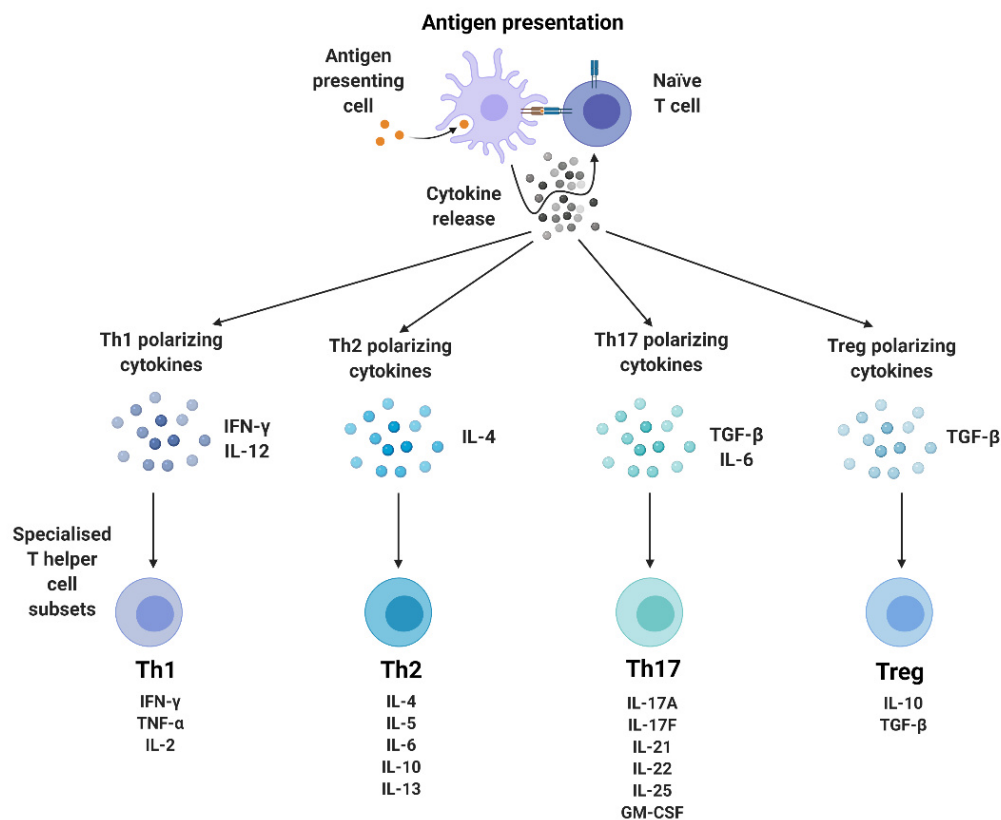


Figure 2.1: A simplified view of T helper cell differentiation [created by the author using Biorender.com, based on information contained in Ivanova and Orekhov (2015) and Zhou et al. (2009)]. Abbreviations: IFN: interferon; TNF: tumour necrosis factor; IL: interleukin, TGF: transforming growth factor; GM-CSF: granulocyte-macrophage colony-stimulating factor.

The cytokines that induce the Th1, Th2 and Th17 differentiation, and those that are subsequently produced by the differentiated cells, have been grouped accordingly in Figure 2.1. In this Chapter, the focus will be on seven cytokines that are typical of Th1, Th2, and Th17 responses, and which form part of the kit that will be used in this study (see Chapter 3). The Th1 cytokines of interest are IL-2, IFN- γ , and

TNF- α ; the Th2 cytokines are IL-4, IL-6, and IL-10; and the Th17 cytokine is IL-17A. A summary of the roles of these cytokines is given in Table 2.1. It is important to note that while these cytokines have traditionally been associated with specific T helper cell subsets, the pleiotropic nature of cytokines imply that they play numerous and sometimes conflicting roles, which depend on the specific immunological situation (see Table 2.1).

Table 2.1: A summary of the role of selected Th1/Th2/Th17 cytokines in the immune response in general.

Cytokine	Associated T helper cell subset	Biological role	Reference(s)
IFN- γ	Th1	Pro-inflammatory; induces Th1 differentiation (in combination with IL-12) and is the main cytokine responsible for the Th1 pro-inflammatory responses: stimulates phagocytosis, the oxidative burst, upregulates MHC class I and II molecule expression (for antigen presentation), induces cytokine expression in other cell types, activation of macrophages.	Spellberg and Edwards (2001); Turner <i>et al.</i> (2014)
TNF- α	Th1	Pro-inflammatory; induces expression of adhesion molecules and cytokines, stimulates the characteristics of inflammation (vasodilation oedema, fever), stimulates the oxidative burst, and is involved in the activation of macrophages.	Cooper and Khader (2008); Turner <i>et al.</i> (2014); Zelová and Hošek (2013)
IL-2	Th1	Pro- and anti-inflammatory; T cell growth factor, upregulates the cytolytic activity of killer cells, stimulates differentiation of Th1, Th2, Th9, Treg, and inhibits differentiation of Th17 and T follicular helper cells.	Spolski <i>et al.</i> (2018)
IL-4	Th2	Anti-inflammatory; induces Th2 and inhibits Th1 differentiation (major regulator of the immune response), differentiation and stimulation of B cells, regulates lymphoid cell apoptosis, involved in the proliferation and differentiation of various non-immune cell types.	Gadani <i>et al.</i> (2012); Ivanova and Orekhov (2015)
IL-6	Th1, Th2	Pro- and anti-inflammatory; T cell activation, regulation of Th2 and Treg subsets, B cell stimulatory factor, haematopoiesis, production of acute-phase proteins.	Luo and Zheng (2016); Turner <i>et al.</i> (2014)
IL-10	Th2, Treg	Anti-inflammatory; immunoregulation to prevent immunopathology associated with excessive Th1 and CD8 T cell responses. Also regulates Th2 responses in coordination with Th1 cytokines.	Couper <i>et al.</i> (2008)
IL-17A	Th17	Pro-inflammatory; primarily acts on non-hematopoietic cells, inducing cytokine and chemokine production for the recruitment of neutrophils and macrophages, and anti-pathogenic peptide production by responder cells.	Jin and Dong (2013); Tsai <i>et al.</i> (2013)

Cytokine: IFN: interferon; TNF: tumour necrosis factor; IL: interleukin.

Associated T helper cell subset: Th1: T helper cell type 1, Th2: T helper cell type 2, Treg: T regulatory cells; Th17: T helper cell type 17.

Biological role: MHC: major histocompatibility complex.

2.2.1 Measuring cytokines

Cytokines typically occur in the pictogram per millilitre range in body fluids and tissues under homeostatic conditions (Schenk *et al.*, 2001). The levels of these proteins increase by several orders of magnitude upon the activation of cytokine-associated pathways such as the inflammatory response (Dinarello, 2007). Though these increases are transient, the measurement of cytokines has played an important part in the understanding of disease progression in various disease states (Bienvenu *et al.*,

2000). The properties and function of cytokines set certain requirements for detection methods; high selectivity (numerous cytokines of different molecular weight and isoelectric points exist), multiplexing capability within a single sample (due to the networked function of cytokines) and high sensitivity (low concentrations) (Stenken & Poschenrieder, 2015).

Typical methods used for cytokine measurement include bioassays, the enzyme-linked immunosorbent assay (ELISA), the enzyme-linked immunospot (ELISPOT) assay (as well as the fluorescence-based variation, FluoroSPOT), and indirect methods such as polymerase chain reaction (PCR; by determining gene expression) (Stenken & Poschenrieder, 2015). The major disadvantages associated with these methods include the need for a large sample volume and the time required for data generation. Although the widely-used ELISA is reproducible, the experience and skill of the analyst can affect the results (Leng *et al.*, 2008; Sudbury *et al.*, 2020). As cytokines are released as part of a larger regulatory network, the measurement of a single cytokine can only provide limited insights into the specific mechanisms employed in a particular disease state (Talat *et al.*, 2011). As such, multiplexed methods achieve more of the requirements for cytokine analyses described above, with the main advantage of multiplexed methods being that multiple cytokines can be measured in a small sample volume, with reduced expense and higher throughput (Tighe *et al.*, 2015). However, this comes with the caveat that cross-reactions must be carefully avoided in the complex mixture of antibodies and antigens (Sudbury *et al.*, 2020). Bead-based immunoassays and protein arrays are commonly used multiplexing methods. Multiplexing will be explained in the context of bead-based immunoassays, as pertinent to this study.

Bead-based immunoassays fulfil the requirements for cytokine measurement set out above by employing monoclonal anti-cytokine antibodies (selectivity), *in situ* separation based on the properties of different bead sets (multiplexing) and fluorescent detection by flow cytometry, which offers a wide dynamic range (sensitivity) (Stenken & Poschenrieder, 2015). Microspheres/beads of approximately seven micrometres in diameter are dyed with a fluorochrome, which, upon excitation with an argon laser, fluoresces at 650 nm. Different populations of such beads are created by varying the intensity of the embedded fluorochrome such that an immunoassay can be created for each cytokine of interest. This is achieved by covalently attaching an antibody against the cytokine of interest to all the beads of a particular bead population. The combination of the antibody and the bead forms the capture bead for the specific analyte, which retains the specific signal intensity of the bead population selected. This unique signal intensity allows the *in situ* separation of each analyte's bead population at the end of the assay. Detection antibodies, conjugated to a fluorochrome such as phycoerythrin (PE), and specific to each analyte makes quantification possible, as the signal produced upon excitation is proportional to the concentration of the analyte in the sample. Quantification is achieved by setting up calibration curves using cytokine standards for each analyte of interest, from which concentrations are calculated by mathematical interpolation using dedicated software. Thus, analytes are identified by the intensity of the bead

population signal and quantified based on the intensity of the detection antibody-fluorochrome complex signal (Stenken & Poschenrieder, 2015).

Flow cytometry is often used in cytokine secretion studies, either to characterise specific subsets of cytokine-producing cells or to facilitate the measurement of the cytokines themselves, such as in bead-based immunoassays. This technique allows for the measurement of the optical and fluorescence characteristics of particles, such as cells, which pass through a light source in a fluid stream (Macey, 2007). This is possible due to light scattering and fluorescence emission as the light from the source hits a particle at the interrogation point. The resulting signals are filtered and directed to detectors placed at various angles from the interrogation point, allowing the isolation of each type of signal and thereby gaining insights into specific characteristics of the cell. A flow cytometer gives information relating to the size and complexity of particles via the scattering of light. Forward scatter (FSC; which represents size) results from light passing around the particle, while side scatter (SSC; which represents complexity) results from light passing through the particle and being deflected by internal structures or denser regions (Adan *et al.*, 2017).

More complex information can be gained by flow cytometry by the measurement of inherent and/or experimentally added fluorescent properties of cells or particles. For instance, by using a sandwich immunoassay, the presence, absence, or quantity of a particular biological structure can be measured. In such an assay, two different antibodies are added to a sample, one to bind to the biological structure of interest, and another (which is conjugated to a fluorophore), to bind to the first antibody. Upon laser excitation of the fluorophore, the fluorescent signal produced is directed to a particular detector in the flow cytometer. Many fluorophores can be combined in a single assay, depending on the capacity of the flow cytometer used, which makes the simultaneous measurement of many characteristics not only possible but relatively simple.

The biological roles of cytokines and their potential as biomarkers for various diseases have been studied extensively, including in the clinical setting. However, the particular cut-off values for use in diagnostics and treatment monitoring have not been well established (Monastero & Penttala, 2017). Indeed, the pleiotropic nature of cytokines and the complexity of the network in which they function, have led to various inconsistencies in the literature. In a review that focused on cytokines as potential biomarkers for distinguishing active from latent TB, Sudbury *et al.* (2020) identified various factors that contribute to varying results. These factors, both biological and analytical, also apply to the heterogeneity observed in the majority of other cytokine studies on HIV infection, TB, and HIV/TB co-infection. These factors include different case definitions and therefore inconsistent participant classification, varying disease severity (which is not always assessed), differences in host immunogenetics, varying infection and disease prevalence in study settings, the use of different methods (as previously discussed), and varying experimental conditions (such as incubation time in antigen stimulation studies) (Sudbury *et al.*, 2020).

Furthermore, environmental and socio-economic factors also influence the cytokine milieu in an individual at a particular point in time. As the interplay and balance between pro- and anti-inflammatory responses at the time of infection are crucial for establishing an effective immune response and can determine the trajectory of the disease, intra-individual variables such as these contribute to the inconsistency of findings (Couper *et al.*, 2008; Sudbury *et al.*, 2020). Additionally, the fluidity in cytokine functions that do not fit into the simplistic Th1/Th2 hypothesis contributes to contradictory results. For example, some authors conclude that Th2 responses are predominantly based on increased levels of IL-10 (Winek *et al.*, 2009), while IL-10 is not only linked to the Th2 response but also has various anti-inflammatory and immunomodulatory effects (Sudbury *et al.*, 2020).

The cytokine profiles of HIV infection, TB, and HIV/TB co-infection have been extensively investigated, however, the results are conflicting. Although cytokine profiles have been linked to metabolic profiles for HIV infection (Peltenburg *et al.*, 2018) and TB (Weiner *et al.*, 2012), this has not been done for HIV/TB co-infection. To reduce variation due to the factors discussed above, and because of the number and diversity of studies investigating cytokines in the context of HIV infection, TB, and HIV/TB, only selected studies will be discussed here. The focus will be on studies that measured more than one cytokine in the serum or plasma of HIV-positive, TB, and HIV/TB co-infected patients, without antigenic stimulation (to emphasise the inherent cytokine profile) as well as with and without treatment (see Sections 2.4.2, 2.5.2, and 2.6.2). Studies outside this scope will be mentioned or discussed selectively. Due to the systemic nature of blood, the cellular sources of cytokines in serum or plasma cannot be distinguished. The observed cytokine profiles are thus from various immune cell types both in blood and various other tissues.

2.3 Metabolites as indicators of the immune response to infection

Any factor that threatens the homeostasis of an organism triggers a range of responses that culminate in metabolic alterations (Wilson & Matschinsky, 2021). When a pathogen invades the host, the metabolic alterations observed are the result not only of the metabolic reprogramming that accompanies the activation and modulation of the immune response but also the effect of the presence of the pathogen itself, in that some non-viral pathogens are continually metabolically active (Amiel & Perona-Wright, 2021; Shi *et al.*, 2016). Additionally, the pathogen actively induces certain processes via various mechanisms, including metabolism, to manipulate the host to reproduce and in some cases, persist. This disturbance of homeostasis results in pathology (Zhang *et al.*, 2018). In chronic diseases, this becomes a cyclical process, where the pathogen and immune response-induced alterations, due to their persistent nature, induce other alterations that are not necessarily directly linked to the infection.

The central dogma of molecular biology explains the flow of information in living organisms. Within this sequence, alterations at the metabolic level most accurately reflect what is currently occurring in the

organism as a consequence of all of the preceding changes at the DNA, RNA, and protein levels. As such, the metabolic alterations in an organism most closely resemble its phenotype at a specific moment in time, and the direction and magnitude of these alterations depend on a multitude of factors (Dettmer *et al.*, 2007). Besides influencing metabolite levels, factors such as the variable expression of enzyme-encoding genes, post-translational modification of proteins, energy availability, hormones, and other signalling molecules such as cytokines are influenced by metabolite levels (Metallo & Vander Heiden, 2013). The “metabolome” has been described as the collection of all the metabolites that participate in the chemical reactions within an organism that is necessary for the growth, maintenance, and function of its cell(s) (Harrigan & Goodacre, 2003). This collection of metabolites includes amino acids, carbohydrates, organic acids, fatty acids, lipids, and vitamins, which participate in various overlapping metabolic pathways (Færgestad *et al.*, 2009). Alterations to individual metabolites, and therefore the flux in one or more pathways, underlie biological mechanisms of energy production, structural changes and signalling (Vinayavekhin *et al.*, 2010). Thus, metabolic alterations are both a consequence and a cause of pathology, which makes the measuring of metabolic alterations clinically relevant.

2.3.1 Measuring metabolites

The characteristics of biofluids as indicators of health and disease have been used since ancient times. For example, charts were used to diagnose medical conditions based on characteristics of the patient’s urine sample (such as smell, taste, and odour) (Cassiday, 2009; Lindon & Nicholson, 2008). As technology improved, various techniques were developed and improved upon over centuries to measure either one or a few metabolites at a time or measure another parameter as a proxy to metabolites. These techniques include measurements of body composition (weight, skinfold measurements, circumferences) (Duren *et al.*, 2008), respirometry by measurement of gas exchanges, energy expenditure using calorimetry (Speakman, 1998), and determination of nitrogen flux by the administration and subsequent measurement of isotopically labelled amino acids (Paton *et al.*, 2003). By the early to mid-1900s, mass spectrometry techniques and the understanding of enzyme function developed to such a degree that researchers applied it to biofluids to relate changes in the metabolic profile to disease states (Van der Greef *et al.*, 2013). Mass spectrometry was also often coupled to chromatographic techniques, which made clinical applications more practical, as the components of a sample could now be separated before identification, which improved the accuracy of component identification (Pocsfalvi *et al.*, 2016). These technologies were especially useful in the study of genetic defects in metabolic pathways since such defects typically lead to large changes in the levels of individual or discrete groups of metabolites. Application of these technologies to chronic diseases yield less definitive results as regulatory mechanisms to maintain homeostasis over long periods obscure metabolic changes directly related to the disease. As such, assessments of nutritional status, body mass index, body composition studies, and measurements of single or representatives of specific classes of metabolites were used to investigate metabolic changes during disease, but these only provided a fragmented view of metabolism. Various advances in separation and

detection techniques as well as data analysis and statistical tools during the late 20th century allowed for metabolic profiling in various settings. The advancements in data handling during the 1990s represented the first steps in the maturation of the field that is referred to as “metabolomics” (Van der Greef *et al.*, 2013).

Metabolomics refers to the analysis of small molecules (i.e. metabolites) present in a biological system to infer biological functions or changes in response to specific internal or external stimuli (Dunn *et al.*, 2005). Metabolomics-based analysis involves the identification and quantification of metabolites by techniques such as nuclear magnetic resonance (NMR) spectroscopy and liquid or gas chromatography-mass spectrometry (LC-MS or GC-MS, respectively), whereafter the data is typically subjected to biostatistics to identify significant alterations to the metabolome (Shulaev, 2006). Metabolomics is used to study disease phenotypes (Wishart *et al.*, 2012) and includes two main approaches; untargeted and targeted metabolomics. Untargeted metabolomics aims to characterise as much of the metabolome as possible by identifying and quantifying most of the metabolites and hence is referred to as a hypothesis-generating approach (Dunn, 2008). Conversely, when a pre-defined set of metabolites are detected and quantified using isotopes, the approach is hypothesis-driven or targeted, and this often succeeds an untargeted approach. These two approaches can also be combined into a semi-targeted approach, in which the hypothesis is not necessarily defined but involves the investigation of a pre-defined class of metabolites, such as amino acids for example, while the ability to detect several hundred other molecules at the same time still exists (Du Preez *et al.*, 2019).

GC is commonly used in metabolomics and exploits the principle that compounds exhibit differing degrees of partitioning between a gaseous mobile phase and a solid stationary phase at different temperatures. As such, GC separates volatile compounds in a mixture based on their chemical (polarity) and physical (volatility) properties (Rahman *et al.*, 2015). A gas chromatograph consists of an inlet (which is set to a temperature high enough to immediately volatilise the sample), a column packed with a stationary phase within an oven, and a supply of gaseous mobile phase (typically hydrogen or helium). Application of a temperature gradient in the oven during the separation allows compounds to elute from more volatile to less volatile. A typical mass spectrometer comprises three parts, each with a distinct function: the ion source ionises compounds, the mass analyser resolves these ions according to their m/z ratios, and the detector records the ions and their relative abundances (Kaklamanos *et al.*, 2020). MS is most often used as a detection system coupled to GC, which allows for the identification and quantification of the separated compounds in the mixture.

When the goal is to obtain a comprehensive picture of the metabolome, GCxGC-TOFMS is ideal – and will be used in this study. This system uses two gas chromatographic columns with different physical characteristics to achieve superior separation (Ong & Marriott, 2002). The first column is typically longer and contains a more apolar stationary phase, while the second is typically shorter and more polar. These

columns are connected by a modulator, which traps compounds eluting from the first column in discrete bands, which are then released into the second column at defined intervals for further separation before reaching the detector (Nolvachai *et al.*, 2020). A temperature gradient is typically used in the primary oven which allows for the separation of compounds in the first column based on volatility (Tolley *et al.*, 2014). In the secondary oven, the temperature is typically higher (at a defined offset, usually 5°C) than that of the primary oven throughout the entire gradient to ensure that all compounds are volatilised and separation based on polarity then only occurs in the second column.

In recent years, metabolomics has been applied to study various aspects of HIV and *Mtb* infection separately, yielding new insights into the infection-induced metabolic alterations and adaptations experienced by the host. Despite the contributions that metabolomics has made to the understanding of these diseases to date, it has not yet been systematically applied to investigate the HIV/TB co-infected state.

2.4 HIV infection and immunometabolism

2.4.1 Basic pathophysiology, diagnosis, prognosis and treatment

HIV belongs to the genus *Lentivirus* within the *Retroviridae* family and is transmitted through contact with infected body fluids or mucous membranes (O'Cofaigh & Lewthwaite, 2013). There are two main types of HIV, referred to as HIV-1 and HIV-2 (Clavel *et al.*, 1986). HIV-1 is the main strain responsible for the pandemic, while HIV-2 is less pathogenic and is significantly less prevalent (Esbjörnsson *et al.*, 2019). As such, all references to HIV in this dissertation implies HIV-1. Upon entering its host, HIV targets immune cells expressing the CD4 glycoprotein on their cell surface, namely CD4 T cells, macrophages, dendritic cells, and monocytes (Yoshimura, 2017) — all vital components of the host's immune defences. Infection and subsequent dysfunction or destruction of these cells by HIV leads to the characteristic immunodeficiency associated with acquired immunodeficiency syndrome (AIDS) (Lyles *et al.*, 1999; O'Cofaigh & Lewthwaite, 2013).

HIV infection is generally established 2–4 weeks after exposure and may be asymptomatic or present with flu-like symptoms. As the infection progresses, four clinical stages become apparent based on CD4 cell count and the overall clinical symptoms portrayed. Although clinical stage 1 may be asymptomatic, it frequently presents with persistent lymphadenopathy (swollen or enlarged lymph nodes), while stage 2 is characterised by severe weight loss and various other co-infections, such as herpes zoster and recurrent respiratory tract infections. During stage 3, opportunistic co-infections become more common and persistent (especially pulmonary TB and oral candidiasis), and symptoms become more serious, which may include persistent fever and diarrhoea (World Health Organization, 2007). The final stage referred to as AIDS, is defined as the severe depletion of T cells as well as susceptibility to various neoplastic and conventional diseases, including various infections (e.g. extrapulmonary TB), central

nervous system diseases, and cancers such as Kaposi's sarcoma (O'Cofaigh & Lewthwaite, 2013). Furthermore, AIDS is also characterised by HIV wasting syndrome (cachexia; persistent weight loss not explained by poor feeding). These four stages are linked to four levels of immunosuppression, measured by CD4 cell count, as not significant ($> 500/\text{mm}^3$), mild ($350\text{--}499/\text{mm}^3$), advanced ($200\text{--}349/\text{mm}^3$), and severe ($<200/\text{mm}^3$), respectively (World Health Organization, 2007).

Diagnostic tests for HIV are based on the detection of viral antigens and antibodies or HIV-specific nucleic acids in the blood. The HIV-1/HIV-2 enzyme immunoassay is typically used due to its high sensitivity, which allows for the detection of seroconversion within a few weeks of infection. However, this test is subject to false positives and therefore results have to be confirmed through western blotting and/or PCR (Fearon, 2005). Following diagnosis and treatment, CD4 cell count and viral load (VL; the number of copies of HIV-1 RNA per millilitre) are used as the gold standard for clinical monitoring of disease progression (Langford *et al.*, 2007; Williams *et al.*, 2011). However, there is natural intra-individual variation associated with CD4 count (Touloumi & Hatzakis, 2000) and VL measurements are often unavailable or not accessible due to its expense in resource-limited areas, which are typically the high-burden regions (Lawn *et al.*, 2009). Such high-burden areas are in desperate need of new markers with which to characterise HIV/AIDS, a topic of intense research in the last two decades.

Various designations are used to distinguish HIV-positive individuals based on disease progression, as this tends to vary significantly among individuals. Three main groups are identified based on the development of AIDS, namely rapid progressors (AIDS develops 2–5 years after seroconversion), typical progressors (HIV-RNA levels increase, CD4 cell count decreases, and AIDS develops 6–10 years after infection), and long-term slow-progressors. The latter refers to individuals that have normal CD4 cell counts ($> 500 \text{ cells}/\text{mm}^3$) and low or stable HIV-RNA for more than 10 years without treatment intervention and that therefore remain healthy for a prolonged period (hence they have no opportunistic co-infections and do not develop AIDS) (Nissen *et al.*, 2018). In the context of viremia, individuals are referred to as “controllers” when there is no detectable HIV-RNA in the blood ($< 50 \text{ HIV-1 RNA copies}/\text{mm}^3$) for at least 12 months, without antiretroviral therapy (ART), or as “non-controllers” when this condition is not met. The mechanisms underlying this spontaneous virological control remain incompletely understood, but host factors such as genetics and the immune response, as well as defective viral variants, have been proposed (Deeks & Walker, 2007). When segregating based on immunological treatment response, the terms “responder” and “non-responder” are used. An individual is considered a responder when a CD4 count above $250 \text{ cells}/\mu\text{l}$ is reached after a specific period of ART and as a non-responder when this is not achieved (Rosado-Sanchez *et al.*, 2019).

Treatment of HIV/AIDS involves combinations of antiretroviral (ARV) drugs (Sitole *et al.*, 2013), termed combination antiretroviral therapy or highly reactive antiretroviral treatment (cART or HAART, respectively). In this dissertation, the term “ART” will be used to refer to treatment with either a single

ARV or a combination of ARVs, for consistency. ART prevents HIV propagation by blocking the cell-to-cell transmission of the virus but it cannot remove the provirus, or prevent its transcription (Bell & Noursadeghi, 2018). Thus, the immune system is reconstituted to some extent upon ART, but the effects of the virus cannot be eliminated. With the up-scaling of ART, HIV-positive individuals were only eligible for ART if they were in clinical stage 3 or 4 (as defined above) or had a CD4 count below 200 cells/mm³ (World Health Organization, 2003). However, the CD4 threshold was relaxed when the “Test and Treat” policy was recommended in 2016, under which all HIV-positive individuals receive ART, regardless of CD4 count (World Health Organization, 2016b). Although these treatments are not a cure, it has ultimately increased the lifespan of individuals, if initiated early (Klatt *et al.*, 2013). The success of ART has shifted the cause of death to serious non-AIDS events, such as TB or chronic non-communicable diseases (Palmer *et al.*, 2016). Moreover, the use of ART is associated with cardiometabolic disorders (such as dysglycaemia, dyslipidaemia, insulin resistance and hypertension) as well as body fat redistribution, which is distinct from the metabolic effects of the virus itself (Yoshimura, 2017). There is no licenced prophylactic vaccine for HIV (Barouch *et al.*, 2018), although two major clinical trials were recently initiated [Imbokodo (NCT03060629; shown to be ineffective in August 2021) and Mosaico (NCT03964415; still underway)]. Despite the success of ART, a vaccine would significantly expedite the process of finally eliminating HIV/AIDS. While the search for a vaccine continues, advances are also being made in the field of HIV prevention and treatment, with research primarily focused on long-acting injectable ARVs (Lazarus *et al.*, 2021) and broadly neutralising antibodies (Hsu *et al.*, 2021). Although the ultimate goal remains an absolute cure, no generally applicable way of eradicating the virus from the body has been found. As such, the next most desirable goal is a “functional cure”, which refers to a state where the VL is sustained below the detection limit for a specific period (as yet undefined), without ongoing treatment (Janssens *et al.*, 2021; Ndung'u *et al.*, 2019). For this, it will be necessary to better understand the immune and metabolic consequences linked to the persistence of HIV in the host.

2.4.2 HIV infection and cytokines

Given the association of HIV/AIDS with immunological dysregulation and deterioration, it is not surprising that cytokines have been investigated extensively as immunological markers and determinants. There is a large body of literature on cytokine alterations in HIV infection in various contexts and using several sample types. As previously mentioned, this discussion will focus on selected studies using unstimulated, blood-based samples. Those studies in which the cytokines of interest to this study (see Section 2.2) were measured, will be preferentially discussed but other studies will be mentioned as necessary to cover essential concepts.

Earlier studies on the cytokine responses during HIV infection strongly suggested that a Th1 to Th2 shift is an essential feature of the aetiology of HIV infection (Clerici & Shearer, 1993; Klein *et al.*, 1997). This was contradicted by other studies (Fakoya *et al.*, 1997; Graziosi *et al.*, 1994) which showed no such shift,

as well as a later study implicating Th2 responses during acute HIV infection (Gorenec *et al.*, 2016). This highlights the complex nature of T helper cell subsets and cytokine production, especially in the context of a chronic and immunodeficiency-inducing infection such as HIV infection.

The studies of Williams *et al.* (2013) and Mlambo *et al.* (2019) are highly similar in design, and are most similar to this study, although the data generated were used in different ways. In both studies, the BD Th1/Th2/Th17 cytometric bead array (CBA) kit was used to measure seven cytokines in the serum of treatment-naïve HIV-positive patients and healthy controls. Williams *et al.* (2013) focused on the differences in cytokine levels between groups and the ability of the identified markers to classify patients, whereas Mlambo *et al.* (2019) focused on determining whether the measured cytokines correlate with VL and CD4 and CD8 T cell count. Despite the significant overlap in the cytokine profiles of the infected and uninfected groups, Williams *et al.* (2013) found that elevated IL-6 and IL-10 best discriminated HIV-positive from HIV-negative individuals. The majority of the HIV-positive cohort in this study had not progressed to AIDS and due to the overlap in the cytokine profiles, it was concluded that the majority of the cohort was in a stable clinical state. When those that progressed to AIDS were included in the model, the discriminatory power of IL-6 and IL-10 improved, which implies that with decreasing CD4 count and increasing VL, the discriminatory power of cytokines improve. Higher VLs are typically associated with higher IL-6 levels, with VL determining the magnitude of cytokine alterations (Kamat *et al.*, 2012; Williams *et al.*, 2013). Increased levels of IL-6 and IL-10 have been repeatedly reported in HIV-positive individuals and are typically associated with disease progression (Borges *et al.*, 2015; Kamat *et al.*, 2012; Roberts *et al.*, 2010). The results of Mlambo *et al.* (2019) confirmed the correlation of elevated IL-6 with higher VL.

The depletion of Th17 cells in the gut is a prominent feature of HIV infection and is linked to the loss of intestinal barrier function. This depletion has a significant effect on immune defences at mucosal surfaces, such as the gut. It has been suggested that Th17 cells hold value in predicting opportunistic infections in HIV-positive individuals (El Hed *et al.*, 2010). IL-17, produced by Th17 cells, was found to be increased in HIV-positive individuals but did not significantly distinguish the groups in the study of Williams *et al.* (2013).

Even when HIV viremia is undetectable and CD4 cell number restored to healthy levels with ART, low levels of inflammation persist, which is visible in the cytokine profiles of such patients (Borges *et al.*, 2015; Kamat *et al.*, 2012). It has also been reported that not only do cytokine levels in virally suppressed HIV patients on ART not normalise to levels similar to healthy controls but that they are further modulated (Vanpouille *et al.*, 2016). There has, however, been reports of cytokines returning to healthy levels after four (Haissman *et al.*, 2009) and six (Sachdeva *et al.*, 2010) months of ART. These studies represent short term ART, at which point the detrimental effects of ART may not have set in and the “improvement” in the cytokine profiles may reflect the decrease in viral replication occasioned by the

initiation of ART. Haissman *et al.* (2009) and Sachdeva *et al.* (2010) reported a decrease in TNF- α and IL-6 in treated compared to untreated HIV-positive individuals. The latter authors additionally reported decreased IFN- γ , IL-1 β and increased IL-2, IL-4 and IL-10. Regidor *et al.* (2011) found that the levels of most cytokines decreased, although not to the levels of healthy controls, after two years of ART. IL-6 and C-reactive protein were the exceptions, as the levels of these cytokines were unchanged after ART.

There are various instances of directly opposing results from different HIV cytokine studies, which may be related to the immunological milieu during the acute stage of infection (Katsikis *et al.*, 2011), duration of HIV infection (when the clinical status is similar) (Vanpouille *et al.*, 2016), the degree of disease progression, and the sensitivity of the assay used. The Th1/Th2 balance hypothesis has also been applied to characterise the cytokine changes during HIV infection and has been most frequently associated with the pro-inflammatory Th2 phenotype (Vanpouille *et al.*, 2016). However, as mentioned earlier, this hypothesis is an oversimplification of the dynamics of T helper cell phenotypes and effector functions, as suggested by various studies that show evidence that there is no clear Th1 to Th2 shift during HIV infection (Graziosi *et al.*, 1994; Maggi *et al.*, 1994; Sarih *et al.*, 1996). This highlights the complexity of the networked function of cytokines and that a simplistic model might lose even more of its explanatory power in a chronic, immune cell-targeting infection such as HIV. For instance, Lisco *et al.* (2012) investigated cytokine alterations in treatment-naïve HIV patients compared to healthy controls and found that during HIV infection, existing correlations between cytokines are strengthened and new correlations are formed between individual cytokines. This implies a less fluid cytokine network and a decreased ability of the HIV-positive immune system to respond to threats (Lisco *et al.*, 2012).

From the studies reviewed here, HIV infection primarily affects circulating levels of IL-6, IL-10, and IL-17. Although ART results in some cytokines trending towards healthy values, the therapeutics may also further modulate the measured cytokine profile.

2.4.3 HIV infection and metabolites

Metabolomics investigations to date indicate that large amounts of energy are rapidly and continuously expended during HIV infection due to constant immune activation, inflammation, and viral replication. Alterations to amino acid and lipid metabolism are primary factors distinguishing treatment-naïve HIV-positive individuals from healthy controls (Li *et al.*, 2018; Peltenburg *et al.*, 2018; Sitole *et al.*, 2019; Tarancon-Diez *et al.*, 2019). Amino acid metabolism is central to the function of the immune system. As the infection becomes chronic, these alterations become more complex as various other areas of metabolism become impaired and amino acids often antagonise each other's effects (Li *et al.*, 2007). These alterations, in combination with changes in acylcarnitines (Li *et al.*, 2018), antioxidants, and muscle metabolism (Peltenburg *et al.*, 2018), are suggestive of an oxidative inflammatory state and mitochondrial dysfunction. Mechanisms leading to HIV-induced mitochondrial dysfunction, oxidative stress (OS), and

progression to AIDS are still unclear and complicate the interpretation of metabolomics findings. At the time of diagnosis, when treatment-naïve samples are typically collected, overlapping biochemical processes have already been set in motion and become so interconnected that no single measure of causality can be attributed. These alterations and their interpretations will now be discussed in light of the most recent findings [post the reviews of Sitole *et al.* (2013) and Williams *et al.* (2017)]. The studies referred to here are summarised in Table 2.2.

Table 2.2: Metabolomics studies that characterise the effects of HIV infection and/or ART on the human host, published after 2017.

Research Model	Metabolome fraction targeted	Analytical apparatus	Groups compared	Statistical approaches	Reference(s)
Disease characterisation – the effect of HIV infection without ART					
Plasma	Total metabolome (GC), TCA cycle metabolites (GC), lipids (LC)	GC- and UHPLC-qTOFMS	HIV+/Tn- (TC and PC groups)	Mann-Whitney U, Spearman rank, FC, heatmaps, HCA, Wilcoxon signed-rank, PCA, RFA, AUC of ROC	Tarancon-Diez <i>et al.</i> (2019)
The effect of HIV infection and the effect of ART					
Plasma	Total metabolome	GC- and LC-MS	HIV-/Tn- *HIV+/Tn- *HIV+/Tn+	Welch's t-test, PCA, RFA, AUC of ROC	Li <i>et al.</i> (2018)
Plasma	Biogenic amines, lipids and signalling lipids	LC-MS and UPLC-TQMS	HIV-/Tn- HIV+/Tn- *HIV+/Tn- *HIV+/Tn+	FC, t-test	Peltenburg <i>et al.</i> (2018)
Serum	Total metabolome, amino acids	NMR and GC-MS	HIV-/Tn- HIV+/Tn- HIV+/Tn+	PCA, OPLS-DA	Sitole <i>et al.</i> (2019)
The effect of ART					
Plasma	Lipids, lipoproteins, and low-molecular-weight metabolites	NMR	*HIV-/Tn- *HIV+/Tn+ (NL and DL groups)	FC, Mann-Whitney U test, RFA, AUC of ROC	Rodriguez-Gallego <i>et al.</i> (2018a)
Plasma	Lipids, lipoproteins, and low-molecular-weight metabolites	NMR	*HIV+/Tn- *HIV+/Tn+ (INR and IR groups)	FC, Mann-Whitney U test, RFA, AUC of ROC	Rodriguez-Gallego <i>et al.</i> (2018b)
Plasma	Lipoproteins (NMR) and total metabolome (LC)	NMR and UHPLC-(ESI)qTOF	*HIV+/Tn- *HIV+/Tn+ (INR and IR groups)	Kruskal-Wallis, Mann-Whitney U, Wilcoxin t-test (paired samples), RFA	Rosado-Sanchez <i>et al.</i> (2019)
Plasma	Total metabolome	UHPLC-MS/MS	HIV-/Tn- HIV+/Tn+	t-tests, PCA and OPLS-DA.	Babu <i>et al.</i> (2019)

Metabolome fraction: TCA: tricarboxylic cycle.

Analytical apparatus: NMR: nuclear magnetic resonance spectroscopy; GC-MS: gas chromatography-mass spectrometry; UHPLC-MS/MS: ultra-high-performance liquid chromatography-tandem mass spectrometry; UPLC-TQMS: ultra-pressure liquid chromatography triple quadrupole mass spectrometry.

Groups: HIV-/Tn-: healthy controls; HIV+/Tn-: treatment-naïve HIV-positive individuals; HIV+/Tn+: HIV-positive individuals on ART; IR: immunological responders (CD4 T cell count equal to or greater than 250 cells/mm³ after 36 months of ART); INR:

*immunological non-responders (CD4 T cell count below 250 cells/mm³ after 36 months of ART); *: longitudinal study; NL: normolipidaemic (normal amount of lipids in the blood); DL: dyslipidaemic (abnormal amount of lipids in the blood); TC: transient controllers; PC: persistent controllers.*

Statistical approaches: PCA: principal component analysis; OPLS-DA: orthogonal projections to latent structure-discriminant analysis; IPA: ingenuity pathway analysis; FC: fold change; RFA: random forest analysis; AUC of ROC: area under curve of receiver operating characteristic.

In terms of characterising the metabolic effects of untreated HIV infection, Tarancon-Diez *et al.* (2019) investigated the mechanisms underlying the loss of virological control in elite controllers (i.e. no detectable HIV-RNA in the blood for at least 12 months, without ART). The metabolic alterations were primarily mapped to aerobic glycolysis, the tricarboxylic acid cycle (TCA), as well as amino acid and lipid metabolism. These changes are generally associated with increased energetic and biosynthetic demands as the immune system responds to the increased viral load (Plaza *et al.*, 2016). A key metabolic reprogramming event in activated T cells is the Warburg effect, characterised by the increased production of lactate due to an increased glycolytic flux, despite the presence of oxygen. This process contributes to the glycolytic and TCA cycle alterations observed during HIV infection. TCA intermediates found to be increased before the loss of virological control were those supplemented by anapleurotic intermediates (i.e. alpha-ketoglutarate and succinate). This supports the concept that the increased energy requirement induces the shuttling of metabolites such as amino acids towards energy production via oxidation in the TCA cycle (Tarancon-Diez *et al.*, 2019). The Warburg effect generally indicates dysregulation of mitochondrial oxidative metabolism, which suggests that mitochondrial dysfunction, a well-known consequence of HIV infection (Pinti *et al.*, 2010), is an early event. Mitochondrial dysfunction affects multiple systems (Nunnari & Suomalainen, 2012) contributing to the loss of lipid homeostasis, as the organelle plays key roles in the biosynthesis of phospholipids for membranes, as well as the catabolism of fatty acids (Tilokani *et al.*, 2018). Immune cell function is impacted, as phospholipids are essential in the structural remodelling, differentiation, and cell signalling of immune cells, all of which are crucial to mounting an effective immune response (O'Donnell *et al.*, 2018). A general decrease in lipids was observed pre-loss of virological control relative to post-loss, as well as in the profiles of transient controllers (having at least two consecutive measurements of detectable viral RNA in 12 months), compared to persistent controllers (who maintained their elite controller status for the entire follow-up period). Increased low-density lipoprotein (LDL) particles and a concomitant increased ratio of LDL to high-density lipoprotein (HDL) particles were also observed in the pre-loss profiles of transient controllers (Tarancon-Diez *et al.*, 2019). Such lipoprotein profiles are associated with a poor prognosis in HIV-positive individuals and a higher risk of cardiovascular disease (CVD) (Riddler *et al.*, 2008). The changes observed by Tarancon-Diez *et al.* (2019) serve as an analogy of what occurs during early HIV infection in patients without virological control (compared to healthy controls) and therefore the occurrences underlying and preceding the development of the typical HIV-positive metabolic profile.

Increased tryptophan catabolism was observed in the HIV-positive cohort of Li *et al.* (2018) and Peltenburg *et al.* (2018), respectively, exemplified by an increased kynurenine/tryptophan ratio. This ratio reflects the activity of indoleamine-2,3-dioxygenase 1 (IDO1), which is involved in the catabolism of tryptophan to kynurenine [known to be immunosuppressive (Dagenais-Lussier *et al.*, 2016)]. In terms of the branched-chain amino acids (BCAAs), Peltenburg *et al.* (2018) observed decreased levels of leucine and isoleucine [the latter corroborated by Sitole *et al.* (2019)], in addition to decreased carnosine, which the authors attributed to impaired muscle metabolism. BCAAs play major roles in immunity (Nie *et al.*, 2018), reflected by the increased levels of these and their catabolic intermediates before the loss of virological control in transient controllers (Tarancon-Diez *et al.*, 2019). During the early stages of infection, BCAA levels increase to provide building blocks and energy to mount the immune response [as seen in the cohort of Tarancon-Diez *et al.* (2019)], before the loss of virological control, with CD4 T cell counts > 600 cells/mm³. When the infection is chronic and the host energy reserves become depleted, BCAA levels decrease [as seen in the cohorts of Li *et al.* (2018), Peltenburg *et al.* (2018), and Sitole *et al.* (2019), comprising HIV-positive patients with CD4 T cell counts of 147 cells/mm³, 441 cells/mm³, and 124 cells/mm³, respectively]. Increased levels of valine, which were found to be highly correlated between blood and cerebrospinal fluid (CSF) samples of HIV-positive individuals, and associated with mild cognitive impairment (Maher *et al.*, 2011), were the best predictor of the loss of virological control (Tarancon-Diez *et al.*, 2019). This suggests that valine plays an important role in immune competency. Furthermore, reduced levels of ornithine and putrescine suggest a decreased urea cycle and reduced ammonia clearance in HIV-positive individuals (Peltenburg *et al.*, 2018). Amino acids play various roles in immune activation and inflammation and hence these changes are not exclusively associated with HIV infection. Similar trends have been reported for various other inflammatory conditions, including TB (Section 2.5.3). These observations indicate general inflammatory and immune activation events such as impaired energy metabolism, OS, mitochondrial dysfunction, and a dysbiosis of the microbiota, which contribute to the immune dysfunction and deficiency characteristic of HIV infection and other infectious diseases (Li *et al.*, 2007). Additionally, alterations to amino acid metabolism have been posited as contributors to the aetiology of HIV-associated neurocognitive disorders (Peltenburg *et al.*, 2018; Sitole *et al.*, 2019). As a last resort, free amino acids, as well as stored amino acids released from muscle tissue, is used to produce much-needed energy via the TCA cycle during extended periods of high energy expenditure, such as chronic infection. Glutaminolysis, a mitochondrial pathway which generates energy via the degradation of glutamine (Seo *et al.*, 2014), has been postulated as a mechanism by which the TCA cycle is replenished during viral infection (Plaza *et al.*, 2016). This is reflected in the increased alpha-ketoglutarate and decreased glutamate observed by Tarancon-Diez *et al.* (2019) and would explain the decreased glutamine observed by Sitole *et al.* (2019). This also suggests that glutaminolysis is an early metabolic alteration in HIV infection. Continual oxidation of amino acids combined with HIV-associated malabsorption (Grinspoon & Mulligan, 2003), further contributes to alterations in amino acid metabolism. Cachexia is associated with progressive HIV infection and is

characterised by the loss of muscle mass. As such, the mobilisation of amino acids for energy production is a major contributor to HIV-based metabolomics findings.

Alterations to acylcarnitine metabolism during HIV infection are suggestive of mitochondrial dysfunction (Li *et al.*, 2018) and were first reported in a simian immunodeficiency virus-based metabolomics study (Wikoff *et al.*, 2008). Viral infections are associated with fatty acid synthesis (Plaza *et al.*, 2016), which, in addition to various other factors such as mitochondrial dysfunction, contribute to a profile of increased unsaturated triglycerides and decreased sphingomyelin species, as observed by Peltenburg *et al.* (2018).

Typically, lipid alterations become more pronounced with the initiation of ART due to the modulatory effects of these drugs on the mitochondria and genes involved in lipid metabolism — thus, investigations frequently focus on lipids in a treated as opposed to untreated HIV context. ART prevents HIV propagation by blocking the cell-to-cell transmission of the virus but it cannot remove the provirus, or prevent its transcription (Bell & Noursadeghi, 2018). Thus, the immune system is reconstituted to some extent upon ART, but the effects of the virus cannot be eliminated. The introduction of ART since the 1990s has altered the course of HIV infection such that opportunistic infections, and therewith mortality, significantly decreased and the infection effectually became chronic (Salas-Salvado & Garcia-Lorda, 2001). However, since HIV-induced metabolic alterations are augmented with the initiation of ART (Jain *et al.*, 2005), this often results in conditions such as dyslipidaemia, CVD, insulin resistance and type 2 diabetes, non-alcoholic fatty liver disease (Ahmed *et al.*, 2018), and HIV-associated neurocognitive disorders (Dickens *et al.*, 2015). HIV infection significantly alters the host's metabolic profile. The profile is also distinct following treatment as compared to that of both healthy and treatment-naïve, HIV-positive individuals (Hewer *et al.*, 2006).

Upon reviewing metabolomics studies investigating the effects of ART one must consider the following: which metabolic alterations are caused solely by ART, which HIV-induced metabolic alterations are restored after ART, and which are compounded? The metabolic alterations associated with ART resemble those seen in individuals with metabolic syndrome and/or the abovementioned inflammation or ageing-related diseases. As previously mentioned, these metabolic changes typically involve alterations to amino acid and lipid metabolism. When comparing treatment-naïve, HIV-positive metabolic profiles to those of individuals on ART, authors refer to those metabolites altered during HIV infection, which return to the level of healthy controls, or at least trend in that direction, as “recovered”, implying a return to a healthier state. Whether this is truly the case, or whether the observed effects are additional metabolic derangements due to treatment, comorbidities or co-infections, can only be determined by observing the “recovery” over time (while including controls for co-infections and comorbidities). ART duration was not a focus in previous HIV metabolomics reviews and therefore studies included in past reviews are reviewed here, in that context, and are indicated in Table 2.3.

Table 2.3: HIV metabolomics studies arranged according to ART duration and research focus.*

Research Model	Duration of ART (years)	ARV combination	Key metabolic findings	Reference(s)
Plasma	< 1	PI	Recovery of His, Kyn, ketone bodies, lysolipids. Butyrylcarnitine in combination with myristic acid predict dyslipidaemia	Li <i>et al.</i> (2018)
Serum	< 1	Two NRTIs and an NNRTI	Partial recovery of Glu and Asp, Trp did not recover (not longitudinal)	Sitole <i>et al.</i> (2019)
Plasma	1	NRTI and NNRTI or NRTI and PI or NRTI only	His and Trp recovered. Increased methionine sulfone (OS indicator)	Peltenburg <i>et al.</i> (2018)
Urine	≤ 1	Two NRTIs, a PI booster, and another PI	Metabolites or parent compounds of drugs, decreased bile acids, androgens, nucleoside, and lipid metabolites	Chetwynd <i>et al.</i> (2017)
Plasma	2	Two NRTIs and an NNRTI and/or a PI or a combination of both.	Late immune recovery associated with increased inflammation, glutaminolysis, and changes to the structure and composition of lipoproteins	Rosado-Sanchez <i>et al.</i> (2019)
Cerebrospinal fluid	~ 2	NRTIs with or without PIs	Neurotransmitters, markers of glial activation and mitochondrial dysfunction, oxidation products, and metabolic waste products	Cassol <i>et al.</i> (2014)
Plasma	2-3	PIs (test cohort) and NNRTIs (validation cohort)	Increased bile acids, acylcarnitines, LPS, and Kyn as well as decreased sulphated steroids, PUFAs, saturated LCFA, eicosanoids, and Trp metabolites	Cassol <i>et al.</i> (2013)
Plasma	3	NNRTIs	VLDL lipoprotein particles, total triglycerides, and lactate predict dyslipidaemia	Rodriguez-Gallego <i>et al.</i> (2018a)
Plasma	3	NNRTIs	Poor immune response associated with high levels of non-HDL particles	Rodriguez-Gallego <i>et al.</i> (2018b)
Plasma	> 5	Two NRTIs and an NNRTI or one NRTI and an NNRTI with an NA	Decreased essential amino acids, altered energy metabolism, as well as alterations to phospholipids and complex lipids related to immune function	Babu <i>et al.</i> (2019)

*Only studies where the mean or median duration of ART was provided were included here.

ARV combination: NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; PI: protease inhibitor; NA: nucleoside analogue.

Key metabolic findings: His: histidine. Kyn: kynurenine; Gln: glutamine; Asp: aspartate; Trp: tryptophan; OS: oxidative stress; LPS: lipopolysaccharide; PUFA: polyunsaturated fatty acid; LCFA: long-chain fatty acids; VLDL: very-low-density lipoprotein; HDL: high-density lipoprotein.

One year of ART use typically leads to increased CD4 T cell counts and decreased VLs, suggesting an improved immunological state. The metabolic dysregulation induced by HIV is, however, only minimally corrected (Li *et al.*, 2018; Peltenburg *et al.*, 2018). ART induces further metabolic alterations, particularly relating to OS (Peltenburg *et al.*, 2018; Sitole *et al.*, 2019) and lipid metabolism (Li *et al.*, 2018). Amino acid recovery following ART has been reported, particularly the recovery of essential and aromatic amino acids. For example, increased levels of histidine and tryptophan, with decreased levels of kynurenine

(Peltenburg *et al.*, 2018), even to the level of healthy controls (Li *et al.*, 2018). In contrast, tryptophan levels did not improve upon ART in the cohort of Sitole *et al.* (2019), while glutamine and aspartate partially recovered. Such inconsistencies in the amino acid profiles may be due to different stages of infection in the respective cohorts and warrant further, targeted investigations where CD4 T cell count, VLs, and treatment duration are controlled.

Li *et al.* (2018) did not observe a significant difference between the pre- and post-ART lipid profiles of patients on protease inhibitors (PI) for less than one year. Lipids remained elevated relative to healthy controls, with a concomitant increase in acylcarnitine derivatives. Peltenburg *et al.* (2018) reported a general increase in lipids in individuals treated with a combination of ARV classes (see Table 2.3) relative to treatment-naïve individuals, except for signalling lipids. Specifically, the phosphatidylcholines, which were decreased in response to HIV infection in the independent cohort, were increased in the longitudinal cohort (pre- and post-ART), suggesting recovery. These results concur with conventional studies that lipid metabolism remains aberrant in HIV-positive individuals on ART.

Chetwynd *et al.* (2017) analysed the urine metabolome of patients who had been on ART for at least one month at the time of sampling as well as those of healthy controls. The authors found that 13 endogenously derived metabolites were significantly different between the healthy and the HIV-positive individuals on ART. These metabolites, which could be linked to known modulatory effects of ART on enzymes and transporters, were indicative of a potential disruption in bile metabolism and transport, altered androgen transport or binding dynamics in plasma, and reflected immune activation.

Cassol *et al.* compared the plasma (2013) and CSF (2014) metabolic profiles of HIV-positive individuals on ART for two to three years to that of healthy controls. The metabolic alterations reported by these authors represent alterations due to HIV and/or its treatment rather than the effects of ART alone, as the authors did not include an HIV-positive treatment-naïve group. The metabolic profiles in both studies indicate mitochondrial dysfunction, OS, and inflammation, with lipid alterations being particularly prominent in the plasma profile (see Table 2.3). Nucleoside reverse transcriptase inhibitors and PIs are respectively known to cause mitochondrial dysfunction and lipid alterations. While it is not possible to assess which HIV-induced metabolic alterations recovered after two or more years of ART, the effects of ART toxicity are apparent in these results.

Rosado-Sanchez *et al.* (2019) and Rodriguez-Gallego *et al.* (2018b) investigated the mechanisms associated with poor immunological recovery of patients on ART. The patients in these cohorts received ART for two and three years, respectively. Patients were referred to as “immunological non-responders” (INR) when CD4 T cell counts did not improve sufficiently after a period of ART, despite successful viral suppression (Rosado-Sanchez *et al.*, 2019). Immunological recovery was defined, in both studies, as reaching a CD4 T cell count above 250 cells/ μ l after a specific period of ART. Patients who achieved this

were referred to as “immunological responders” (IR). In terms of the relationship between amino acid metabolism and poor immune recovery, many of the amino acid alterations typically associated with HIV infection were more pronounced in INR individuals, including glutaminolysis (Rodriguez-Gallego *et al.*, 2018b; Rosado-Sanchez *et al.*, 2019). Biological processes most enriched when assessing predictive capacity were microbial translocation and the inflammatory and acute-phase response, which are indicative of continued immune activation and inflammation, and subsequent high energy expenditure (Rosado-Sanchez *et al.*, 2019). Although Rodriguez-Gallego *et al.* (2018b) found that higher levels of large HDL particles, HDL cholesterol, and larger LDL particles were predictive of good immunological recovery (i.e. differentiated the INR group from the IR group at baseline or pre-ART), this was not replicated by Rosado-Sanchez *et al.* (2019). In the latter study, no differences between the lipoprotein particles of the INR and IR groups were observed at baseline, although differences in lipid metabolism were observed. A decrease in phosphatidylcholines in the INR group relative to the IR group predicted poor immunological recovery. Interestingly, these lipids were restored after one year of ART (Peltenburg *et al.*, 2018), as mentioned earlier. The post-ART profiles identified HDL particles and glutamate as parameters with the highest correlation with immune recovery after cART (Rosado-Sanchez *et al.*, 2019). In INR, a profile with high levels of almost all LDL and very-low-density lipoprotein particles (VLDL) and low levels of HDL particles corresponds with the HIV-associated atherogenic profile identified by Riddler *et al.* (2008), which further confirms the likelihood of a poor prognosis and higher risk for comorbidities, such as dyslipidaemia, in these cases.

Several metabolomics studies have focused on the characterisation of HIV-associated comorbidities over the years. The studies of Li *et al.* (2018) and Rodriguez-Gallego *et al.* (2018a) are the newest contributors to this avenue of research. Both studies investigated predictive markers of ART-induced dyslipidaemia as it plays an important role in the development of CVD. Using NMR, Rodriguez-Gallego *et al.* (2018a) identified VLDL concentrations, sizes, and numbers, as well as total triglycerides and lactate as predictors of dyslipidaemia in patients receiving non-nucleoside reverse transcriptase inhibitors (NNRTIs) for at least three years. These predictive metabolites suggest that mitochondrial dysfunction is part of the aetiology of NNRTI-induced dyslipidaemia and mirror dyslipidaemias in non-infected individuals. Furthermore, the combination of butyrylcarnitine and myristic acid (a long-chain fatty acid) was most predictive for dyslipidaemia in the study of Li *et al.* (2018). Although these HIV-positive individuals were on PIs for less than a year, the results implicated markers of impaired mitochondrial function as well as impaired signalling between adipose and other tissues as predictive for ART-induced dyslipidaemia. Developing such predictive profiles could aid in selecting the most appropriate ART regimen in an individualised manner, both for those normolipidaemic at the initiation of ART and those already suffering from HIV-induced dyslipidaemia, possibly reducing the risk factors and thereby the likelihood of CVD (Non *et al.*, 2017).

Babu *et al.* (2019) investigated the long-term effects of ART by comparing the metabolic profiles of HIV-positive individuals who had been on ART for five years or more, had low viremia and had stable CD4 T cell counts for two years, to healthy controls. All essential amino acids, except histidine, were lower in the HIV-positive group (untreated and treated). However, histidine returned to normal values in studies investigating short-term ART (Li *et al.*, 2018; Peltenburg *et al.*, 2018). After excluding malnutrition as a possible cause, Babu *et al.* (2019) linked the decreased amino acids to possible malabsorption due to the detrimental effects of HIV on intestinal health. Alterations to energetics included decreased TCA cycle intermediates, except for alpha-ketoglutarate, which was elevated. This suggests that glutaminolysis was active and supports both the decreased amino acid levels and decreased urea cycle intermediates measured in this study. The lipids that were significantly altered have important roles in immunity and reflect, in conjunction with altered cytokines, and various other proteins measured, that despite CD4 T cell counts being stable for an extended period, immunological and metabolic dysfunction persists even when viral replication is very low. The metabolic profile of HIV-positive individuals on ART as observed by Babu *et al.* (2019) is similar to that seen in age-related diseases but appears earlier in these individuals. This occurrence has also been observed by other metabolomics studies (Cassol *et al.*, 2014; Cassol *et al.*, 2015) with the potential to accelerate the ageing process in HIV-positive individuals.

From the studies reviewed here, progressive HIV infection typically shows increased lactate, glutaminolysis, triglycerides, lipids, LDL, and tryptophan catabolism as well as decreased signalling lipids, BCAAs, and urea cycle activity. ART restores some of these changes to the levels seen in healthy controls. It is unclear whether the metabolic alterations ascribed to “recovery” is due to a true return to a healthier state in one or more areas of metabolism or due to one or all of the following factors: residual viral activity, permanent HIV-induced damage, and/or toxicity of ART. To better understand the underlying mechanisms associated with the persistent immune and metabolic derangements in HIV-positive individuals on ART, longitudinal studies over longer periods would be ideal, wherein the effects of short- and long-term ART use on the same patients are investigated. However, the practicality of such studies is limited by poor adherence to therapy and lack of funding, especially in high prevalence areas, which could bias results.

The studies reviewed here not only confirm previous results but also provide new insight into the metabolic mechanisms underlying the effects of HIV infection and ART on the host. Furthermore, these studies highlight differences between infection and treatment-induced effects and provide some indication of the temporal nature of these changes.

2.5 Tuberculosis disease and immunometabolism

2.5.1 Basic pathophysiology, diagnosis, prognosis and treatment

Human TB is a highly contagious disease, most often caused by *Mtb*, a member of the *Mycobacteriaceae* family of bacteria. *Mtb* is transmitted via aerosolised infectious droplets, expectorated by infected individuals when speaking or coughing, which are then inhaled by others (Dheda *et al.*, 2016). Most individuals can contain *Mtb* infection in a latent (non-infectious and asymptomatic) stage via the formation of a fibroblast capsule around the bacilli, known as a granuloma (Cardona, 2018). Approximately 10% of all infections with *Mtb* lead to active TB. The host immune competency is the most significant factor for the development of active TB, the specific manifestations and the progression of the disease (Achkar & Jenny-Avital, 2011). Active TB manifests with symptoms such as persistent cough with mucus, fever, night sweats, weight loss, haemoptysis, pleurisy, dyspnoea, wheezing, fatigue (Luies & Du Preez, 2020), as well as organ-specific symptoms depending on the site of infection (Dheda *et al.*, 2016). Ultimately, TB patients develop a wasting state known as cachexia (similar to that mentioned for HIV/AIDS), which is why TB was known as “consumption” in previous centuries (Semba *et al.*, 2010). The different states of *Mtb* infection were seen as a simple dichotomy (active versus latent TB) for some time, but it has recently been recognised that diverse pathological states exist within and between these designations and that *Mtb* infection is rather a continuous spectrum (Barry *et al.*, 2009).

To prevent progression to active disease, diagnostic efforts have focused on detecting latent TB, especially in high-burden settings and in HIV-positive individuals (Dheda *et al.*, 2016). Latent TB is diagnosed with immunological tests based on a delayed hypersensitivity reaction to *Mtb* proteins (Heemskerk *et al.*, 2015), namely the tuberculin skin test (TST) and the interferon-gamma release assay (IGRA). Currently, acid-fast smear microscopy is the most common method to detect active TB, especially in high-burden settings; however, bacterial culture remains the gold standard (Tiberi *et al.*, 2017). Other techniques used include molecular methods (such as the GeneXpert MTB/RIF test currently recommended by the World Health Organization (WHO) due to its rapid, cost-effective diagnostic ability and detection of drug resistance (Dheda *et al.*, 2016; World Health Organization, 2018)), serological tests (based on the detection of antibodies in the blood) and phage-based assays (Dheda *et al.*, 2016). Chest X-rays are typically used as a diagnostic aid and to assess the extent of pulmonary TB, however, there has been difficulty in developing a reliable scoring system for this application (Pefura-Yone *et al.*, 2017). In addition to developing improved diagnostic techniques, there is also a need to develop simple and reliable techniques as indicators or predictors of disease progression, ideally at an early stage.

As TB is a curable disease, accurate, timely detection and treatment are imperative for a good prognosis, especially in HIV-positive individuals, and in light of the spread of drug-resistant TB. Currently, drug-sensitive TB is treated using the directly observed treatment, short-course (DOTS) regimen, which

involves a four-drug combination (isoniazid, rifampicin, pyrazinamide and ethambutol); of which each dose is taken under direct medical supervision over six months. DOTS is highly successful if adhered to properly (Glaziou *et al.*, 2014). However, treatment is complicated in HIV-positive individuals on ART and in cases of drug resistance also involve a longer period of treatment, has greater risks of adverse effects, and is more expensive (Tiberi *et al.*, 2017). Various treatment regimens are available for latent TB, involving either or both isoniazid and rifampicin (or rifapentine), typically administered for three months. However, if isoniazid is taken alone, the duration of treatment extends to six months (World Health Organization, 2019). No TB vaccine is currently available that can truly act prophylactically, and although the Bacille Calmette Guerin vaccine can prevent severe forms of childhood TB, it is ineffective in adults (Dheda *et al.*, 2016). Various vaccines are currently in clinical trials (World Health Organization, 2020), and hence a means to curb TB transmission, a factor central to controlling this pandemic, could be attained soon.

2.5.2 Tuberculosis and cytokines

As with HIV, cytokines have been studied extensively in the context of TB, but the focus here will be on studies measuring cytokines in a multiplexed manner in unstimulated, blood-based samples. A large proportion of the literature focuses on the constituents of a protective versus non-protective immune response to TB. A Th1 immune response involving the release of IFN- γ and TNF- α is necessary, but not sufficient, for protection against *Mtb* infection (Kumar *et al.*, 2019; Sudbury *et al.*, 2020). This is further supported by the fact that a failure to produce another Th1-associated cytokine, IL-2, results in the rapid spread and multiplication of *Mtb* (Handzel *et al.*, 2007). A Th2 response has generally been considered an unsuitable reaction to *Mtb* infection and increases the likelihood that latent *Mtb* infection will progress to active TB (Gourgouillon *et al.*, 2012; Ruhwald & Ravn, 2009). The role of the Th2 response in TB has not been fully elucidated (da Silva *et al.*, 2015; Sudbury *et al.*, 2020), however, it has been reported that increased Th2 responses downregulate Th1 responses as the classical antagonist to Th1 responses (da Silva *et al.*, 2015) rather than indicating susceptibility to infection (Gourgouillon *et al.*, 2012). It has also been suggested that it is the overproduction of Th2 cytokines and not the failure of Th1 responses that result in the development of TB (Winek *et al.*, 2009). While such responses have previously been associated with the immune-evasive mechanisms of *Mtb* (Sudbury *et al.*, 2020; Van Crevel *et al.*, 2002), it is not clear whether Th2 cytokines cause reactivation of TB or whether their release is a consequence of progression to active disease.

Verbon *et al.* (1999) compared the serum cytokine concentrations of patients with untreated active TB (pulmonary and extrapulmonary), with active TB during treatment (at least two weeks after treatment commenced), and after treatment, to those of household contacts and healthy controls. The levels of IFN- γ , IL-6, and IL-10 were increased in untreated TB. While the levels of IFN- γ declined during and after treatment, IL-6 and IL-10 did not return to levels comparable to healthy controls. The levels of IFN- γ and

IL-6 were also increased in patients experiencing fever, malaise, and anorexia (Verbon *et al.*, 1999). Similarly, increased levels of IFN- γ , IL-6, and IL-17 in the plasma of patients with active TB compared to healthy controls have been reported, using various assays to measure different cytokines (multiplexed Luminex assay, ELISAs, and ELISPOT assays). Although the level of IL-17 decreased significantly after three weeks of treatment, it was still higher than that in healthy controls. In the study of Xu *et al.* (2016), the levels of IFN- γ and IL-6 showed a decreasing trend after treatment, but this was not significant.

Kumar *et al.* (2019) used ELISAs to measure the plasma cytokine levels in individuals with latent TB, active pulmonary TB before and after anti-TB treatment, and healthy controls. Active TB was associated with increased systemic levels of pro-inflammatory cytokines (IFN- γ , TNF- α , IL-17A, IL-17F, IL-6, IL-1 β) relative to the latently infected and healthy groups, which decreased after six months of anti-TB treatment. The results from the active TB group in the study of Kumar *et al.* (2019) were also stratified based on time to culture conversion following treatment. Culture conversion is typically defined as the point at which the first of two negative sputum cultures is attained, at two consecutive clinic visits, without an intervening positive result (Rambaran *et al.*, 2021). Slow responders (those still culture-positive after two months of anti-TB treatment) had higher levels of pro-inflammatory cytokines (IFN- γ , TNF- α , IL-17A, IL-1 β) than fast responders (Kumar *et al.*, 2019). Interestingly, higher levels of the same four cytokines were also associated with increased disease severity (as measured by stratification on cavitary and bilateral disease, separately) in the study of Kumar *et al.* (2019). These results were not replicated in the study of Rambaran *et al.* (2021), who measured these and other cytokines in the plasma of active TB patients using a multiplex assay. Instead, the latter study found that increased levels of IL-6 and IL-1 receptor antagonist were associated with cavitary disease. Similarly, Kumar *et al.* (2019) associated increased levels of IFN- γ , TNF- α , IL-17A, and IL-17F with bacterial burden, while Rambaran *et al.* (2021) found no clear dose-response between bacterial burden and cytokine levels. This discrepancy might be ascribed to the fact that the patients of Kumar *et al.* (2019) had no history of previous TB, while those of Rambaran *et al.* (2021) had recurrent TB (a new episode of TB after being previously cured). The variation in the blood cytokine profiles of primary versus recurring TB is substantiated by the results of Handzel *et al.* (2007), who showed that cytokine secretion was overall lower in the serum of patients with recurrent TB compared to that of patients with primary disease, household contacts, and healthy controls.

Several studies (including antigen stimulation studies) have indicated that there is no definite Th1/Th2 skewing in TB patients (Handzel *et al.*, 2007; Winek *et al.*, 2009). Collectively, these results indicate that the cytokine profile of TB patients varies with disease presentation and severity, which highlights the complexity of cytokine interactions. The importance of the delicate regulation necessary to maintain the balance between a protective immune response and immune-mediated pathology is highlighted by the fact that similar responses were also associated with bacterial clearance after treatment than with disease severity in the study of Rambaran *et al.* (2021).

From the studies reviewed here, TB primarily affected circulating levels of IFN- γ , TNF- α , IL-10, and IL-17. Anti-TB treatment tends to reverse the trends induced by TB, although not consistently in all cohorts.

2.5.3 Tuberculosis and metabolites

Mtb infection places immense strain on the host in terms of energy expenditure — hence nutritional and metabolic alterations have long been associated with TB. Metabolomics has been extensively applied to various aspects of TB research and has contributed to, among others, a better understanding of the pathogen, its interaction with the host, and its effect on the physiology of the host. Many studies have focused on the discovery of biomarkers for specific TB-related applications; however, the results are often not extensively interpreted in a biological context. One of the main factors hampering the development of new or improved diagnostic and/or prognostic techniques is an insufficient understanding of the mechanisms regulating the pathogen, the host response to it, as well as the interactions between these processes. Though many potential biomarkers for TB have been identified, without a proper understanding of their biological origins and roles as a starting point, validating these for clinical implementation, is complicated (Walzl *et al.*, 2018). The role of metabolomics in various aspects of TB research has been extensively reviewed (Table 2.4) but the focus here will be on the most recent studies using human samples (Table 2.5).

Table 2.4: A summary of reviews relating to the role of metabolomics in various aspects of TB research.

Reference	Aim
General	
Mirsaeidi <i>et al.</i> (2015)	To review the advantages and disadvantages of the application of metabolomics to mycobacterial disease in general.
Du Preez <i>et al.</i> (2019)	To review metabolomics studies investigating <i>Mtb</i> and pulmonary TB in terms of intended research outcomes and future clinical applications.
Diagnostics	
Du Preez and Loots (2014)	To review the conventional TB diagnostic techniques and the role that metabolomics has played in identifying novel TB biomarkers.
Loots (2016)	Editorial; provides an overview of the achievements, advantages, and limitations, and goals of the applications of metabolomics to the understanding and diagnosis of TB.
Du Preez <i>et al.</i> (2017)	To review the state of metabolomics biomarkers and their role in the diagnosis and characterization of TB, as well as their clinical application.
Therapeutics	
De Villiers and Loots (2013)	To evaluate metabolomics as a tool for better understanding the mechanisms underlying TB treatment failure.
Luies <i>et al.</i> (2017a)	To review the contribution of metabolomics to TB treatment research — drug mechanisms, metabolism, and toxicity, as well as the prognostic value of metabolite biomarkers for prediction of treatment outcome, as well as the development of new and less toxic TB drugs and regimens.
Du Preez and Loots (2018)	To review the contribution of pharmacometabonomics in the understanding of the mechanisms of action of first-line TB drugs.

Table 2.5: New investigations not included in previously published TB-related metabolomics reviews.

Research Model	Metabolome fraction targeted	Analytical apparatus	Groups compared	Statistical Approaches	Reference(s)
Disease characterisation and diagnostics					
Human: serum	Total metabolome	NMR	TB-/Tn- LTB+/Tn- TB+/Tn-	Fold change, t-test, PCA, OPLS-DA, unique structures plots, MSE, logistic regression	Albors-Vaquer <i>et al.</i> (2020)
Human: macrophages	Total metabolome	RPLC-Q-TOFMS (untargeted), NMR (targeted)	Infection with <i>Mtb</i> , stimulation with LPS, or left untreated	Fold change, PCA, PLS-DA, random intercept models	Vrieling <i>et al.</i> (2020)
Human: serum	Biogenic amines, acylcarnitines, glycerophospholipids, sphingomyelins	LC-MS/MS and FIA-MS	TB-/Tn- LTB+/Tn- TB+/Tn-	PCA, PLS-DA, Kruskal-Wallis, Mann-Whitney U test	Cho <i>et al.</i> (2020)
Human: plasma	Amines, acylcarnitines	LC-MS/MS	TB-/Tn- TB+/Tn- TB+/DM+/Tn-	HCA, PLS-DA, AUC of ROC, linear regression models, Kruskal-Wallis	Vrieling <i>et al.</i> (2018)
Human: plasma	Lipids	NMR	TB-/Tn- *TB+/Tn- *DM+/Tn- *TB+/DM+/Tn-	PCA, HCA, PLS-DA, AUC of ROC, linear regression and mixed effect models, Kruskal-Wallis	Vrieling <i>et al.</i> (2019)
Human: pleural effusion	Targeted (in-house database)	LC-MS/MS (+ and - ESI)	TPE vs MPE	Spearman rank correlations, t-test, PCA, OPLS-DA, AUC of ROC	Che <i>et al.</i> (2018)
Human: plasma	Lipids	UPLC-MS/MS	TB-/Tn- TB+/Tn- TB-/lung cancer TB-/CAP	PCA, OPLS-DA, K-means clustering, AUC of ROC	Han <i>et al.</i> (2020)
Human: plasma, Mouse: serum, zebrafish: larvae	Amines	UPLC-MS (ESI+)	Human: *TB+/Tn- *TB+/Tn+ Mouse: TB+/Tn- Zebrafish: <i>M. marinum</i> infection	PLSDA	Ding <i>et al.</i> (2020)
Human: pleural effusion	Total metabolome	UPLC-MS and -MS/MS (ESI+ and -)	TPE vs MPE	Fold change, PLS-DA, AUC of ROC	Cao <i>et al.</i> (2021)
Human: feces	Total metabolome, SCFA	GC-MS	TB-/Tn- TB+/Tn-	PCA, OPLS-DA, random forest modelling, t-test, Mann Whitney U	Wang <i>et al.</i> (2021a)
Elucidation of drug metabolism and drug action mechanisms					
Human: urine	Organic acids	GCxGC-TOFMS	TB+/Tn- *TB+/Tn+	Repeated-measures ANOVA, ASCA	Combrink <i>et al.</i> (2019)
Human: serum	Total metabolome	UPLC-Q-TOF-MS	TB-/Tn-TB+/Tn+ (DS) TB+/Tn+ (MDR)	PCA, OPLS-DA, ANOVA, t-test, AUC of ROC	Huang <i>et al.</i> (2020)

Human: hair	11 MDR-TB drugs	LC-MS/MS	TB+/Tn+ (MDR) TB+/Tn+ (pre-XDR) TB+/Tn+ (XDR)	Sensitivity and specificity analyses	Metcalf <i>et al.</i> (2021)
Human: urine	Acylcarnitines and amino acids	LC-MS (acylcarnitines) and GC-MS (amino acids)	TB+/Tn- *TB+/Tn+ (cured) *TB+/Tn+ (uncured)	Unfolded PCA, ASCA, t-test	Opperman <i>et al.</i> (2021)

Research model: *Mtb*: *Mycobacterium tuberculosis*; *DPI*: diphenyleneiodonium, an inhibitor of superoxide production; *BMDMs*: bone marrow-derived macrophages.

Analytical apparatus: *UPLC-qTOF*: ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry; *ESI*: electrospray ionization; *ESI+*: *ESI* positive ion mode; *ESI-*: *ESI* negative ion mode; *NMR*: nuclear magnetic resonance spectroscopy; *RPLC-Q-TOFMS*: reversed-phase high-performance liquid chromatography quadrupole time-of-flight mass spectrometry; *LC-MS/MS*: liquid chromatography-tandem mass spectrometry; *UPLC-MS*: ultra-high-performance liquid chromatography-mass spectrometry; *UPLC-MS/MS*: ultra-high-performance liquid chromatography-tandem mass spectrometry; *FLA-MS/MS*: flow injection analysis tandem mass spectrometry; *GCxGC-TOFMS*: two-dimensional gas chromatography time-of-flight mass spectrometry; *UHPLC-HRMS*: ultra-high-performance liquid chromatography and high-resolution mass spectrometry.

Groups compared: *TB*: active tuberculosis; *N/A*: not applicable; *TPE*: tuberculous pleural effusion; *MPE*: malignant pleural effusion; *DM*: diabetes mellitus, *: longitudinal study (before and after treatment); *LTB*: latent *Mtb* infection; *LPS*: lipopolysaccharide; *Tn-*: treatment negative; *Tn+*: treatment positive; *CAP*: community-acquired pneumonia; *DC*: drug-susceptible; *MDR*: multidrug-resistant; *XDR*: extensively drug-resistant.

Statistical approaches: *PCA*: principal component analysis, *OPLS-DA*: orthogonal projections to latent structure-discriminant analysis; *AUC of ROC*: area under the curve of receiver operating characteristic; *HCA*: hierarchical cluster analysis; *PLS-DA*: partial least squares-discriminant analysis; *MSEA*: metabolite set enrichment analysis; *ANOVA*: analysis of variance; *ASCA*: *ANOVA* simultaneous component analysis.

Research has recently focused on better understanding the latent stage of *Mtb* infection and the mechanisms underlying its conversion to active TB, as this might inform better prophylactic action in future. The relationship between latent and active TB was investigated by Albors-Vaquer *et al.* (2020), who compared the serum metabolic profiles of patients with active TB, household contacts of active TB patients, and healthy controls. Within the context of this study, household contacts of TB patients were considered as potential latent TB cases, regardless of TST results, as they were asymptomatic but exposed to *Mtb* for prolonged periods. The metabolic profiles of the active TB group and the household contact group (compared to the healthy controls) had a large degree of overlap, with 13 of the 15 metabolites that characterised active TB, also being significant in the household contact profile. Of these overlapping metabolites, many amino acids were decreased while other metabolites were mainly increased. In a similar study, Weiner *et al.* (2018) observed a more oxidative state and increased inflammation, indicating loss of immunological control over *Mtb* by the host (decreased histidine and glutamine and increased kynurenine and cortisol) during the conversion from latent to active TB. As these trends have been reported by various studies for active TB patients, the authors concluded that the conversion from latent to active TB is reflected in a quantitative rather than qualitative change in the metabolic profile (Weiner *et al.*, 2018). In the study of Albors-Vaquer *et al.* (2020) the metabolites with the highest predictive capacity for the development of active TB were decreased glutamine and citrate. While glutamine was significantly

decreased in the exposed and active TB groups, citrate was only significant in the household contact group. Decreased glutamine was also found by Weiner *et al.* (2018), months before progression to active disease, but was not found to be predictive in that study. This indicates that the activation of glutaminolysis is also an early event in TB, as was suggested for HIV (Section 2.4.3). The nature of the interaction of metabolites like citrate and glutamine in the metabolism, being part of multiple overlapping processes, means that they are not ideal for use as predictive biomarkers. Besides the fact that these metabolites are in constant flux, other disease states or health conditions also alter their levels (e.g. glutamine in HIV infection). The studies related to latent TB discussed here confirm that latent *Mtb* infection is detectable using metabolomics and can be distinguished from active TB. However, as the affected metabolites overlap largely with the profiles of other diseases, similar studies with a longitudinal design and including other disease controls (especially those with latent or non-progressive phenotypes) will be necessary to determine the clinical relevance of these results.

Vrieling *et al.* (2020) investigated the impact of *in vitro* *Mtb* infection on primary human macrophage metabolism. The analyses indicated an increased glycolytic flux upon stimulation with *Mtb*. Untargeted LC-MS metabolomics confirmed and complemented these results, indicating that various other areas of metabolism besides glycolysis are affected upon *Mtb* infection. As only tentative annotation is possible using untargeted metabolomics, a targeted NMR approach was then used to characterise the metabolic profile of M2 (macrophage colony-stimulating factor-derived) macrophages upon *Mtb* infection. *Mtb*-infected macrophages exhibited increased nicotinamide adenine dinucleotide and glutathione synthesis, decreased glutamine and increased glutamate, increased macrophage phagocytic activity (indicated by increased levels of creatine phosphate), and also confirmed an increased glycolytic flux, evident by an increased lactate/glucose ratio (Vrieling *et al.*, 2020). Findings at the cellular level evaluate the effect of *Mtb* infection on the host in a simpler metabolic environment (in terms of aetiology) than that of biofluids such as blood and urine. This provides a clearer indication of the mechanisms underlying the changes observed in biofluids.

Cho *et al.* (2020) and Ding *et al.* (2020) confirmed previously reported alterations to the biogenic amine profile of active TB patients, in serum and plasma, respectively. Increased levels of glutamate with concurrently decreased glutamine resulted in a decreased glutamine/glutamate ratio. Though previous studies do not always find these metabolic partners together (likely due to the untargeted nature of studies), increased levels of glutamate have been reported in the blood of active TB patients (Feng *et al.*, 2015; Frediani *et al.*, 2014; Zhou *et al.*, 2013), as has decreased levels of glutamine (Vrieling *et al.*, 2018; Weiner *et al.*, 2012; Zhou *et al.*, 2013). Furthermore, an increased aspartate/asparagine ratio was reported (Cho *et al.*, 2020; Ding *et al.*, 2020), confirming the finding of increased aspartate by Weiner *et al.* (2012). The balance of glutamine and glutamate, as well as asparagine and aspartate, have various roles in the immune system. An imbalance in these ratios affects the immune-competence of T cells and macrophages

(Yoneda *et al.*, 2009). Furthermore, these amino acids have been linked to processes related to *Mtb* growth and its success as an intracellular pathogen (Cho *et al.*, 2020; Frediani *et al.*, 2014; Thandi *et al.*, 2020).

OS is well established as a feature of active TB. Indicators of OS were found in the form of decreased methionine levels with concomitantly increased sulfoxymethionine (Ding *et al.*, 2020; Suzuki *et al.*, 2016). An increased tryptophan/kynurenine ratio was absent from the biogenic amine profile reported by Cho *et al.* (2020) and Ding *et al.* (2020) (although tryptophan was decreased in the latter), yet it was markedly altered in the publicly available dataset of Weiner *et al.* (2012), resulting in a significantly higher diagnostic capacity. The severity of disease in the cohort of Weiner *et al.* (2012) was higher than in that of Cho *et al.* (2020), indicating that the severity of systemic inflammation influences the metabolic pathways affected. There is no widely applied TB prognostic biomarker to match subjects based on disease severity. Most often, the extent of TB disease is assessed by chest X-rays. This might explain the difference in the profiles obtained by different groups (in addition to differences due to analytical and statistical platforms).

Vrieling *et al.* (2018) investigated the severity of TB and its correlation to sputum time to positivity (indicative of mycobacterial load). The authors identified metabolite markers relating to BCAAs and ketone bodies. The same group also correlated metabolites to the chest X-ray score, which is indicative of the severity of lung lesions. Chest X-ray scores correlated with four acylcarnitines (hexadecenoylcarnitine, hexanoylcarnitine, dodecenoylcarnitine, and tetradecenoylcarnitine) and 3-methoxytyrosine, however, no overlap in the metabolic markers was evident (Vrieling *et al.*, 2019). Thus, further investigations regarding metabolites correlating to disease severity are necessary to confirm these results. As the immunological status of a patient is the primary determinant of their control of the bacterium, correlation of immune markers to metabolites to investigate TB disease severity may also yield valuable results and is currently of great research interest.

Che *et al.* (2018) investigated the specific effects of TB on the lung metabolome by comparing the metabolic profiles of pleural effusion caused by TB (TB pleural effusion; TPE) to that caused by lung cancer (malignant pleural effusion; MPE), a condition which is also characterised by inflammation and altered energy metabolism. In general, the results indicated increased energy expenditure and more severe inflammation in the TPE group relative to the MPE group. The TPE profile was similar to metabolic profiles previously reported for active TB; decreased free fatty acids, amino acids, urea, and TCA cycle intermediates, with a concomitant increase in acylcarnitines and bile acids. The specific decrease in tryptophan together with increased levels of the immunosuppressive kynurenine, which has been consistently reported in TB metabolomics studies, was also observed. The association of TPE with increased levels of tryptophan catabolic products, decreased fatty acids, as well as increased acylcarnitines and bile acids were confirmed by Cao *et al.* (2021). In the latter study, which was virtually identical in design to that of Che *et al.* (2018), the combination of four acylcarnitines and two fatty acids

discriminated TPE from MPE with high sensitivity and specificity. Similarly, Wang *et al.* (2017) compared 58 pleural effusion samples, of which 20 were MPEs, 20 were TPEs, and 18 were transudative pleural effusions (i.e. those resulting from low oncotic pressure or low hydrostatic pressure). The authors observed increased lipids in TPE compared to transudative pleural effusion and associated this with a large number of lipids in *Mtb*, which accounts for 20–40% of the dry weight of these bacteria, and 60% of the dry weight of the cell wall. This was further substantiated since no significant difference was observed between the TPE and MPE samples since tumours are also associated with increased lipid content. In a plasma-based study, TB patients were distinguished from patients with lung cancer and with community-acquired pneumonia by increased cholesterol esters and decreased phospholipids. These changes were associated with the utilisation of host lipids by *Mtb* as a carbon source and in various immune evasion mechanisms. Furthermore, the combination of two phosphatidylcholines, a cholesterol ester and sphingomyelin, was able to distinguish TB patients from those with other lung conditions (Han *et al.*, 2020). Collectively, these studies suggest that lipids could be used as biomarkers for distinguishing TB from other lung diseases, but this requires further investigation.

Inflammatory diseases (such as TB) are often associated with insulin resistance, hence diabetes mellitus (DM) is a known risk factor for TB. Evidence for insulin resistance has also been found in TB patients without a clinical diagnosis of DM, indicating that the condition is caused by the diseased state (Philips *et al.*, 2017). Two recent metabolomics studies by Vrieling *et al.* (2019; 2018) characterised the metabolic alterations underlying TB and DM, as well as how these change when DM presents as a comorbidity in TB patients. Besides the amino acid changes discussed above, these studies confirmed previous reports of decreased serum histidine (Weiner *et al.*, 2012), decreased serum alanine (Zhou *et al.*, 2013), and increased serum (Weiner *et al.*, 2012; Zhou *et al.*, 2013) and urinary (Luies & Loots, 2016) phenylalanine levels in TB patients. These alterations have been associated with the inflammatory state and OS, as well as cachexia. Furthermore, decreased urea (Weiner *et al.*, 2012), ornithine (Vrieling *et al.*, 2019), and citrulline, with concurrently increased aspartate (Ding *et al.*, 2020; Vrieling *et al.*, 2019; Weiner *et al.*, 2012) are indicative of dysregulation of the urea cycle in TB patients. Although the exact mechanisms underlying these alterations are still unclear, they may be related to antimicrobial mechanisms of immune cells. For example, Thandi *et al.* (2020) recently showed that ornithine inhibits *Mtb* growth and enhances autophagy in mouse alveolar macrophages.

Ding *et al.* (2020) found that ten amine metabolites were common in the metabolic profiles of human TB patients (plasma), mice infected with *Mtb* (serum), and zebrafish larvae infected with *Mycobacterium marinum*. Five of these metabolites (methionine, asparagine, tryptophan, citrulline, and phenylalanine, or their metabolic partners) have been consistently reported as distinguishing TB patients from healthy controls. The authors concluded that these metabolites are fundamental to mycobacterial infection but whether these metabolites distinguish mycobacterial infections from other infections, still needs to be investigated.

Although it has been established that the composition of the gut microbiome of TB patients is distinct from healthy controls and that these microbes are important in the immune response to *Mtb*, the study of Wang *et al.* (2021a) indicates that the faecal metabolome of TB patients is also distinct from that of healthy controls. In this study, the dysbiosis of the gut microbiome correlated with various metabolic alterations, primarily involving energy, amino acid, and lipid metabolism. These altered metabolites are related to immunological mechanisms such as increased inflammation, and appetite loss (Wang *et al.*, 2021a). Future metabolomics studies using stool samples could lead to a better understanding of the interaction of the gut microbiome, metabolism, and the immune response during *Mtb* infection.

Combrink *et al.* (2019) investigated the urine organic acid profiles of TB patients during the first month of anti-TB treatment. Samples were collected before treatment and at one, two, and four weeks after treatment initiation, which allowed unique insights into the early effects of treatment on the host. The markers identified were grouped as drug metabolites, metabolites indicative of a decreased bacterial load, and alterations to the host metabolome. The trends in the latter group of metabolites suggested a progressive decrease in OS with treatment duration, indicating that the extent of OS associated with anti-TB treatment (Sodhi *et al.*, 1997) is significantly smaller than that caused by TB. This was also confirmed by Vrieling *et al.* (2019), who investigated the plasma metabolic profiles of patients after two and six months of anti-TB treatment. TB-associated amino acids (histidine, glutamine, tryptophan, phenylalanine, ornithine, citrulline, and glycylglycine) returned, completely or partially, to values comparable with those of a healthy control group after two months of treatment. However, specific metabolic alterations associated with increased OS and hepatotoxicity were observed: increased levels of methionine sulfone (formed by the oxidation of methionine sulfoxide, the initial oxidation product of methionine) and putrescine. After six months of treatment, all of the above-mentioned amino acids were comparable to healthy control values. In contrast, 3-methoxytyrosine, which was increased in TB patients and showed the highest predictive capacity for TB, did not normalise after treatment. Although these results seem to contradict those of Combrink *et al.* (2019), it is hypothesised that TB-induced OS is decreased after one month of treatment through control of the bacterium, but that the oxidative effects of the anti-TB treatment itself are maximal after two months of treatment. This hypothesis is substantiated by the fact that the first two months of anti-TB treatment are more intensive than the last four. Furthermore, methionine sulfone and putrescine returned to normal levels upon completion of anti-TB treatment, reflecting the decreased intensity of the anti-TB treatment regimen during the last months. Combrink *et al.* (2019) confirmed their findings using a targeted metabolomics approach to investigate the acylcarnitine and amino acid profiles of patients with either cured or failed treatment outcomes, at diagnosis and at various time points after treatment initiation (Opperman *et al.*, 2021). The patients with a failed treatment outcome experienced the same treatment-induced metabolic alterations as the cured patients, but to a higher extent and at an earlier time point following treatment initiation. This was linked to possible vitamin B6 deficiency, fluctuating levels of OS and urea cycle intermediates, and drug-induced

alterations to enzyme function. Furthermore, the authors proposed that genetic and phenotypic differences in drug metabolising enzymes, and therefore altered drug-enzyme interactions, relates to the diverging treatment outcomes. These results highlight the need for a holistic and personalised approach to TB treatment (Opperman *et al.*, 2021).

The gamma-glutamyl cycle (γ -GC) forms an integral part of glutathione recycling. Gamma-glutamyl dipeptides, by-products of glutathione synthesis, have been reported to be lower in the serum of TB patients compared to healthy controls (Vrieling *et al.*, 2019; Weiner *et al.*, 2012). Similarly, 5-oxoproline, a metabolite directly downstream from the gamma-glutamyl amino acid in the γ -GC, shows the same trend (Che *et al.*, 2013; Combrink *et al.*, 2019) and correlates with pathological lung damage (Che *et al.*, 2013). The imbalance of the γ -GC in active TB patients suggests decreased antioxidant capacity because of the prolonged OS. The increased levels of gamma-glutamylalanine, gamma-glutamylglutamine (Vrieling *et al.*, 2019), and 5-oxoproline (Che *et al.*, 2013; Combrink *et al.*, 2019) observed in TB patients after treatment reflects an increase in antioxidant capacity (due to increased glutathione synthesis) resulting from a lowered mycobacterial burden and relief of the diseased phenotype.

Patients infected with *Mtb* strains that are resistant to a specific set of drugs are considered to have multidrug-resistant or extensively drug-resistant tuberculosis (MDR- and XDR-TB, respectively). These strains increase the morbidity and mortality associated with TB, as treatment options are limited, and diagnosis of MDR-TB can be time-consuming. Huang *et al.* (2020) searched for serum metabolic biomarkers of MDR-TB, which may provide alternative diagnostic options in future. Decreased NADH metabolites, phosphatidylcholines, caprylic acid, and increased D-xylulose in serum distinguished MDR-TB patients from drug-susceptible TB patients and healthy controls. The authors speculated that these alterations represent mechanisms of drug resistance, especially relating to cell membrane remodelling in resistant strains of *Mtb* (Huang *et al.*, 2020). Metcalfe *et al.* (2021) developed a method to determine prior exposure to anti-TB drugs from hair samples, based on the detection of drug-derived metabolites, which could inform clinicians' decisions regarding treatment regimens for specific patients.

The studies reviewed here provide many new insights and confirm previous results relating to the metabolic mechanisms associated with latent *Mtb* infection and conversion to active disease, *Mtb* infection at the cellular level, active TB, comorbidities such as DM, as well as TB treatment. The main metabolic changes associated with TB include altered glutamine/glutamate and asparagine/aspartate ratios, increased tryptophan catabolism, markers of oxidative stress, acylcarnitines, as well as decreased TCA intermediates, amino acids, fatty acids and urea cycle activity. Many of the metabolic alterations discussed are similar to those discussed for HIV infection and in many cases, the HIV- and TB-associated metabolic alterations overlap with those of other disease states. Determining the similarities and differences between the HIV/AIDS and TB-associated metabolic states may aid in understanding the mechanisms that underlie the synergistic effect observed during HIV/TB co-infection.

2.6 HIV/TB co-infection and immunometabolism

2.6.1 Basic pathophysiology, diagnosis, prognosis and treatment

Although HIV-positive individuals suffer from many serious secondary infections, TB is the most common disease, especially in sub-Saharan Africa. Due to the immunosuppressive effects of HIV on the host, HIV-positive individuals have a 26–31 times greater risk of developing active TB compared to HIV-negative individuals (Wood *et al.*, 2000; World Health Organization, 2012). This increased risk, along with various other factors, contributes to the significant global burden of HIV/TB co-infection. Although pulmonary TB is the most common form of active TB in HIV/TB co-infected individuals, this population is also more prone to the development of independent or concurrent extrapulmonary TB, especially when CD4 T cell counts are low (Chaisson, 1987; Elliott *et al.*, 1995; Schutz *et al.*, 2010). When co-infection with these pathogens is established, they act synergistically to increase the disease burden in the host (Kwan & Ernst, 2011). As expected, the pathophysiology of *Mtb* infection differs in HIV-positive individuals and even more so in those who have progressed to severe immunosuppression, i.e. AIDS, typically showing an accelerated clinical course (Badri *et al.*, 2001; Schutz *et al.*, 2010). In general, HIV/TB co-infected patients present with characteristic symptoms of active pulmonary TB (Burman & Jones, 2003; Luies & Du Preez, 2020) but often presents with more severe systemic symptoms such as fever and experience weight loss. However, a mucus-producing cough is less common, which is linked to HIV-induced immunosuppression, as a less vigorous immune response results in fewer pulmonary complications (Garcia *et al.*, 2007; Schutz *et al.*, 2010; Selwyn *et al.*, 1998). In 2019, 208 000 of the approximately 1.4 million TB-related deaths were among HIV-positive individuals (World Health Organization, 2020).

The incidence of smear-negative (culture-negative, symptomatic) and subclinical (culture-positive, asymptomatic active) TB is also higher among co-infected patients. This leads to decreased sensitivity in screening tests and subsequent diagnostic delays, as the most widely applied TB diagnostic tests are based on sputum examination (Schutz *et al.*, 2010; Tiberi *et al.*, 2017). This manifests as higher mortality rates for smear-negative HIV-positive TB patients, the discovery of subclinical TB only after the initiation of antiretroviral therapy (ART) (Sterling *et al.*, 2010), and poor treatment outcomes for these patients. Therefore, it is recommended that all HIV-positive individuals are screened for *Mtb* infection, latent or otherwise (Tiberi *et al.*, 2017). TB can be cured in these individuals, although treatment is complicated by various factors (Tiberi *et al.*, 2017). Co-infected individuals also have an increased rate of recurrence, mainly due to reinfection as opposed to relapse (Charalambous *et al.*, 2008; Sonnenberg *et al.*, 2001).

Various aspects of the pathology of HIV infection, TB, and HIV/TB co-infection remain incompletely understood, which hampers the development of improved or novel diagnostic and management strategies. There is a need to better understand and characterise the complex immune and metabolic

changes induced by co-infection since many deaths can be prevented with timely diagnosis and treatment.

2.6.2 HIV/TB co-infection and cytokines

HIV and *Mtb* display the same outcome in their interactions with the host: effective replication without killing the host for as long as possible, to improve the chances of transmission to a new host and perpetuation of this process. During co-infection, the core of the synergism, which amplifies the disease burden, lies in the deterioration of immunological functions (Pawlowski *et al.*, 2012; Sharan *et al.*, 2020). The overall immune activation is worse in patients with HIV/TB co-infection relative to those with TB only (Vanham *et al.*, 1996). The variability in the cytokine literature on HIV infection and TB makes a direct comparison of findings between the disease states difficult, but it is clear that in neither of these studies a clear Th1 or Th2 shift is present, and that the Th1/Th2 hypothesis is indeed a gross oversimplification of helper cell dynamics during infection. However, a predominant Th2 response is typically associated with disease progression in both HIV infection and TB (Gourgouillon *et al.*, 2012; Ruhwald & Ravn, 2009; Williams *et al.*, 2013). From the studies discussed in Sections 2.4.2 and 2.5.2, HIV infection primarily affected IL-6 and IL-10, and IL-17 to some extent, whereas TB affected IFN- γ , TNF- α , IL-6, and IL-17. In multiple cases, alterations to the same cytokines are found in both HIV infection and TB, but these changes are not always statistically significant for both diseases. Statistical significance depends on multiple factors and complicates the identification of areas of similarity and difference. Although many other cytokines and chemokines are affected by these diseases and may be affected during dual infection, the above-mentioned cytokines are those of interest to this study. As with HIV infection and TB, there is a plethora of cytokine literature related to HIV/TB co-infection, but only blood-based studies measuring multiple cytokines without antigen stimulation will be discussed here, and are summarised in Table 2.6.

The studies of Subramanyam *et al.* (2004) and Benjamin *et al.* (2013) are similar in design. These studies used ELISA-based assays to measure cytokine levels in the plasma of healthy controls, HIV-negative individuals with active TB (mono TB), HIV-positive individuals without TB (mono HIV), and HIV/TB co-infected individuals. None of the infected patients had been treated for HIV or TB. Both of these studies measured IL-10, and IFN- γ , while IL-18 was additionally measured by Subramanyam *et al.* (2004) and IL-2, IL-4, IL-12 P70, and TNF- α by Benjamin *et al.* (2013). Subramanyam *et al.* (2004) noted that IL-10 varied within groups but did not find a significant difference in IL-10 between groups. The authors ascribed the finding to the healthy controls originating from a region where various parasitic, helminthic, and tropical diseases are endemic and may have been latently infected with *Mtb*, as they were workers at a TB hospital. Benjamin *et al.* (2013) additionally indicated that the levels of IL-10 were similar between mono HIV and HIV/TB co-infected patients, while it was elevated in the latter group compared to both healthy controls and mono TB patients.

Table 2.6: Studies investigating the inherent blood-based cytokine profile of HIV/TB co-infected individuals, in a multiplexed manner.

Research Model	Cytokines targeted	Analytical method	Groups compared	Statistical approaches	Reference(s)
PBMC supernatant and plasma	IL-10, IL-12, IL-18 and IFN- γ	ELISA	HIV-/TB-/Tn- HIV+/TB-/Tn- HIV-/TB+/Tn- HIV+/TB+/Tn-	ANOVA, t-test	Subramanyam <i>et al.</i> (2004)
Plasma	IL-12 P70, IL-2, IL-4, IL-10 TNF- α and IFN- γ	ELISA	HIV-/TB-/Tn- HIV+/TB-/Tn- HIV-/TB+/Tn- HIV+/TB+/Tn-	Mann-Whitney U, t-test	Benjamin <i>et al.</i> (2013)
Plasma	43 cytokines/chemokines, including IFN- γ , TNF- α , IL-2, IL-4, IL-6, IL-10, and IL-17	Milliplex® MAP Human Cytokine/Chemokine 41-plex kit and Bio-Rad Bioplex Pro Human Cytokine 2-plex kit (Luminex technology)	HIV+/TB/Tn $\pm^{**}$ HIV+/TB+/Tn $\pm^{**}$	Mann-Whitney U, random forest modelling, machine learning prediction using SuperLearner, AUC of ROC	Farr <i>et al.</i> (2018)
Plasma	IL-1 α , IL-1 β , IL-6, IL-12 P40, IL-12 P70, TNF- α , IFN- α 2, IL-8, IP-10, MCP-1, MCP-3, MIP-1a, MIP-1 β , IFN- γ , IL-4, IL-15, IL-17 α , IL-10, IL-1RA, VEGF	Milliplex® MAP Human Cytokine/Chemokine Panel I and IV (Luminex technology) and ELISA	HIV-/TB+/ATT+ and HIV+/TB+/ATT+: culture conversion vs non-conversion	Logistic regression, AUC of ROC, Cox proportional hazards, ANOVA, Kruskal-Wallis test	Rambaran <i>et al.</i> (2021)
Plasma	38 cytokines/chemokine panel, including IFN- γ , TNF- α , IL-6, IL-10, and IL-17A	Milliplex® MAP Human Cytokine/Chemokine kits, Bio-Rad Bioplex kits (Luminex technology), and ELISA	Discovery cohort: HIV+/TB-/ATT-/ART $\pm^*$ HIV+/TB-/ATT+/ART $\pm^*$ Validation cohort: HIV+/TB+/Tn $\pm$ HIV+/TB-/Tn $\pm$	Mann-Whitney U and Kruskal-Wallis, HCA, FC, Spearman's rank, ANOVA, AUC of ROC, network analysis	Du Bruyn <i>et al.</i> (2021)

Research model: PBMC: peripheral blood mononuclear cell.

Cytokines targeted: IL: interleukin; TNF: tumour necrosis factor; IFN: interferon; MCP: monocyte chemoattractant protein; RA: receptor antagonist; IP: human interferon-inducible protein; MIP: macrophage inflammatory protein; VEGF: vascular endothelial growth factor-C.

Analytical apparatus: ELISA: enzyme-linked immunosorbent assay; ICS: intracellular cytokine staining; xMAP: multi-analyte profiling.

Groups compared: HIV: human immunodeficiency virus; TB: tuberculosis; Tn: treatment; ATB: active pulmonary tuberculosis; +: positive; -: negative; $\pm$: some patients positive and some negative; *: longitudinal study; **: 65% of patients were on ART; TST: tuberculin skin test; ART: antiretroviral therapy; ATT: anti-tuberculosis treatment.

Statistical approaches: AUC of ROC: area under the curve of receiver operating characteristic; ANOVA: analysis of variance; HCA: hierarchical cluster analysis; FC: fold change.

Subramanyam *et al.* (2004) found that IFN- γ was elevated in HIV/TB co-infected patients, mono HIV, and mono TB patients relative to healthy controls. When stratified based on CD4 T cell count, HIV/TB co-infected patients with CD4 T cell counts below 200 cells/ml had significantly higher IFN- γ levels than the mono HIV and mono TB patients. HIV/TB co-infected patients with CD4 T cell counts above 200 cells/mm³, had IFN- γ levels lower than mono HIV, but higher than mono TB patients. While IFN- γ was also higher in HIV/TB co-infected patients and mono TB patients, it was decreased in mono HIV patients relative to healthy controls in the cohort of Benjamin *et al.* (2013). In both these studies, however, the IFN- γ levels of HIV/TB co-infected patients were intermediate, between those of individuals with mono HIV or TB.

Benjamin *et al.* (2013) used the IFN- γ /IL-10 ratio as an indicator of Th1/Th2 skewness. An increased ratio suggested Th1 predominance in mono TB patients, while a decreased ratio suggested Th2 predominance in patients with mono HIV. The ratio for HIV/TB co-infected patients was intermediate (although much closer to the TB ratio, indicating some skewness towards Th1). The authors suggested a compromised pro-inflammatory environment during HIV/TB co-infection. The results of Subramanyam *et al.* (2004) are also suggestive of a Th1 predominance, especially when HIV infection is advanced in co-infected patients. However, this is an oversimplification, as discussed earlier, and in light of the pleiotropic nature of cytokines, cannot be a definite conclusion.

Farr *et al.* (2018), Rambaran *et al.* (2021), and Du Bruyn *et al.* (2021) used multiplexed kits to analyse more than 20 cytokines and chemokines at once, the latter two studies supplementing these kits with ELISA assays for specific cytokines/chemokines not included in the multiplexed kits. Given the difficulties in diagnosing TB in HIV-positive individuals, the aim of Farr *et al.* (2018) was to identify inflammatory markers that can distinguish between HIV-positive individuals with and without TB. The combination of IL-6 and IFN- γ was found to have the highest discriminatory power, while the authors stressed that inclusion of IFN- γ induced protein 10 (IP-10, also known as CXCL-10) or an alternative indicator of IFN- γ activity, such as monokine induced by IFN- γ , must be included when biomarker panels are constructed. This is based on the strong performance of IP-10 in their study as well as previous studies (Farr *et al.*, 2018).

The importance of IP-10 in TB was further demonstrated when Rambaran *et al.* (2021) found that increased levels of this chemokine were associated with early bacterial clearance after two months of anti-TB treatment in mono TB and HIV/TB co-infected patients (the latter after adjusting for covariates). Furthermore, increased IL-6 was associated with faster bacterial clearance and cavitary disease (an indication of TB severity) highlighting how a single cytokine can induce protective immune responses, but also contribute to immunopathology (Rambaran *et al.*, 2021).

Although Farr *et al.* (2018) and Rambaran *et al.* (2021), both included IL-17 in their multiplexed panels, only Du Bruyn *et al.* (2021) found this cytokine to have significance in the HIV/TB co-infection context. Varied findings may be ascribed to cohort differences. In addition, the statistical approach was focused on network analysis, rather than discrimination of groups or simpler correlations. IL-17 was found to have the highest nodal connectivity in HIV/TB co-infected patients compared to those with mono TB and in ART-naïve HIV/TB co-infected patients compared to ART-experienced co-infected patients. Furthermore, VL was found to drive network connectivity in co-infected patients, thus mediating ongoing systemic inflammation, which is reversed with ART. Increased nodal connectivity of IL-17 was not associated with TB severity but was associated with increased mortality in co-infected patients. These results imply that IL-17 plays an important role in HIV-induced immune activation and inflammation, especially in viremic patients and those with more rapid progression (Du Bruyn *et al.*, 2021).

The studies discussed here underscore the importance of IFN- γ (and the chemokines it induces), IL-10, IL-6, and IL-17 in the immune response during HIV/TB co-infection. Thus, representatives from the Th1, Th2 and Th17 cytokine groups are important in the context of co-infection, highlighting the complex interplay of immune functions during concomitant chronic infections. These cytokines are important in HIV infection and TB, respectively (mentioned at the start of this section), indicating that the HIV/TB co-infected cytokine profile reflects the immune response to both HIV infection and *Mtb*.

2.6.3 HIV/TB co-infection and metabolism

During HIV and *Mtb* infection, the collective actions of the innate and adaptive immune responses result in a concentrated effort to eliminate the pathogenic threats (Kwan & Ernst, 2011). A state of chronic inflammation is established (Aounallah *et al.*, 2016; Semba *et al.*, 2010) and the balance between the immune system and metabolism is disrupted. This is mainly due to the significant energy demands and biosynthesis required to mount and sustain an immune response (Aounallah *et al.*, 2016). As such, the infected host experiences anorexia via effects on the hypothalamus (Gautron & Laye, 2010), which collectively results in a negative energy balance, and the consequent utilisation of stored fat and eventually protein (from muscle tissue) (Powanda & Beisel, 2003) for energy. This often culminates in malnutrition and the condition known as cachexia (Moldawer & Sattler, 1998), an established feature of both HIV infection and TB. Consequently, there is an inability to control infection, resulting in increased mortality (Paton *et al.*, 2006; Zachariah *et al.*, 2002).

To date, metabolic changes during HIV/TB co-infection have typically been measured by conventional assays [such as resting energy expenditure measurement by indirect calorimetry (Melchior *et al.*, 1991; Schwenk *et al.*, 2003), body composition measurement by dual-energy X-ray absorptiometry (Meininger *et al.*, 2002; Schwenk *et al.*, 2003) and nitrogen flux by the administration of, for example, a glycine isotope (Paton *et al.*, 2003)]. Comparing the findings of studies on HIV infection and TB using these techniques,

HIV infection was characterised by alterations in energy, glucose, lipid, and protein metabolism (Salas-Salvado & Garcia-Lorda, 2001), while active TB was characterised by altered protein metabolism (MacAllan *et al.*, 1998), low serum cholesterol (Taylor & Bamgboye, 1979), and glucose intolerance (Oluboyo & Erasmus, 1990). In HIV/TB co-infected patients, protein metabolism was mainly affected. The net protein balance in the fed state was shown to be impaired, compared to a strongly anabolic protein balance in those with HIV infection or TB only (Paton *et al.*, 2003). Serum albumin levels serve as indicators of protein status. HIV/TB co-infected patients were shown to suffer from hypoalbuminemia relative to TB patients (Van Lettow *et al.*, 2003). The distribution and magnitude of body composition changes were not altered in HIV/TB co-infection relative to that of TB patients (Paton & Ng, 2006).

It is worth mentioning that the metabolic characteristics of HIV/TB co-infection derived from conventional methods mainly focused on the synergistic effects of HIV infection, TB, and malnutrition, which together have been termed “triple trouble” with reference to the public health term “double trouble” when referring to HIV/TB co-infection (Van Lettow *et al.*, 2003). The risk of malnutrition (Swaminathan *et al.*, 2008) and the severity of wasting (Lucas *et al.*, 1994; Villamor *et al.*, 2006) are exacerbated during HIV/TB co-infection relative to either disease state alone, although the mechanisms underlying this are not fully understood. HIV infection is associated with the loss of gut mucosal integrity. The CD4 T cells of the gut mucosa are preferentially depleted, leading to impaired immune function in the gut (Ibarrondo *et al.*, 2013; Sharan *et al.*, 2020). This leads to a “leaky gut” and the translocation of gut microbial products into the blood, which exacerbates systemic immune activation (Brenchley *et al.*, 2006; Sharan *et al.*, 2020). Furthermore, it is well established that HIV induces changes in the gut microbiota composition (Vujkovic-Cvijin & Somsouk, 2019). How these processes are affected by *Mtb* during HIV/TB co-infection are not yet clear (Waters *et al.*, 2020), although it is known that patients with TB also have an altered gut microbiota composition (Hu *et al.*, 2019; Luo *et al.*, 2017; Winglee *et al.*, 2014).

The effects of HIV infection on the gut and the high energy requirement during chronic infection culminates in micronutrient deficiencies [reviewed in detail by Semba *et al.* (2010)] and alterations to the macronutrient status (e.g. increased utilisation of protein and fat for energy). Micronutrients have major roles in maintaining homeostasis. Deficiencies have far-reaching complications for the immune system and metabolism (Katona & Katona-Apte, 2008), of which tracing the source might be extremely difficult due to the confounding effect of the inflammatory state and the inherent interconnectedness of metabolism.

Although aspects of metabolism and nutrition improve by treating HIV-positive or TB patients with ART and anti-TB drugs, respectively (Drain *et al.*, 2007; Kennedy *et al.*, 1996), these treatments do not restore patients to a healthy state. HIV-positive individuals often still suffer from wasting even after the initiation of ART (Tang *et al.*, 2002). ART also induces metabolic alterations separate from HIV infection (Jain *et al.*, 2005; Van der Valk *et al.*, 2002), while anti-TB treatment fails to completely recover protein reserves

(Onwubalili, 1988; Schwenk *et al.*, 2004) and induces OS (Sodhi *et al.*, 1997). These effects are reflected in the metabolomics literature for HIV infection (Babu *et al.*, 2019; Hewer *et al.*, 2006; Li *et al.*, 2018) and TB (Combrink *et al.*, 2019; Luies *et al.*, 2017b; Opperman *et al.*, 2021). The causes of malnutrition and wasting in HIV-positive and TB patients are multifactorial and are influenced by disease progression. It is known that the development and degree of HIV-induced wasting is influenced by factors such as CD4 T cell count (Forrester *et al.*, 2001), VL (Lyles *et al.*, 1999; Zackin *et al.*, 1999), inflammatory markers [reviewed by Chang *et al.* (1998)], and nutrition [reviewed by Koethe and Heimbürger (2010)]. The role of these factors and the HIV-induced gut complications mentioned above, in the co-infected individual, remains open for investigation.

The signalling pathways involved in pathogenic control are closely involved in the regulation of nutrients and metabolic homeostasis (Aounallah *et al.*, 2016) and are mediated by cytokines, chemokines, and various reactive oxygen species (ROS). Besides directly damaging the invading microbe, ROS stimulates the production of pro-inflammatory cytokines, which exacerbates the inflammatory state and perpetuates immunometabolic alterations (Zhang *et al.*, 2018). Increased levels of ROS and pro-inflammatory cytokines are the hallmarks of chronic inflammation (Awodele *et al.*, 2012), and have been studied in HIV infection (Williams *et al.*, 2013), TB (Yao *et al.*, 2017), and co-infection (Verma *et al.*, 2018). Although these interactions have been linked to various metabolic changes and are more pronounced during co-infection (Awodele *et al.*, 2012; Madebo *et al.*, 2003), these are complex and requires further investigation (Zhang *et al.*, 2018).

No single factor can be attributed to the cause of the co-infected phenotype as the interactions between the host and the two pathogens are multidirectional: either oppositional or synergistic and to various degrees. Metabolomics has aided in better understanding HIV infection and TB, respectively (Section 2.4.3 and 2.5.3). Metabolomics, however, has not yet been systematically applied to characterise the co-infected state. Given that conventional assays are less sensitive, less specific, and laborious to perform (Williams *et al.*, 2011), researchers have turned to newer, high-throughput omics techniques to better match the dimensionality of the problem to the data obtained and thus allow for a more comprehensive profile of host metabolism, as it relates to HIV/TB co-infection.

2.6.3.1 HIV/TB co-infection and metabolomics

Only three metabolomics investigations that study a very specific aspect of HIV/TB co-infection have been published to date (Table 2.7). To the best of our knowledge, no metabolomics study has hitherto characterised the HIV/TB co-infected metabolic profile, in an explorative context, relative to healthy individuals or either HIV-positive or TB-positive individuals, or in terms of a treatment-associated profile.

Table 2.7: Metabolomics studies published to date investigating a specific aspect of HIV/TB co-infection.

Research Model	Aim	Metabolome fraction targeted	Analytical apparatus	Groups compared	Statistical approaches	Reference(s)
Human: plasma	To investigate whether IDO1 activity (measured as Trp/ Kyn) could be used as a diagnostic/predictive marker of TB in HIV-positive individuals	Tryptophan and kynurenine	UPLC-MS	*HIV+/TB+/ART+ *HIV+/TB+/ART+/ATT+ HIV+/TB-/pneumonia+	t-test, Mann-Whitney U, Kruskal-Wallis, AUC of ROC, Spearman correlations	Adu-Gyamfi <i>et al.</i> (2017)
Human: plasma	To investigate whether individuals with and without TB-IRIS could be distinguished based on metabolic differences and if these differences are detectable before the onset of TB-IRIS	Total metabolome	LC-MS	HIV+/TB+/ART+/non-IRIS HIV+/TB+/ART+/IRIS+	t-test with Bayesian adjustment, FC, PCA, pathway analysis in MetaboAnalyst, Lasso regression models	Silva <i>et al.</i> (2019)
Human: plasma	To determine whether tryptophan metabolism is among the highest regulated metabolic pathways in the response of the host to <i>Mtb</i> infection, TB, and treatment	Total metabolome	LC-MS, MS/MS	HIV-/LTBI- (healthy controls) *HIV-/LTBI+/TB-/DOTS+ *HIV-/TB+/DOTS+ *HIV-/MDR-TB+/MDR-Tn+ *HIV+/MDR- TB+/MDR-Tn+	Wilcoxon rank, AUC of ROC, pathway enrichment analysis	Collins <i>et al.</i> (2020)

Aim: IDO 1: indoleamine 2,3-dioxygenase 1; Kyn: kynurenine; Trp: tryptophan; TB: tuberculosis; IRIS: immune reconstitution inflammatory syndrome.

Analytical apparatus: (UP)LC-MS: (ultra-performance) liquid chromatography-mass spectrometry; MS/MS: tandem mass spectrometry.

Groups: HIV: human immunodeficiency virus; TB: tuberculosis; ART: antiretroviral treatment; ATT: anti-TB treatment; IRIS: immune reconstitution inflammatory syndrome; +: positive; -: negative; *: longitudinal study; LTBI: latent mycobacterium tuberculosis infection; DOTS: directly observed treatment, short-course; MDR: multidrug-resistant tuberculosis; MDR-Tn: treatment for MDR-TB.

Statistical approaches: AUC of ROC: area under the curve of the receiver operating characteristic; FC: fold change; PCA: principal component analysis.

The optimal physiological function is reliant on a balanced tryptophan metabolism (Wang *et al.*, 2021b), as the immune, central nervous, and reproductive systems respond to alterations in its catabolic flux (Ball *et al.*, 2014). The enzymes tryptophan 2,3-dioxygenase and IDO1 catabolise tryptophan via the kynurenine pathway, producing a range of immunomodulatory and neuroactive catabolites. IDO1 activity in particular has been widely investigated for its role in immune tolerance and suppression (Wang *et al.*, 2021b). Increased IDO1 activity, induced by IFN- γ (Katz, 2008), and the resulting alterations to the kynurenine/tryptophan (K/T) ratio have been investigated as biomarkers for various disease states (Li *et al.*, 2019; Mancuso *et al.*, 2015; Pett *et al.*, 2017; Sucher *et al.*, 2010). This includes HIV infection and

TB measured conventionally (Almeida *et al.*, 2009; Dagenais-Lussier *et al.*, 2016; Gautam *et al.*, 2018; Gostner *et al.*, 2015; Jenabian *et al.*, 2013; van Laarhoven *et al.*, 2018) and via metabolomics (Isa *et al.*, 2018; Li *et al.*, 2018; Luies & Loots, 2016; Peltenburg *et al.*, 2018; Sitole *et al.*, 2019; Weiner *et al.*, 2012).

Of the three studies mentioned, two investigated the effects of IDO1 activity on host metabolism during HIV/TB co-infection. Adu-Gyamfi *et al.* (2017) monitored HIV-positive individuals longitudinally for the development of active TB. At the time of diagnosis, IDO1 activity was higher in those individuals who developed TB compared to those who did not, an effect that was reversed after six months of anti-TB treatment. The IDO1 activity of HIV/TB co-infected individuals was also four-fold higher when compared to that of HIV-positive individuals with confirmed pneumonia. Collins *et al.* (2020) confirmed that individuals with HIV/TB co-infection have higher IDO1 activity compared to those with a single disease. Collins *et al.* (2020) further found that HIV-positive individuals with MDR-TB also had an increased K/T ratio relative to those with TB alone. After receiving treatment for MDR-TB for two years, there was no significant change in the K/T ratio, which is in concurrence with a slower bacterial clearance rate in MDR-TB patients (Collins *et al.*, 2020). Indoleamine 2,3-dioxygenase 2 (IDO2) plays a smaller role in tryptophan catabolism and has been suggested to promote pro-inflammatory B cell responses, as opposed to the immunosuppressive effects of IDO1, through the induction of T regulatory cell responses (Manches *et al.*, 2008) and other mechanisms (Mellor *et al.*, 2002; Merlo *et al.*, 2020; Munn *et al.*, 1999). The levels of *IDO2* transcripts is higher during co-infection compared to individuals with TB alone, suggesting that HIV infection or the co-infected state causes increased production of IDO2 in addition to IDO1 (Collins *et al.*, 2020). These studies indicate that increased IDO activity is significantly more pronounced in the HIV/TB co-infected state and that the K/T ratio may be a suitable biomarker for TB in HIV-positive patients (Adu-Gyamfi *et al.*, 2017) and a possible target for host-directed therapies (Collins *et al.*, 2020). Studies are needed to evaluate whether the significantly increased IDO1 activity contributes to the increased TB progression risk in HIV-positive individuals (Collins *et al.*, 2020; Pawlowski *et al.*, 2012) and those studies comparing HIV-positive individuals with other secondary (specifically respiratory) diseases as diseased controls to validate this are also warranted. Diagnostically, it would be ideal to include both HIV-negative TB-positive patients and HIV-positive TB-negative patients as controls in a single study.

Within the context of co-infection, ART is typically initiated based on a patient's immunological status. Patients with $CD4 < 50$ cells/mm³ or significantly advanced disease are prescribed to start ART within two weeks of starting anti-TB treatment, while those with an improved immunological profile ($CD4 > 50$ cells/mm³) start ART two to eight weeks after anti-TB treatment initiation. These guidelines have been proposed in response to the development of an aberrant immune response (immune reconstitution inflammatory syndrome; IRIS) in HIV/TB co-infected patients when ART is initiated early in the course of anti-TB treatment (Manosuthi *et al.*, 2012; Török *et al.*, 2011; World Health Organization, 2016b). IRIS is characterised by a severe inflammatory response that is triggered against a pre-existing condition in HIV-positive individuals shortly after the initiation of ART. When this reaction is directed

against *Mtb*, it is referred to as TB-IRIS. It may be transient and resolve on its own, but it may also be fatal. Since there are currently no diagnostic tests for TB-IRIS, its diagnosis is based on clinical presentation and medical history (Pozniak *et al.*, 2011; Tiberi *et al.*, 2017). Considering this, Silva *et al.* (2019) collected plasma samples from HIV/TB co-infected individuals after the initiation of ART and as close as possible to the onset of IRIS (at a time point compared to this for those who did not develop IRIS). Those individuals who developed IRIS experienced increased arachidonic acid (most notably increased hydroxyeicosatetraenoic acids) and glycerophospholipid metabolism relative to the non-IRIS group at the start of the study, whereas altered sphingolipid and linolenic acid metabolism distinguished the groups during the IRIS window. This study showed that altered polyunsaturated fatty acid (PUFA) metabolism distinguished patients who develop IRIS from those who do not. Since IRIS involves an acute inflammatory response these results are expected, as PUFAs give rise to various inflammatory mediators. This study too underscores the reflection of immunological changes in the metabolism and its potential prognostic application.

The studies reviewed here stress the many gaps that exist in our knowledge of HIV/TB co-infection, including an incomplete understanding of the mechanisms underlying diagnostic problems, the effects of ART and anti-TB treatment on the host, recurrent TB, and the effect of *Mtb* on the gut mucosal damage induced by HIV. These studies also highlight the potential of metabolomics to address such gaps. Comparing the existing HIV infection and TB metabolomics profiles (Tables 2.8–2.10) in the absence of a comprehensive HIV/TB co-infection profile may yield insight into the mechanisms of what is known about host metabolism during HIV/TB co-infection and inform the design of future metabolomics studies.

2.6.3.2 Comparing HIV infection and TB metabolic profiles

To date, metabolomics has been used to investigate various aspects of HIV infection and TB, using various sample matrices, which can be used as a guide in terms of what to expect during co-infection. HIV infection metabolomics studies have often been concerned with characterising the disease state, prognosis, and the effect of ART, using blood-based (serum or plasma), CSF, cell culture, urine, bronchoalveolar lavage fluid (BALF), oral wash/saliva, and tissue samples (Williams *et al.*, 2017). Within the TB metabolomics literature, blood-based, urine, sputum, tissue, pleural effusion, and cell culture samples have been used, with a focus on biomarker discovery for diagnosis and treatment outcome. As *Mtb* has its own metabolism, unlike HIV, bacterial culture metabolomics has also been extensively used to characterise the mycobacterium's metabolic adaptations (Du Preez *et al.*, 2019).

The normal course of the interactions between the immune, metabolic, and endocrine systems is distorted due to the chronic nature of immune activation and inflammation during HIV and *Mtb* infection. At the time of diagnosis, when treatment-naïve samples are typically collected, overlapping biochemical processes have already been set in motion and become so interconnected that no single measure of

causality can be attributed. This makes it difficult to interpret metabolomics data. Despite this, and although the trends are not always consistent, an overlapping pattern of metabolites frequently altered during HIV infection and TB can be identified and linked to known effects of these infections. Tables 2.8–2.10 compare the serum, plasma and urine metabolomics profiles of untreated HIV-positive and TB patients (where these patients were compared to healthy controls), respectively. Other sample matrices were not compared in this way as there is very little overlap in the use of matrices other than blood and urine between the two disease states. In cases where a metabolite was found to be significantly altered during HIV infection and/or TB, but where the trend was inconsistent, the metabolite is marked as “altered”, instead of “increased” or “decreased”.

Table 2.8: A comparison of the findings of HIV infection and TB serum metabolomics investigations.

Similarities		
Metabolic alteration	Proposed origin(s) of metabolic alterations*	Reference(s)
Decreased tryptophan and increased levels of its catabolic products (e.g. kynurenine)	Increased IDO activity	HIV: Sitole <i>et al.</i> (2019) TB: Weiner <i>et al.</i> (2012)
Altered indole-3-acetic acid	Uremic cytotoxic activity, gut microbes	HIV: Williams <i>et al.</i> (2011) TB: Weiner <i>et al.</i> (2018)
Increased glutamate	<i>Mtb</i> nitrogen balance, redox homeostasis, and survival mechanisms. Immune activation and oxidative stress of the host	HIV: Sitole <i>et al.</i> (2019) TB: Cho <i>et al.</i> (2020); Zhou <i>et al.</i> (2013)
Increased aspartate	Immune activation and oxidative stress	HIV: Sitole <i>et al.</i> (2019) TB: Cho <i>et al.</i> (2020); Weiner <i>et al.</i> (2012)
Altered phenylalanine and tyrosine	Decreased activity of phenylalanine hydroxylase	HIV: Sitole <i>et al.</i> (2019) TB: Che <i>et al.</i> (2013); Weiner <i>et al.</i> (2012); Zhou <i>et al.</i> (2013)
Decreased alanine	Energy production	HIV: Sitole <i>et al.</i> (2019) TB: Zhou <i>et al.</i> (2013)
Altered pyroglutamic acid/5-oxoproline	Mitochondrial dysfunction, glutathione deficiency (i.e. oxidative stress), or reduced glutathione cycling	HIV: Williams <i>et al.</i> (2011) TB: Che <i>et al.</i> (2013); Weiner <i>et al.</i> (2012)
Altered phospholipids	<i>Mtb</i> -induced inhibition of phospholipase A2, lipolysis in the granuloma, inflammation, and immune activation	HIV: Williams <i>et al.</i> (2011) TB: Weiner <i>et al.</i> (2018)
Increased fatty acids and/or their catabolic products	Inflammation and immune activation, mitochondrial dysfunction	HIV: Williams <i>et al.</i> (2011) TB: Weiner <i>et al.</i> (2018)
Decreased creatine		HIV: Sitole <i>et al.</i> (2019) TB: Weiner <i>et al.</i> (2018)

Differences					
HIV infection	Proposed origin(s) of metabolic alterations	Reference(s)	TB	Proposed origin(s) of metabolic alterations	Reference(s)
			Altered urea cycle intermediates		Che <i>et al.</i> (2013); Weiner <i>et al.</i> (2012)
			Decreased methionine, increased oxidative products of methionine	Oxidative stress	Cho <i>et al.</i> (2020); Weiner <i>et al.</i> (2012)
			Increased fibrinopeptides	Tuberculous granulomas	Weiner <i>et al.</i> (2012)

**Observed trends are not always attributed to a specific mechanism by the investigators. Thus, metabolite changes may overlap between the HIV infection and TB metabolic profiles, but the proposed mechanism given in the table may represent only that given in either the HIV or TB literature. IDO: indoleamine 2,3-dioxygenase.*

Table 2.9: A comparison of the findings of HIV infection and TB plasma metabolomics investigations.

Similarities		
Metabolic alteration	Proposed origin(s) of metabolic alterations	Reference(s)
Decreased tryptophan and increased levels of its catabolic products (e.g. kynurenine)	Increased IDO activity	HIV: Li <i>et al.</i> (2018); Peltenburg <i>et al.</i> (2018) TB: Collins <i>et al.</i> (2020); Vrieling <i>et al.</i> (2019); Vrieling <i>et al.</i> (2018)
Altered glutamine and/or glutamate	Involved in multiple overlapping metabolic pathways	HIV: Munshi <i>et al.</i> (2013); Tarancon-Diez <i>et al.</i> (2019) TB: Ding <i>et al.</i> (2020); Vrieling <i>et al.</i> (2019); Vrieling <i>et al.</i> (2018)
Increased aspartate	Dysregulated urea cycle, involved in many other metabolic processes	HIV: Munshi <i>et al.</i> (2013); Scarpellini <i>et al.</i> (2016) TB: Ding <i>et al.</i> (2020); Vrieling <i>et al.</i> (2019)
Dysregulation of the urea cycle (decreased ornithine and related metabolites which do not correspond exactly)	Dysregulation of amino acid metabolism by various mechanisms	HIV: Peltenburg <i>et al.</i> (2018) TB: Vrieling <i>et al.</i> (2019)
Decreased alanine	Wasting/cachexia	HIV: Peltenburg <i>et al.</i> (2018) TB: Vrieling <i>et al.</i> (2019); Vrieling <i>et al.</i> (2018)
Decreased histidine	Oxidative stress and/or inflammation	HIV: Li <i>et al.</i> (2018) TB: Ding <i>et al.</i> (2020); Vrieling <i>et al.</i> (2019); Vrieling <i>et al.</i> (2018)
Altered choline		HIV: Munshi <i>et al.</i> (2013) TB: Vrieling <i>et al.</i> (2019)
Increased sarcosine	Muscle wasting and folate deficiency during HIV	HIV: Munshi <i>et al.</i> (2013), Peltenburg <i>et al.</i> (2018) TB: Vrieling <i>et al.</i> (2019)

Differences					
HIV infection	Proposed origin(s) of metabolic alterations	Reference(s)	TB	Proposed origin(s) of metabolic alterations	Reference(s)
Altered BCAAs and their catabolic intermediates (↑ during early stages of infection, ↓ during later stages)	Immune activation, muscle catabolism (wasting)	Li <i>et al.</i> (2018); Peltenburg <i>et al.</i> (2018); Tarancon-Diez <i>et al.</i> (2019)	Altered phenylalanine		Ding <i>et al.</i> (2020); Vrieling <i>et al.</i> (2019); Vrieling <i>et al.</i> (2018)

*Observed trends are not always attributed to a specific mechanism by the investigators. Thus, metabolite changes may overlap between the HIV infection and TB metabolic profiles, but the proposed mechanism given in the table may represent only that given in either the HIV or TB literature. IDO: indoleamine 2,3-dioxygenase; BCAA: branched-chain amino acid; ↑: increased; ↓: decreased.

Table 2.10: A comparison of the findings of HIV infection and TB urinary metabolomics investigations. *

Similarities					
Metabolic alteration		Proposed origin(s) of metabolic alterations		Reference(s)	
Increased neopterin		Product of the catabolism of guanosine triphosphate in immune cells in response to interferon-gamma – indicative of a pro-inflammatory immune status.		HIV: Munshi <i>et al.</i> (2013) TB: Isa <i>et al.</i> (2018)	
Increased catecholamines (e.g. epinephrine, norepinephrine)		Various roles in the immune system and as neurotransmitters and hormones		HIV: Munshi <i>et al.</i> (2013) TB: Das <i>et al.</i> (2015)	
Differences					
HIV infection	Proposed origin(s) of metabolic alterations	Reference(s)	TB	Proposed origin(s) of metabolic alterations	Reference(s)
Increased isoleucine and methionine, decreased alanine	Immune activation, increased amino acid oxidation for energy	Munshi <i>et al.</i> (2013)*	Increased tryptophan catabolic products (e.g. kynurenine)	Increased IDO activity	Luies and Loots (2016), Isa <i>et al.</i> (2018)
Increased formic acid and decreased 2-methylglutaric acid	Altered central energy metabolism; pyruvate and TCA cycle metabolism		Increased intermediates of phenylalanine and tyrosine metabolism	Compromised insulin production and resulting in decreased PAH activity, altered gut microbial activity	Luies and Loots (2016); Das <i>et al.</i> (2015)
			Increased fatty acids and their catabolic products	Compromised insulin production, various bacterial mechanisms	Luies and Loots (2016)
			<i>Mtb</i> -specific metabolites	Cell wall components, intermediates of bacteria-specific metabolic pathways	Luies and Loots (2016)

*There is only one HIV metabolomics study on urine that focused on the characterisation of the disease state. IDO: indoleamine 2,3-dioxygenase 1; PAH: phenylalanine hydroxylase; TCA: tricarboxylic acid cycle.

Despite the potential for metabolic differences between serum and plasma (Liu *et al.*, 2018), the blood-based metabolomics investigations reviewed here show that there is significant overlap between the metabolic alterations, as induced by HIV infection and TB. For example, alterations to tryptophan and its catabolic products, glutamine and/or glutamate, aspartate, as well as alanine are commonly reported for HIV infection and TB in both serum and plasma (see “similarities” in Tables 2.8 and 2.9). Alterations to the following metabolites are similar (being frequently cited as statistically significant and, in some cases, trending in the same direction) between HIV infection and TB but occur only in either the reported serum or plasma metabolic profiles: indole-3-acetic acid, phenylalanine and tyrosine, pyroglutamic acid (also called 5-oxoproline), phospholipids, fatty acids, and creatine in serum (Table 2.8), as well as urea cycle intermediates (other than aspartate), histidine, choline, and sarcosine in plasma (Table 2.9).

The central role of amino acids in the functioning of the immune system and metabolism, especially during chronic infection, was discussed in Sections 2.4.3 and 2.5.3. Specifically, the relation of the oxidation of amino acids for energy, through mechanisms such as glutaminolysis, was highlighted. In addition to this, an imbalance in the ratio of glutamine and glutamate, as well as asparagine and aspartate, affects the immune-competence of T cells and macrophages (Yoneda *et al.*, 2009). Increased serum (Table 2.8) and plasma (Table 2.9) aspartate was reported during HIV infection (Munshi *et al.*, 2013; Scarpellini *et al.*, 2016; Sitole *et al.*, 2019). An increased aspartate/asparagine ratio was also observed in the serum (Table 2.8) and plasma (Table 2.9) of TB patients (Cho *et al.*, 2020; Ding *et al.*, 2020), confirming the finding of increased aspartate by Weiner *et al.* (2012). Aspartate forms part of the urea cycle, where it is combined with citrulline to form argininosuccinate by argininosuccinate synthase. In the liver mitochondria, aspartate is also produced from oxaloacetate by aspartate aminotransferase, with the concurrent conversion of glutamate to alpha-ketoglutarate (Watford, 2003). This highlights the interconnected nature of the observed alterations to glutamine/glutamate and aspartate in HIV infection and TB metabolomics. Since part of the urea cycle occurs in mitochondria, these alterations relate to mitochondrial dysfunction that has previously been associated with HIV infection (Pinti *et al.*, 2010) and TB (Luies, 2017; Luies *et al.*, 2017b). Alterations to the urea cycle were only reported for TB in serum (Table 2.8) but not for HIV infection. Similar urea cycle alterations were reported for both HIV infection and TB in plasma (Table 2.9).

Alterations to plasma phenylalanine are more commonly reported for TB than for HIV infection [see “similarities” (Table 2.8) and “differences” (Table 2.9)], with only one HIV serum investigation reporting on this metabolite (Table 2.8). These changes have been postulated to be related to decreased activity of phenylalanine hydroxylase, which converts phenylalanine to tyrosine (Ghannoum *et al.*, 2013), as a consequence of compromised insulin secretion (Luies & Loots, 2016). Inflammatory diseases (such as TB) are often associated with insulin resistance, as discussed in Section 2.5.3. One serum-based study (Che *et al.*, 2013) reported decreased phenylalanine, although it is typically reported to be increased in serum and urine (Luies & Loots, 2016; Vrieling *et al.*, 2018; Weiner *et al.*, 2012; Zhou *et al.*, 2013). Altered energy

regulation through the endocrine system contributes to the other metabolic alterations discussed here, as various enzymes are influenced by insulin (Rahmatabady, 2013). Furthermore, mitochondrial metabolism, which is modulated by HIV and *Mtb*, is central to coupling amino acids (such as glutamine, glutamate, and alanine) to insulin secretion (Newsholme *et al.*, 2007).

During gluconeogenesis, non-carbohydrate molecules are converted to glucose, an essential energy substrate for leukocytes (Newsholme & Newsholme, 1989) during times of decreased energy availability. In the liver, the glucose-alanine cycle functions as a gluconeogenesis pathway, using alanine as a substrate. The amino group of alanine is transaminated to a keto acid, and the resulting carbon skeleton is used in gluconeogenesis. This process contributes to the decreased levels of alanine (see “similarities” in Tables 2.8 and 2.9) that have been reported in both HIV infection (Peltenburg *et al.*, 2018; Sitole *et al.*, 2019) and TB (Vrieling *et al.*, 2019; Vrieling *et al.*, 2018; Zhou *et al.*, 2013). The decrease in alanine relates to cachexia in HIV-positive [as suggested by (McKnight *et al.*, 2014)], and TB patients, as a TB study that included lung cancer patients related aberrations in glucose and alanine metabolism to the degree of weight loss, rather than the presence of cancer (Leij-Halfwerk *et al.*, 2000). In other tissues, the carbon skeletons of amino acids (except for leucine and lysine) are also used for gluconeogenesis. Such oxidation of amino acids results in the need for increased ammonia secretion through the urea cycle (Rahmatabady, 2013), previously mentioned to be dysfunctional (due to mitochondrial dysfunction or other mechanisms).

Histidine is associated with anti-inflammatory and anti-oxidative processes (Li *et al.*, 2018), mainly through the actions of or effects on cytokines (Hasegawa *et al.*, 2012; Son *et al.*, 2005). As such, decreased levels of this essential amino acid have been associated with inflammatory diseases (Hisamatsu *et al.*, 2012; Yu *et al.*, 2015) including HIV infection (Li *et al.*, 2018) and TB (Vrieling *et al.*, 2019; Vrieling *et al.*, 2018; Weiner *et al.*, 2012) (see “similarities in Table 2.9). The levels of histidine were found to be significantly correlated with CD4 T cell count, which implies a role for this amino acid in CD4 T cell regeneration (Li *et al.*, 2018).

A metabolite directly associated with OS, which was common amongst HIV infection (Williams *et al.*, 2011) and TB (Che *et al.*, 2013; Weiner *et al.*, 2012) metabolomics studies, was pyroglutamic acid (also referred to as 5-oxoproline) (see “similarities” in Table 2.8). Decreased levels of this metabolite were discussed in Section 2.5.3 and are indicative of decreased antioxidant capacity in TB patients. The alterations observed for other amino acids in HIV infection, such as glutamate and aspartate, have also been associated with OS (Sitole *et al.*, 2019). In serum, decreased methionine and increased oxidative products of this amino acid (sulfoxymethionine) have been reported for TB (Ding *et al.*, 2020; Suzuki *et al.*, 2016), but not HIV infection (“differences”, Table 2.8). Decreased methionine has been reported for HIV infection in plasma (Peltenburg *et al.*, 2018) but this is an inconsistent finding. Based on previous reports (using conventional techniques), OS-associated alterations are worse during the co-infected state

(Awodele *et al.*, 2012; Madebo *et al.*, 2003). However, the mechanisms that give rise to these alterations may interact in more complex ways than that which would result in simple compounding of effects.

The role of the mitochondria in lipid biosynthesis and the role of lipids in immune cell remodelling with relation to HIV infection and TB were previously mentioned (Sections 2.4.3 and 2.5.3). As such, it is not surprising that alterations to phospholipids are a common finding in HIV infection and TB metabolic profiles (“similarities”, Table 2.8). Phosphatidylcholines (a class of phospholipids, Table 2.8) have also been implicated as markers of a more progressive/worsened outcome in HIV infection and TB. In a cohort of HIV-positive individuals on ART, a decrease in phosphatidylcholines predicted poor immunological recovery (Rosado-Sanchez *et al.*, 2019). Interestingly, these lipids were restored after one year of ART (Peltenburg *et al.*, 2018). These lipids, among other metabolites (see Section 2.5.3) also distinguished patients with MDR-TB from those with drug-susceptible TB (Huang *et al.*, 2020). Alterations to fatty acids were common between HIV infection and TB in serum (see “similarities” in Table 2.8). The importance of altered fatty acids and acylcarnitines during HIV infection was discussed in Section 2.4.3 [with reference to Li *et al.* (2018) and Peltenburg *et al.* (2018)] and the importance of fatty acids as indicators of TB was exemplified by the studies of Che *et al.* (2018) and Cao *et al.* (2021) in Section 2.5.3. Fatty acids have already been proven to be important in the context of HIV/TB co-infection, as PUFAs, glycerophospholipids, and sphingomyelins distinguished individuals who developed IRIS from those who did not (Silva *et al.*, 2019).

The metabolism of choline, sarcosine and creatinine is closely linked, as all three these metabolites are intermediates in pathways related to glycine metabolism. While the plasma levels of choline were increased during HIV infection (Munshi *et al.*, 2013), they were lower for TB patients and even more so in TB patients with DM (Vrieling *et al.*, 2019) (see “similarities in Table 2.9). Choline is a major component of phospholipids, and as such alterations in the levels of this metabolite is associated with membrane turnover (Munshi *et al.*, 2013), which is high during immune activation. Furthermore, choline is also a precursor for acetylcholine, a neurotransmitter, and has been suggested as a biomarker for the AIDS dementia complex (Munshi *et al.*, 2013). A gut microbe-related choline metabolite has also been related to carotid atherosclerosis progression in HIV-positive individuals (Shan *et al.*, 2018). Decreased levels of choline have been associated with non-alcoholic fatty liver disease due to decreased intake of choline and/or gut microbe dysbiosis (Leermakers *et al.*, 2015; Sharpton *et al.*, 2019). This trend had not been reported in TB patients before the study of Vrieling *et al.* (2019), who advised caution in the interpretation of this finding. Sarcosine is a downstream metabolite of choline and is converted to glycine. It is an important intermediate in one-carbon metabolism, which is involved in many physiological processes, including redox defence (notably through glutathione). Increased levels of plasma sarcosine (see “similarities” in Table 2.9) has been associated with folate deficiencies and muscle wasting during HIV infection (Munshi *et al.*, 2013), but this metabolic alteration has not been interpreted in the context of TB. However, as sarcosine is a product of muscle degradation and cachexia is a characteristic feature of TB

(Semba *et al.*, 2010), there may be a similar mechanism driving the increased levels of sarcosine. Glycine and arginine are precursors for creatine. The majority of all creatine in the body is in muscle, where it serves as the primary fuel to maintain ATP availability for cellular metabolism, especially during periods of high energy demand (Kreider & Stout, 2021). As such, the observed serum creatine in HIV infection (Sitole *et al.*, 2019) and TB (Weiner *et al.*, 2018) relates to the prolonged period of high energy expenditure and cachexia associated with these diseases (see “similarities” in Table 2.8). Another contributor to this alteration may be mitochondrial dysfunction, as creatine crosses the mitochondrial membrane as part of the creatine phosphate shuttle, which facilitates the replenishment of ATP (Kreider & Stout, 2021).

Considering the comparisons (Tables 2.8 and 2.9), the HIV/TB co-infected metabolic profile obtained from blood is likely to be characterised primarily by exacerbated amino acid alterations (especially the essential amino acids and those non-essential amino acids involved in immune function and redox balance such as glutamate and glutamine). There may also be lipid alterations (deduced from similar metabolic changes highlighted in Tables 2.8 and 2.9).

Figure 2.2 represents the overlapping metabolic alterations and maps these changes and their interpretations, which have been identified by HIV infection and TB metabolomics, separately, onto what is known about host metabolism during the co-infected state (dark purple arrow). It shows that HIV infection and TB (to the left and right of the Venn diagram, respectively) is associated with constant immune activation and inflammation as well as alterations to the gut microbiome (metabolites 1 and 2), which increases the host energy expenditure as immune cells differentiate (reflected by metabolites 3–14) and pathogens carry out biosynthetic processes (metabolites 3 and 10). This adds to the oxidative profile (metabolites 2–4, 6, 7 and 14), impacting mitochondrial structure and function (metabolites 5, 9, 11 and 14). Part of the urea cycle occurs in mitochondria, and since this organelle’s structure and function is impacted, there is reduced clearance of ammonia, which translates to urea cycle alterations. In this chronic state of immune activation and inflammation, the individual experiences malaise and anorexia, leaving a malnourished individual who eventually suffers from cachexia (metabolites 7, 13 and 14). Dysfunctional mitochondria impact lipid metabolism reflected as changes in phospholipids, choline, sarcosine and fatty acids — all with interrelated and sometimes compounding effects on the immune system, inflammation and biosynthetic processes. Given that these changes occur during HIV infection and TB respectively, it is highly likely that they will be detected in co-infected samples (represented by the overlapping Venn diagram) and that more quantitative versus qualitative differences will reflect.

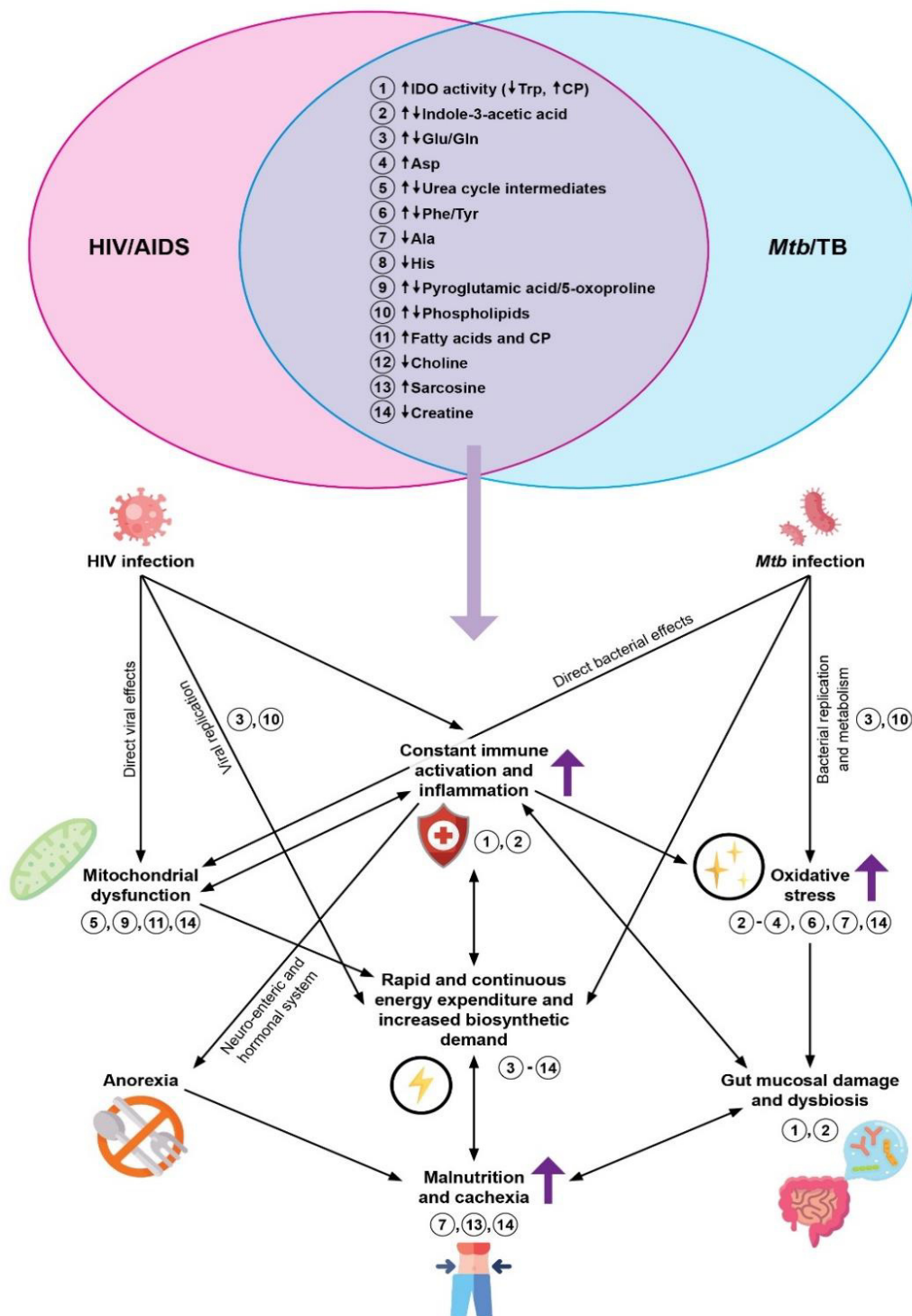


Figure 2.2: The proposed metabolic effects of HIV/TB co-infection, based on the overlapping findings of prior blood-based HIV infection and TB metabolomics studies (as indicated by the Venn diagram at the top). The dark purple arrows represent effects that are known to be exacerbated during HIV/TB co-infection. The light purple arrow represents metabolites hypothesised to be altered during HIV/TB co-infection, based on published literature. The numbers indicate the overlapping metabolites and where they play a role in what is known about host metabolism during HIV/TB co-infection. Abbreviations: HIV: human immunodeficiency virus; AIDS: acquired immunodeficiency syndrome; Mtb: Mycobacterium tuberculosis; TB: tuberculosis; IDO: indoleamine-2,3-dioxygenase; Trp: tryptophan; CP: catabolic products; Glu: glutamate; Gln: glutamine; Phe: phenylalanine; Tyr: tyrosine; Asp: aspartate; Ala: alanine; His: histidine. Figure created by the author with the help of NWU Graphic Services.

Although the lipids did not overlap between the studies compared, the classes affected were similar, and their numerous roles in HIV infection, TB, and the immune response are already established. The exacerbated immune dysfunction during co-infection (Sharan *et al.*, 2020) is likely to be reflected in the metabolic profile in terms of the overlapping HIV infection and TB metabolites (Figure 2.2 and “similarities” in Tables 2.8–2.10). Furthermore, those altered metabolites which have to date only been associated with either HIV infection or TB may also be significant during co-infection (“differences” in Tables 2.8–2.10) and metabolites that have not previously been associated with HIV infection or TB may also be significant in the co-infection metabolic profile.

Features unique to TB pathology have also been reported, such as fibrinopeptides, proposed to originate from the granuloma (Weiner *et al.*, 2012) (see “differences”, Table 2.8). Within plasma, alterations to the branched-chain amino acids were more commonly reported for HIV infection than for TB, although alterations to these amino acids are commonly attributed to immune activation and wasting, which are also features of TB (see “differences”, Table 2.9). Given the limited number of metabolomics studies that focused on HIV/TB co-infection to date, the metabolic mechanisms underlying HIV/TB co-infection remains incompletely characterised. The comparison made here yields insight into how the metabolic changes during HIV infection and TB may interact during the co-infected state and could serve to inform future study design. Compared to blood, urine is used less frequently for characterisation or diagnostic purposes of either HIV infection or TB, although it has been popular for TB drug metabolism elucidation [see Du Preez *et al.* (2019)]. As such, the extensive comparison made for blood could not be made for urine (and other sample matrices), and hence a large piece of the puzzle is still missing.

Given the advantages of urine as a clinical specimen (Du Preez *et al.*, 2019), it has been considered an excellent alternative to sputum for TB diagnostics. Characterising the urine metabolic profile of HIV-positive individuals will be a necessary step if urine were to be developed as a robust TB diagnostic matrix. The HIV infection and TB urine metabolic profiles overlap to a smaller degree than the blood-based profiles. While the urinary metabolic profile of HIV-positive patients indicates altered energy metabolism and the utilisation of amino acids for energy, the urinary TB metabolic profile indicates increased inflammation and compromised insulin production in addition to reflecting *Mtb*-related alterations (see “differences”, Table 2.10). This relatively smaller degree of overlap may be due to the limited number of studies eligible for the comparison (in which participants were untreated for either condition; see Table 2.10). Nevertheless, increased levels of neopterin (which has been detected using conventional assays for both diseases) and catecholamines were points of similarity in the urine metabolic profiles of HIV infection and TB (Table 2.10).

The study of Munshi *et al.* (2013) provides unique insights into HIV infection since it relied on the use of various sample types, namely plasma, urine, and saliva. The only other urinary HIV-related metabolomics study is that of Chetwynd *et al.* (2017), which does not concur with Munshi *et al.* (2013) on all results. For

instance, the authors' observations regarding the extent to which immunosuppression is reflected in urine differs. Chetwynd *et al.* (2017) found that no separation could be achieved within the HIV-positive treatment-naïve group upon stratification by CD4 T cell count (point of division at 250 cells/mm³), which led them to suggest that metabolic markers of immunosuppression are not visible in urine. However, conventional methodologies established that urinary neopterin has an inverse correlation with CD4 T cell count (Immanuel *et al.*, 2005). Increased levels of urinary neopterin have been recognised as a marker of immune activation, a trend which has been observed in urinary HIV infection (Munshi *et al.*, 2013) and TB metabolomics studies, as well as in a small group of co-infected patients included in the study of Isa *et al.* (2018), in which the elevation was more pronounced. These results confirm previous findings [reviewed by Siawaya *et al.* (2007)] and suggest that urinary neopterin may be useful as part of the HIV/TB co-infection biosignature. There have been observations where urinary neopterin decreased in response to ART (Munshi *et al.*, 2013) as well as in serum upon anti-TB treatment. This suggests the additional usefulness of neopterin as a prognostic marker. In co-infected patients, consistently high neopterin levels have been associated with HIV disease progression and poor outcomes (Immanuel *et al.*, 2005). As co-infection metabolic profiles have not yet been systematically studied, this remains a viable avenue for future investigation.

There are a few studies not relevant to the comparisons in Tables 2.8–2.10, but which are important to mention in the context of the co-infected state. Alterations to the human microbiome have been associated with both HIV infection and TB, but it remains unclear exactly how the microbiome is affected during co-infection. The composition of the microbiome of the upper respiratory tract of HIV-positive treatment-naïve individuals was similar to those of HIV/TB co-infected patients except for a lower abundance of species belonging to the *Veillonellaceae* family of bacteria. This family was also less abundant in the treatment-naïve TB group relative to the treated TB group. This suggests such an alteration to the microbiome of HIV-positive patients to be indicative of TB, but this requires further investigation (Htun *et al.*, 2018).

Two metabolomics studies focused on lung health in HIV-positive individuals, which provides the opportunity to evaluate the state of the lungs before *Mtb* infection in HIV-positive individuals from a metabolomics perspective. Cribbs *et al.* (2014) studied the BALF metabolic profiles of HIV-positive individuals compared to healthy controls and found that the primary differences reflected in metabolites have their origin in the lung microbiota, and could further indicate a subclinical infection in the HIV-positive lung. This study established a specific metabolic profile of the HIV-positive lung environment, which gives an indication of lung immune function and could contribute to improving diagnostic and prognostic techniques. In another study, HIV-positive individuals could not be distinguished from healthy controls based on lung microbial population composition (determined by 16S rRNA gene sequencing); however, a distinction could be made based on the BALF metabolic profile (Cribbs *et al.*, 2016). These studies indicate that the interaction of the host and the microbiota contribute to a functional difference

in the HIV-positive lung, rather than the species present. Given that HIV-induced metabolic alterations have their origin in immune dysfunction, it is the most likely cause for the alterations observed by the authors. Such an altered environment may change the way that the HIV-positive host interacts with *Mtb*, and vice versa, contributing to the altered clinical course and phenotype of co-infected individuals.

2.7 Beyond characterisation: Immunometabolism and its potential role in the diagnosis, management and monitoring of HIV/TB co-infection

There are several aspects in the HIV/TB co-infection field that remain unanswered. These gaps were reinforced/emphasised in this analysis of the HIV infection and TB cytokine and metabolomics literature on informing potential changes during co-infection. The overlap in trends of various Th1 and Th2 cytokines in co-infection studies, as well as the overlap in metabolites from the respective HIV infection and TB studies (which confirm what is already known about co-infection), indicates the potential use of multiplexed cytokine assays and metabolomics in further characterising the co-infected state.

The diagnostic problems experienced in HIV/TB co-infected individuals are related to the diagnosis of TB rather than the diagnosis of HIV infection, as mentioned above, due to the limitations of sputum smear and culture-based tests [reviewed by Schutz *et al.* (2010); Tiberi *et al.* (2017)]. Co-infected individuals are also often unable to provide a sufficient sputum sample for diagnostic testing, especially when severely immunocompromised (World Health Organization, 2015). Molecular tests for TB diagnosis (GeneXpert *MTB*/RIF assay and the improved version, GeneXpert *MTB*/RIF Ultra) are highly effective in diagnosing TB in HIV-negative individuals, has a better turn-around time, and can give an early indication of drug resistance. However, access to such tests in high burden areas is limited by cost and there are concerns about the specificity (false-positives) of this test in HIV-negative individuals (World Health Organization, 2019). Similarly, a urinary point-of-care diagnostic assay based on the detection of an *Mtb* cell wall component, lipoarabinomannan (LAM), has not found widespread application partly due to its inapplicability to HIV-negative samples. It is recommended that this test should not be used for screening or diagnosis in HIV-negative individuals nor HIV-positive individuals with relatively high CD4 T cell counts (World Health Organization, 2015). Given the low sensitivity of this test, an improved version (Fujifilm SILVAMP TB LAM) has been developed which retained specificity with improved diagnostic sensitivity (Broger *et al.*, 2019). Despite high efficacy when this test is applied to immunocompromised individuals, a difficulty remains in assigning diagnostic categories [such as definite TB, possibly TB, or not TB, as used by Broger *et al.* (2019)] based on the results of various indicators of TB. For example, several patients could not be diagnostically classified, but tested positive using Fuji-LAM. Consequently, these patients were not started on anti-TB drugs and died within three months of follow-up (Broger *et al.*, 2019).

Considering the sputum diagnostic limitations, the focus for the development of new TB diagnostic techniques should be on the identification of non-sputum biomarkers. Metabolomics can aid in identifying markers with diagnostic application or be combined with existing tests to increase sensitivity and specificity. Urine is an ideal sample in this sense as the procedures for collection and storage are simple and require minimal biosafety considerations (World Health Organization, 2015). This makes identifying markers to be used in conjunction with the LAM assay an attractive avenue of research to improve the early detection of TB and timely intervention in HIV-positive individuals. However, the criteria by which such a marker is selected must be carefully considered, particularly amenability to accurate and sensitive detection by an inexpensive point-of-care platform (Walzl *et al.*, 2018) and specificity to TB (as opposed to a general inflammatory marker). Cytokines may provide a viable option in this regard, as they can be measured using simple assays and various studies have stressed that cytokines must be combined with other markers when used as diagnostic tools (Monastero & Pentyala, 2017). One must also consider the increased incidence of extrapulmonary TB in this regard. Often, extrapulmonary TB is not included in the TB diagnostic testing procedure, and even if it was, sample collection for diagnosis remains difficult (Denkinger *et al.*, 2014). If biomarkers specific to extrapulmonary disease are identified, including these in the standard TB diagnostic repertoire may significantly improve the early diagnosis of TB in HIV-positive individuals.

Metabolomics has been applied to identify metabolic determinants that indicate conversion of latent *Mtb* infection to clinical disease in the studies of Weiner *et al.* (2018) and Albors-Vaquer *et al.* (2020). The efficacy of such a profile has not yet been tested in HIV-positive individuals, for whom the risk of conversion to clinical disease is much greater than for HIV-negative individuals. The use of cytokines as biomarkers for the conversion of latent to active TB in the absence of HIV infection has also been reviewed, although it was concluded that validation studies are lacking (Sudbury *et al.*, 2020). Such validation studies in HIV-negative and HIV-positive populations are a necessary step for the plethora of literature that exists on the topic to find real value. Untreated profiles aid in understanding the underlying mechanisms that drive conversion of latent *Mtb* infection to active TB in HIV-positive individuals and provide early prognostic information, especially regarding TB-IRIS and treatment response. However, this might prove difficult considering sample availability, given the recommendation for early treatment of latent *Mtb* infection (World Health Organization, 2016a) as well as the “Test and Treat” policy for HIV infection, recommended by the WHO (World Health Organization, 2016b). Nonetheless, such basic understanding is necessary for the development of robust and sustainable diagnostic and prognostic procedures.

Even if a highly effective diagnostic technique for TB in HIV-positive persons is developed, its success will depend on the integration of HIV infection and TB services on the ground, especially in high-burden areas, which are unfortunately also most often resource-limited. Once the difficult diagnosis is made, various factors complicating the management of HIV/TB co-infection come into play and are usually

associated with unfavourable treatment outcomes due to various factors [see Tiberi *et al.* (2017) and Letang *et al.* (2020)]. Pharmacometabolomics is a branch of metabolomics wherein the effects of a drug or combination of drugs underlie the observed metabolic alterations (Combrink *et al.*, 2019) and have been applied to HIV infection (Babu *et al.*, 2019; Chetwynd *et al.*, 2017; Li *et al.*, 2018) and TB [Du Preez *et al.* (2019), see Table 2.9]. The interactions between ART and anti-TB drugs, and their interactions with enzymes, have been characterised by high-performance LC-MS (Wenning *et al.*, 2009) and LC-tandem MS (Varshneya *et al.*, 2019), demonstrating how metabolomics aids the understanding of *in vivo* drug metabolism and efficacy, which is essential to the development of better treatment strategies for co-infected patients.

Besides the interactions of the drugs themselves, variations on an individual level, identified as one of the factors contributing to TB treatment failure (De Villiers & Loots, 2013) will also affect the efficacy of drug regimens. The rate of recurrent TB (relapse and reinfection) is greater among HIV-positive compared to HIV-negative individuals (Charalambous *et al.*, 2008; Sonnenberg *et al.*, 2001). The only way to prevent reinfection would be an effective vaccine. Also, by better understanding the mechanisms underlying treatment failure in co-infected patients, the cases of relapse will be minimised by optimising treatment regimens. Cytokines have been shown to predict TB treatment failure (Lee *et al.*, 2015; Siawaya *et al.*, 2009) and recurrent TB in HIV-positive individuals on ART (Sivro *et al.*, 2017). Metabolomics has been used to identify the host factors that contribute to TB treatment failure in HIV-negative TB patients (Luies *et al.*, 2017b). Combined immunometabolic approaches could provide valuable insights, as co-infection is associated with poor treatment outcomes and susceptibility to infection in general, due to the additional immunosuppressive effects of HIV (Guerra-Assuncao *et al.*, 2015). Another aspect of interest from an immunometabolic perspective is how the effect of the second *Mtb* co-infection event on the HIV-positive host would compare to that of the first.

Given that much of the HIV and *Mtb* infection-induced metabolic changes are driven by immune activation, inflammation and OS with subsequent impact on the mitochondria and the gut (Figure 2.2), metabolomics could aid in elucidating the effect that *Mtb* has on the gut mucosal damage as induced by HIV. The metabolic markers obtained are not unique and have been reported in the metabolomics investigations of other inflammatory conditions (Leermakers *et al.*, 2015; Pett *et al.*, 2017). As such, more longitudinal studies, especially in the treatment context, with the inclusion of more disease controls to get more specific markers associated with each condition are thus needed.

For the rational design of novel or improved diagnostic, treatment, and monitoring techniques, the research focus must first fall on better characterising a disease state so that the origin of identified markers is understood, as opposed to unqualifiedly advocating statistically significant markers that have no true specificity for the disease state. It is of extreme importance to develop and validate highly specific biomarkers for HIV/TB co-infection — although biosignatures may be more practical taking into account

the overlapping pathologies of HIV infection and TB. Furthermore, when developing these immune and metabolic signatures, it should be kept in mind that the ultimate goal is point-of-care, individualised assessments that should be accessible even in resource-limited settings, as these are commonly the areas of high incidence and spread. This further requires the complete integration of HIV and TB services and for this, a greater awareness of co-infection should be brought among clinicians and built into infrastructure to ensure that even those with minimal education could apply it.

2.8 References

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CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

Blood is uniquely suited to investigations relating to the disease phenotype of an individual as it suffuses all organs, and therefore reflects metabolic reactions holistically. As such, it gives a “snapshot” of the metabolic state at any given point (Álvarez-Sánchez *et al.*, 2010) and reveals any alterations caused by infection(s) (Du Preez *et al.*, 2019). As HIV infects the immune cells, its effect on the host is systemic and not localised. Thus, many metabolomics investigations have used blood to investigate the effects of HIV infection on the host. Although not many bacterial products are found in blood, blood-based samples have also been successfully used to characterise the systemic effects of *Mtb* infection on the host (Du Preez *et al.*, 2019). Considering this, blood is an ideal medium to study HIV/TB co-infection. Serum is the liquid portion of blood that is separated from other components by a process that allows or facilitates clotting (when using coagulant-coated tubes). It is generally considered a cleaner sample than plasma (liquid portion of blood that has been prevented from clotting via anticoagulants) (Psychogios *et al.*, 2011). However, a disadvantage to the systemic nature of the information obtained is that all effects cannot necessarily be traced to their origins. Furthermore, although blood is relatively simple to process, proteins and lipoprotein particles have to be removed (Du Preez *et al.*, 2019). Blood is also obtained by a semi-invasive procedure, causing anxiety in some individuals that could alter the metabolome (Dunn *et al.*, 2005). The process to obtain serum may alter some metabolite levels, e.g. eicosanoids released as a result of clotting — this implies that the levels measured will not necessarily correspond to the physiological levels (Psychogios *et al.*, 2011). Despite some disadvantages, serum has been successfully used in many HIV infection and TB metabolomics studies (see Chapter 2) with reproducible results.

3.2 Experimental design

As part of a larger collaborative study, serum samples (n=84) were collected by the South African Tuberculosis Vaccine Initiative (SATVI) and Desmond Tutu HIV Centre at the University of Cape Town. These were divided into groups based on HIV, TB, HIV treatment, and co-infection status. All samples were analysed with a pooled quality control (QC) sample, prepared by combining a small volume (10 µl) of each sample into the same tube. This pooled QC was used for cytokine and metabolomics analysis. Cytokine analyses were done in two batches (batches 1–4 and 5–8 of the metabolomics analysis), including one QC aliquot per batch. Metabolomics analysis was done in eight batches, to which samples were assigned randomly. One QC aliquot was used per batch and analysed three times; at the start, middle, and end of the batch. For this investigation, 29 healthy controls (HIV-/TB-/Tn-), 22 untreated HIV-negative, TB-positive individuals (HIV-/TB+/Tn-), 9 untreated HIV/TB co-infected individuals (HIV+/TB+/Tn-) and 12 HIV/TB co-infected individuals receiving ART (HIV+/TB+/Tn+) were analysed. The broad outline of this study is indicated in Figure 3.1.

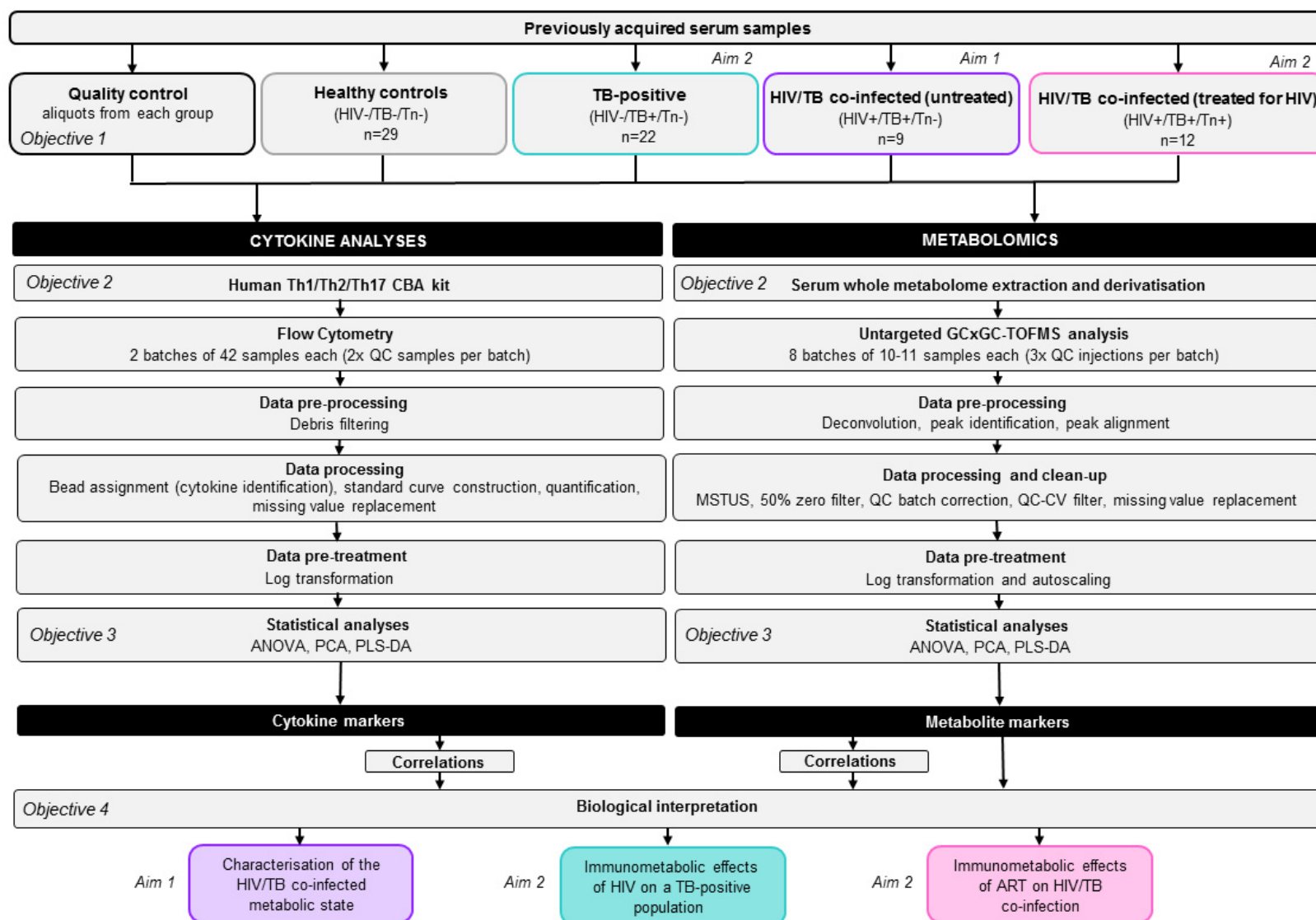


Figure 3.1: Experimental design. A schematic representation of this investigation, showing the groups compared and the workflow followed for the cytokine and metabolomics experiments, data handling and interpretation.

3.3 Materials and methods

3.3.1 Ethical approval

Ethical approval for sample collection, done according to the Declaration of Helsinki and International Conference of Harmonisation guidelines, was obtained from the Ethics Committee of the University of Cape Town (reference no: 680/2013). Ethical approval was also obtained from the Health Research Ethics Committee (HREC) of the North-West University, South Africa, for the larger study focused on the HIV/TB co-infection metabolomics (NWU-00355-20-A1), as well as for this sub-study (NWU-00355-20-A1-01).

3.3.2 Sample collection, transport and storage

Serum samples (n=84 for the larger study, of which only the 72 relevant to the aims of this sub-study were used here) were collected by SATVI and the Desmond Tutu HIV Centre at the Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa, according to standard operating procedures. Samples were routinely and continuously collected as part of the community outreach work of the Desmond Tutu HIV Centre. All patients were South African adults (male and female, between the ages of 18 and 69) residing in the Masiphumelele and Ocean View Townships in Cape Town, in the Western Cape Province of South Africa. All participants were informed verbally (in their mother tongue) that participation is voluntary, of the implications of participation, and that they may withdraw from the study at any time. Additionally, participants had the opportunity to read an information sheet (containing the above-mentioned information), which was also discussed in detail, and to ask questions. After the consent form was explained by a trained field worker, participants gave written consent. Following sample collection as well as upon the follow-up visit to the clinic, participants received a financial incentive (R100). Additionally, participants received counselling and immediate treatment in case of all positive results.

Blood samples were collected by a registered nurse at the Desmond Tutu HIV Centre. Sample collection/measurement procedures were performed in a separate room to ensure the privacy of patients during sample collection. Sample labelling and data were de-identified. The basilica vein at the antecubital fossa (elbow pit) of the patient was used for blood collection. Five different 2 ml tubes were used to collect 0.5 ml blood per tube from each patient, in the order of Cell Preparation Tube (CPT) 1, PaxGene, Clotted, Li Heparin, CPT 2. These tubes were then placed in a bio-carrier and immediately transported to the University of Cape Town, within 2 hours, where serum was prepared through centrifugation using standard protocols and then frozen at -80°C. These serum samples were transported on dry ice to the North-West University, Focus Area of Human Metabolomics: Laboratory of Infectious and Acquired Diseases, where it was again stored at -80°C until the day of analysis.

Based on the clinical information received from the Desmond Tutu HIV Centre, the samples were divided into groups based on HIV, TB, and HIV treatment status (all samples were untreated for TB). TB was diagnosed using GeneXpert, while all HIV/TB co-infected individuals had a serum-confirmed HIV status. Not all samples were tested for HIV/TB co-infection since this is based on clinical symptoms in clinical practice. Given the rural background of patients, and therefore limited access to healthcare, the exclusion criteria were not extensive. Patients were excluded from the study if they: (a) consumed any over-the-counter or chronic medication, except for one patient taking non-steroidal anti-inflammatory drugs (trimethoprim/sulfamethoxazole) and one taking antibiotics (amoxicillin/ clavulanic acid); (b) had another disease that is similar to those under investigation (e.g. asthma) – one patient was diagnosed with nephrotic syndrome and was not excluded; or (c) were pregnant or lactating. Given that the burden of both HIV and TB is highest in resource-limited areas (Glaziou *et al.*, 2014; World Health Organization, 2020), these exclusion criteria increases the likelihood that the results of this study will be robust enough to be repeated in any cohort.

3.3.3 Demographic information

The demographical and clinical summary of the cohort is given in Table 3.1. The serum cytokine and metabolic profiles of all patients (mentioned in Section 3.3.2) were measured. Obtaining untreated HIV, TB and co-infected samples is extremely difficult in the light of the “Test and Treat” policy for HIV and latent TB, as recommended by the WHO (World Health Organization, 2016a; World Health Organization, 2016b), hence the small sample sizes. Regarding the antiretroviral regimens, all patients received a fixed-dose combination of two nucleoside reverse transcriptase inhibitors (NRTIs; Tenofovir and Emtricitabine) and one non-nucleoside reverse transcriptase inhibitor (Efavirenz), except for two patients who received two NRTIs (Abacavir with Lamivudine and Combivir, respectively) and two protease inhibitors (Lopinavir/Ritonavir).

Table 3.1: Cohort demographics and clinical information.

	HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB+/Tn+
No. of patients (%)	29 (40.3)	22 (30.6)	9 (12.5)	12 (16.7)
Age (years), mean $\pm$ SD (range)	33.4 $\pm$ 10.3	34.4 $\pm$ 12.8	31.9 $\pm$ 6.2	40.9 $\pm$ 10.6
Sex, male:female ratio (%)	13:16 (45:55)	13:9 (59:41)	5:4 (56:44)	4:8 (33:67)
Smokers in the group (%)	9 (31.0)	11 (50.0)	2 (22.2)	3 (25.0)
CD4 T cell count (cells/mm ³ blood), mean $\pm$ SD	N/A	N/A	221.4 $\pm$ 252.4	182.7 $\pm$ 162.2
Viral load (copies/mm ³), range *	N/A	N/A	< 20–124	< detectable–513 509
Duration of ART (years), mean $\pm$ SD	N/A	N/A	N/A	4.3 $\pm$ 3.6
Patients on FDC ART combination (%)	N/A	N/A	N/A	10 (83.3)
Patients on different ART combinations (%)	N/A	N/A	N/A	2 (16.7)

*Groups: HIV-/TB-/Tn-: healthy controls; HIV-/TB+/Tn-: untreated HIV-negative patients with active pulmonary TB; HIV+/TB+/Tn-: untreated HIV/TB co-infected patients; HIV+/TB+/Tn+: HIV/TB co-infected patients on antiretrovirals; ART: antiretroviral therapy; SD: standard deviation; FDC: fixed-dose combination. *Viral load was not available for all patients, hence the data shown here are not representative of the entire group. Viral load also varied due to differences in ART duration.*

3.3.4 Cytokine analyses

3.3.4.1 Cytometric bead array

The Human Th1/Th2/Th17 CBA kit (catalogue number: 560484, lot: 0064427 and 0202260, BD Biosciences, San Jose, California) was used to measure the levels of IL-2, IL-4, IL-6, IL-10, TNF- α , IFN- γ and IL-17A simultaneously. Analysis was done in two batches and the same sample order was used as for the metabolomics analysis. Serum samples were thawed and aliquoted (50 μ l) and the CBA analysis was performed as per the manufacturer's instructions and as previously described (Williams *et al.*, 2013). A serial dilution of cytokine standards was performed to set up a standard curve for quantitation of the cytokines (0–5000 pg/ml, dilution factor of 2). Seven sets of capture beads, each set of beads coated with an antibody specific to one of the seven cytokines measured, were mixed to allow multiplexed detection of cytokines. The detection reagent [PE-conjugated detection antibody, 50 μ l] and the mixed capture beads (50 μ l) were added to all samples and standards. This mixture was then incubated for 3 hours at room temperature, protected from light. During this time, samples were agitated (vortexing for ~1 s) every 30 min, to ensure maximum interaction between the capture beads and the sample. This incubation allows sandwich complexes to form between the capture beads, cytokines and the PE-detection antibody. Upon excitation by the laser during flow cytometry, these sandwich complexes allow a signal to be detected that is proportional to the concentration of each of the cytokines. The samples were then washed and the pellet resuspended in wash buffer (300 μ l) for analysis on the flow cytometer.

3.3.4.2 Flow cytometry

The samples were analysed on the BD Accuri C6 Plus flow cytometer using BD Accuri C6 Plus CSampler software (version 1.0.23.1). Before data acquisition on the day of analysis, instrument performance was assessed by performing an automated QC procedure using Cytometer Setup and Tracking beads (catalogue number: 661414, lot: 9155868). On both days of analysis, the instrument passed this assessment. The template available from the website of BD Biosciences was used to collect data. Thresholds were set on 500 000 for forward and side scatter, which allows for the exclusion of debris and background signal. A dot plot of FSC versus SSC and a histogram of event count versus allophycocyanin (APC) (indicating the seven bead populations) allowed the bead population to be defined and gated. Using a medium flow rate (35 μ l/min), 3 000 events were collected per sample within the defined gate. After every four samples, the instrument was backflushed to purge the flow cell and avoid the build-up of any background signal. A dot plot of PE (detection antibody) and APC (bead populations) showed the seven bead populations as well as their shifts in concentration. When all samples were acquired, the data was exported as flow cytometry standard (FCS) files, which were used for data processing.

3.3.4.3 Data processing and clean-up

The data was processed using BD FACS Array Version 3.0 software. The bead library corresponding to the Human Th1/Th2/Th17 cytokine kit as well as the FCS files of all samples and standards were imported. The scatter parameter was FSC-H, while APC-H and PE-H were the clustering and reporting parameters, respectively. The software uses the scatter parameter to identify the total bead population, which is then plotted on a histogram of event count versus the clustering parameter (APC-H). This allows the seven bead populations (corresponding to the seven cytokines) to be distinguished. The bead library is then used to assign specific beads to specific peaks in one of the FCS files that are chosen as a reference sample (typically a standard). This assignment is then used as a template to identify the seven bead populations in each sample's FCS file. Although the identification of bead populations was not affected by background noise and no manual clustering was necessary, debris filtering was used to remove any remaining noise. This was done per bead population by plotting FSC-H against APC-H, and gating on each population individually. The standard concentrations were defined and five parameter logistic regression standard curves (as per convention) were plotted for each bead population, using a R^2 cut-off of 98% for fitting accuracy. The calculated concentrations were then exported as an Excel sheet and used to assess whether a batch effect was present using principal component analysis (PCA).

A batch effect is any variability due to non-biological systematic errors (Wang *et al.*, 2013) observed in the results obtained from a set of samples analysed in an uninterrupted sequence (within batch) as well as variability between such sets (between batch), that negatively affect the accuracy or precision of such data. Therefore, to make these measurements directly comparable (Wehrens *et al.*, 2016) and to be able to make accurate inferences about the biological variation of interest, this non-biological variation must be removed (De Livera *et al.*, 2015). As the QC samples analysed in each batch are identical in composition, the variation in these measurements should be low. Therefore, these can be used to assess whether a batch effect is present and if so, serve as references for its correction (Wehrens *et al.*, 2016). Based on the distribution of the data in Figure 3.2, the QC samples cluster together, indicating that a batch effect was not present.

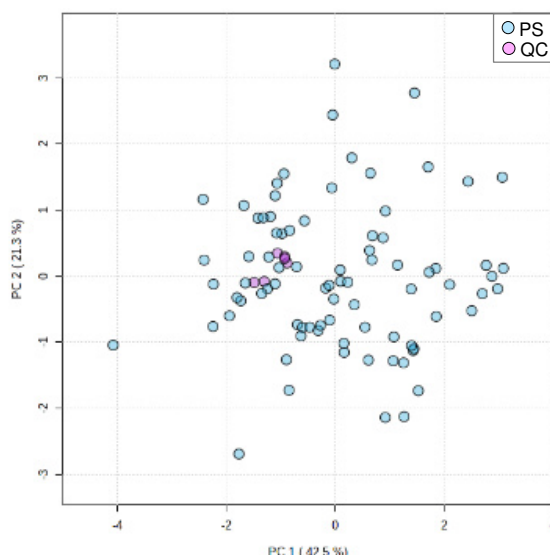


Figure 3.2: PCA of cytokine data, indicating that no batch effect was present [blue: patient samples (PS); purple: pooled quality control (QC) samples].

3.3.5 Metabolomics

3.3.5.1 Reagents and chemicals

The components of the internal standard stock solution (3-phenylbutyric acid, acetaminophen, nonadecanoic acid, and norleucine), as well as the derivatisation reagents (methoxyamine hydrochloride and N,O-bis(trimethylsilyl)trifluoroacetamide [BSTFA] with 1% trimethylsilyl chloride [TMCS]) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Pyridine was purchased from Merck (Darmstadt, Germany). Ultra-pure Burdick and Jackson brand (Honeywell International Inc., Muskegon, USA) acetonitrile, methanol and chloroform were used.

3.3.5.2 Whole metabolome extraction and derivatisation

Extraction and analysis of samples were done as described by Stander *et al.* (2018). Briefly, all samples were thawed and 50 μ l aliquots were prepared. Each batch of serum samples (50 μ l aliquots in 1.5 ml LoBind Eppendorf tubes) and one QC aliquot (50 μ l) per batch was thawed at room temperature and vortexed for 2 min. Thereafter, the experimental and QC samples were subjected to a whole metabolome extraction, using 50 μ l of a 0.45 μ g/ml internal standard stock solution (dissolved in a chloroform:methanol:water [1:3:1] solution). To precipitate proteins, 300 μ l of ice-cold acetonitrile was added to samples on ice. The samples were then vortexed for 2 min and centrifuged at 3500 $\times$ g for 10 min at 4°C. The supernatants were then transferred to GC-MS sample vials and dried under a light stream of nitrogen gas at 40°C for approximately 45 min. Following this, samples were incubated at 50°C for 90 min with 50 μ l of methoxyamine hydrochloride dissolved in pyridine (15 mg/mL). After methoxymation, 80 μ l BSTFA with 1% TMCS was added and samples were incubated for 60 min at 60°C for derivatisation by

silylation. To prepare for injection, each derivatised sample was transferred to a new GC-MS vial (containing a vial insert) and capped.

3.3.5.3 GCxGC-TOFMS analysis

Derivatised samples were placed into the tray of the Gerstel Multi-Purpose Sampler (Gerstel GmbH and co.KG, Eberhard-Gerstel-Platz 1, D-4573 Mülheim an der Ruhr, Germany) coupled to a GCxGC-TOFMS (Pegasus 4D; LECO Corporation, St. Joseph, MI, USA). The latter uses an Agilent 7890A gas chromatograph (Agilent, Atalanta, GA) and a time-of-flight mass spectrometer from LECO Corporation (St. Joseph, MI, USA) and is coupled to a cryogenic cooler. One microliter of each QC (one per sample batch) and all samples were injected with a 1:5 split ratio, at an inlet temperature of 270°C (kept constant throughout the run). Initially, one injection of a mixture of fatty acyl methyl esters (FAMES; to assess system performance using a sample with a known response) and three QC injections (to prime the system) were done. The following sequence was then followed and repeated until all samples in each batch had been injected: an injection of hexane (to assess the cleanliness of the system and wash away any carry-over), a QC injection, 5–6 randomised samples, hexane again, three QC injections, and a FAMES injection.

A Restek Rxi-5Sil-MS primary capillary column (28.759m, 0.25 mm internal diameter and 0.25 µm film thickness) and a Restek Rxi-17 secondary capillary column (1.380m, 0.25 mm internal diameter and 0.25 µm film thickness) were used to achieve compound separation. An initial primary oven temperature of 70°C was maintained for 2 min, after which the temperature increased 4°C per minute until the maximum temperature of 300°C was reached, at which it was maintained for 2 min. The secondary oven was initially kept at 85°C for 2 min, followed by an increase of 4.5°C per min, to a final temperature of 300°C, which was held for 4.5 min. The modulator was programmed to an initial temperature of 100°C for 2 min, where after it was increased by 4°C per min to a temperature of 310°C, which was held for 12 min. To control the effluent emerging from the primary to the secondary column, cryomodulation and a 1.5 s hot pulse of nitrogen gas every 3 s were used. Regarding the TOFMS, the filament was kept off for the first 400 s, to exclude solvent peaks, which have the potential of saturating the detector. After this, the filament bias was set to -70 eV for fragmentation and the temperature of the transfer line was kept at 270°C. The masses 50–800 m/z were collected at an acquisition voltage of 1600V and an acquisition rate of 200 spectra/second.

3.3.5.4 Data processing and clean-up

The data generated was processed using ChromaTOF software version 4.32 (LECO Corporation), which included peak detection, deconvolution, alignment of all samples, as well as compound identification. To calculate the baseline, the baseline offset was set to be just above the noise. To find peaks, the first dimension peak width was set to 32, the second dimension peak width was set to 0.15, and a minimum of

three apexing masses with a signal-to-noise ratio of 110 was required. A minimum spectral match of 70% was required to combine modulated peaks. The National Institute of Standards and Technology (Food and Drug Administration) libraries and the library of the Potchefstroom Laboratory for Inborn Errors of Metabolism were searched to identify peaks, with the requirement of 70% spectral overlap before a name was assigned. To calculate peak areas, each peak's unique mass was used. For peak alignment, the criteria for a spectral match was a mass threshold of 10 and a minimum similarity match of 60%. For the retention time match, no difference in retention time was allowed and peaks had to be a maximum of one modulation period apart to be matched. To find peaks not identified by the initial search, the signal-to-noise ratio was decreased to 20. Analytes were kept only if they appeared in a minimum of five samples and in at least 50% of the samples in a class.

The dataset was then exported from ChromaTOF and was normalised using total useful signal (Warrack *et al.*, 2009) to reduce technical variation and make samples more comparable. The normalised dataset was subjected to various clean-up steps, including a 50% zero filter, batch effect correction, and QC coefficient of variation (CV) filter (Luies & Loots, 2016). Figure 3.3 is a two-dimensional PCA scores plot of the QC data, which was used to assess whether a batch effect was present. Based on the distribution of the data (Figure 3.3A), all of the batches do not overlap with each other, indicating a batch effect. Although all possible measures are always taken to prevent the occurrence of a batch effect, it may be caused by factors beyond the analyst's control. To correct for this in the QC and patient samples, quantile equating (Wang *et al.*, 2017) was used. Following this, there was much better overlap between the batches (Figure 3.3B, the PCAs were created in MetaboAnalyst), indicating that all variation observed in the data from here onwards can be attributed to biological differences and not a systematic error.

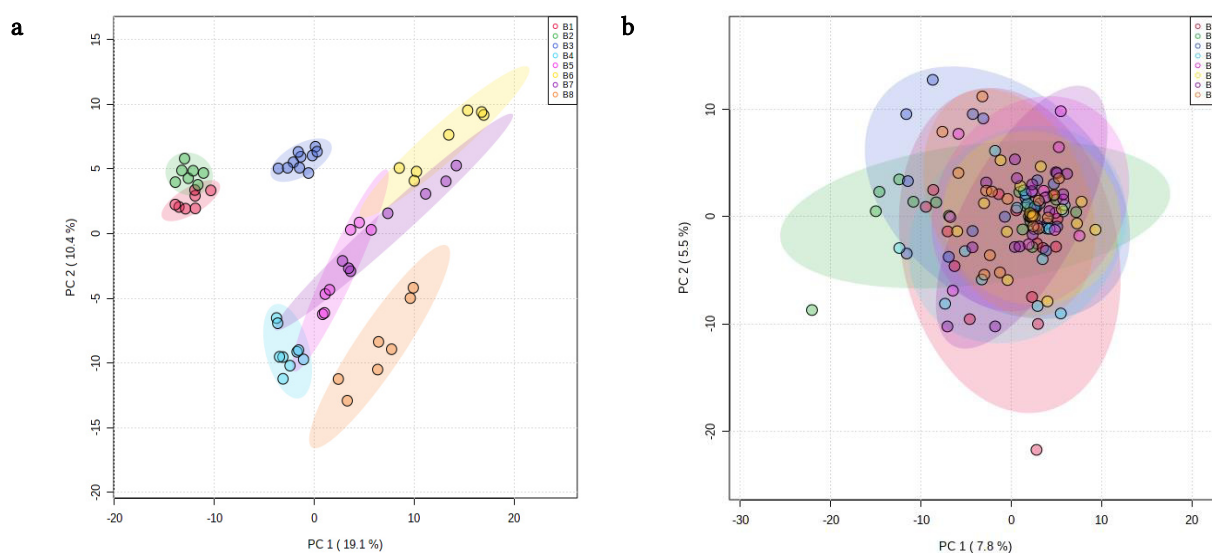


Figure 3.3: Batch correction. GCxGC-TOFMS quality control data represented in a two-dimensional principal component scores plot, (a) before and (b) after batch correction by quantile equating.

3.4 Statistical analyses

The distributions of nominal variables were assessed using Fisher's exact tests in IBM SPSS Statistics 27 software. Missing value replacement, data pre-treatment, and statistical analysis were performed using MetaboAnalyst (version 5.0). As per default, for both the cytokine and metabolomics datasets, missing values were replaced by one-fifth of the minimum value in the dataset for each variable and is based on the idea that these missing values are unlikely truly zero but rather the result of compound abundances falling below the detection limit of the instrument (Schoeman *et al.*, 2012). The cytokine dataset was log-transformed, while the metabolomics dataset was log-transformed and autoscaled to achieve a normalised distribution, as this is a prerequisite for the accurate usage of various statistical analyses. Univariate analyses included one-way analysis of variance (ANOVA) with correction for multiple testing by the Benjamini-Hochberg (BH) approach [variables were considered statistically significant if the adjusted p-value was < 0.05] and Fisher's least significant difference for post-hoc analysis. The BH method was also used to correct for multiple testing for all other statistical tests done in this experiment. Pearson's r correlations were also performed for cytokines and metabolites. Cytokine correlations were considered significant when the BH-adjusted p-value was < 0.05 . The metabolic correlations were done selectively due to the size of the dataset. Metabolites indicated as significant by ANOVA with a particular biological significance were correlated to all other metabolites. Metabolic correlations were only considered significant if the adjusted p-value was < 0.005 , to further reduce the number of metabolite markers to a usable amount for data interpretation. Correlation analysis was done between the CD4 T cell counts (for all HIV-positive patients), all cytokines and all metabolite markers.

Multivariate analyses included PCA (used to assess the inherent structure of the data) and partial least squares-discriminant analysis (PLS-DA). PLS-DA models were subjected to leave-one-out cross-validation and permutation testing (precision accuracy during training) but given the small sample size, was not used to identify predictive markers. Instead, PLS-DA variable influence on projection (VIP) values, which directly correlate with the importance of a metabolite in differentiating the groups (De Villiers & Loots, 2013), were used in an explorative sense. That is, to assess the distribution of groups and determine whether the cytokines and metabolites identified as statistically significant in the univariate analyses retained significance in a multivariate statistical setting. Quantitative metabolite set enrichment analysis (MSEA), using the Small Molecule Pathway Database (SMPDB) was also performed on those metabolites identified as significant during ANOVA.

3.5 Quality assurance/Repeatability

Repeatability concerns the variation observed between repeated measurements that are made on the same subject and under the same conditions. This can be assessed using the CV value, calculated as the ratio between the standard deviation and the average, which measures the spread/variability of data points

around the mean of that data series, notwithstanding differences in the means of these data series (Eaton, 2007). To achieve the first objective, the machine and analyst repeatability was determined, as this conveys information about the reliability of the measurements and subsequent data interpretation (Bartlett & Frost, 2008).

3.5.1 Cytokine analyses

One QC sample was prepared with each batch of samples ($n=2$) and each of these was injected repeatedly ($n=3$). For analyst repeatability, the QC samples of the two days were compared, while machine repeatability was determined by comparing the repeated injections of each QC sample to each other. The limit of detection for each cytokine, according to the BD CBA manual (<https://www.bdbiosciences.com/en-eu/products/reagents/immunoassays/cba>) is indicated in Table 3.2, which further shows that the average concentrations of six of the seven cytokines that were measured, were below the detection limit of the assay. The samples used in this study were collected from 2015 to 2017, and had therefore been in storage for four to six years prior to cytokine analyses. Furthermore, during this time, the samples had undergone multiple freeze-thaw cycles as the metabolomics analyses were performed twice by the analysts of the NMP, and the cytokine analyses also had to be repeated due to an unapparent instrumental error (explained in more detail below). It has been shown that cytokines degrade over time, even when stored at -80°C , and especially so when samples have been in storage for two or more years (Keustermans *et al.*, 2013) and that freeze-thaw cycles affect the reliability of measurements (Huang *et al.*, 2017). This could explain the low levels of cytokines in these samples.

Table 3.2: Limit of detection and average concentrations of the cytokines in the QC samples for each of the cytokines in the BD Human Th1/Th2/Th17 CBA kit.

Cytokine	Limit of detection (pg/ml)	Average concentration in QC samples (pg/ml)	CV for concentration values (%)	CV for MFI values (%)
IL-17A	18.9	13.37	18.23	2.62
IFN-γ	3.7	1.34	45.43	5.88
TNF	3.8	0.74	42.93	5.03
IL-10	4.5	1.48	39.74	11.25
IL-6	2.4	39.33	3.70	5.67
IL-4	4.9	1.29	45.34	5.08
IL-2	2.6	0.16	116.31	18.02

Abbreviations: IL: interleukin; IFN- γ : interferon-gamma; QC: quality control; CV: coefficient of variation; MFI: mean fluorescent intensity.

The cytokine analyses were originally run on a machine which passed its performance checks (as verified by a specialist on the day of analysis). Following data processing, it was clear that the data was not usable, although there were no apparent issues during data acquisition. Troubleshooting finally indicated a

misaligned laser in conjunction with some build-up in the fluidics lines (after the machine had stood unused during the COVID-19 lockdown), which explained the high CVs observed to some extent. Hereafter, a replacement machine was acquired and test samples were run to optimise the assay and determine if the repeatability could be assessed more extensively. However, the CBA kits are very expensive, and as such a limited number of tests are available (especially given the number of samples). Furthermore, each kit allows for only two separate experiments since it comes with only two vials of standards, which must be prepared fresh each time. Therefore the opportunities for optimisation were not extensive. Because of this, analyst repeatability could not be properly assessed, as only two batches of samples could be run with the second kit. Seeing as this kit should theoretically be highly repeatable, determining analyst repeatability was not imperative. Moreover, the repeat analysis resulted in limited sample volume left for the second analysis, hence the assay (which does not allow much room for alterations) could not be extensively tested to optimise it for these samples.

The CVs obtained in the second analysis were better compared to those of the first analysis, but were still too high when assessed on the extrapolated concentration values (Table 3.2) — the raw data CV values were indeed very low. The standards also served as controls to determine if the assay was working properly. The 0 pg/ml standard served as negative control, while all other concentrations of cytokines could be seen as positive controls. All standards had a recovery between 90 and 115% (where the ideal range is 80-120%). This indicated that the assay was indeed working properly. Although the cytokines that fell below the detection limit could still be quantified by extrapolation using the standard curves, it is less repeatable than when working in a range where the exact response for a specific concentration is known. The effect of this can be seen when the CVs calculated for concentrations are compared to those calculated for the mean fluorescent intensity (MFI; the measured response or raw data). Considering this, and the fact that many of the values fell beneath the limit of detection, a biostatistician was consulted, whom advised to keep the cytokine and metabolomics datasets separate, and only interpreting the cytokine data based on trends (as opposed to the original intent of correlating this to the metabolomics results).

It should also be noted that the CVs calculated for analyst repeatability are based on only two QC samples (i.e. two repeats, as the QC samples are identical, but were prepared and analysed on different occasions), while the requirement is typically three repeats for a reliable standard deviation. There is an inherent trend in CV values where those at the extremes of the working range typically have higher CVs (Reed *et al.*, 2002). As such, compounds detected in low concentrations will naturally have higher CV values and vice versa (Luies, 2017). This is because the CV is sensitive to small changes in the average when the average is near zero (NIST, 2017). This is clear in the high CVs for IL-2 and the low CVs for IL-6 (Table 3.2). The CVs based on MFI are consistently low, except for IL-2. The levels of IL-2 were too low to be quantified in the QC sample of batch 1, and as there are only two points of comparison (two batches;

one value being a zero and the other an extrapolated value), the CV value is remarkably high – for all other cytokines an extrapolated value could be determined in both batches.

3.5.2 Metabolomics analysis

For extraction or analyst repeatability, the QC sample was extracted repeatedly on different days (n=8) and each extract was injected once into the GCxGC-TOFMS for analysis. In contrast, the repeatability of the machine was determined by extracting one QC aliquot and injecting this into the machine multiple times (n=10). The resulting datasets were processed (including batch correction) and CV values of all compounds, and for ten compounds representing different chemical classes eluting throughout the chromatographic run, were calculated for both datasets (shown in Table 3.3, along with the average peak intensity of each compound). This was done on the batch corrected dataset, since this data is used for statistical analyses and therefore represents the repeatability of the data used for interpretation.

Table 3.3: Ten compounds detected throughout the entire GCxGC-TOFMS chromatographic run of the repeatability study. The average total signal, first and second dimension retention time, and CV value for each metabolite after batch correction are also indicated to show machine and analyst repeatability.

Compound name	Average total signal (standard deviation)	Average first dimension retention time	Average second dimension retention time	Machine repeatability (CV value) (%)	Analyst repeatability (CV value) (%)
2-Ethyl-3-hydroxypropionic acid	0.053 (0.005)	769.021	1.301	9.810	1.853
L-Isoleucine	2.195 (0.150)	852.131	1.348	6.719	0.276
L-5-Oxoproline	8.136 (0.391)	1178.102	1.616	4.710	0.280
L-Hydroxyproline	0.201 (0.017)	1328.572	1.255	8.717	0.211
4-Hydroxyphenyllactic acid	0.054 (0.008)	1932.622	1.387	11.657	0.738
Tetrahydrofuran	0.111 (0.019)	2028.897	1.398	14.819	0.300
D-Erythro-pentofuranose	0.128 (0.024)	2367.479	1.301	16.251	0.832
Uridine	0.071 (0.006)	2625.855	1.600	8.470	0.234
Palmitic acid	8.290 (0.648)	2692.980	1.057	7.174	0.841
Arachidonic acid	0.681 (0.050)	3135.755	1.604	7.490	0.981

The Food and Drug Administration recommends a CV cut-off of 15% for targeted chromatographic analyses [except near the lower limit of quantification, where 20% is acceptable (Food and Drug Administration, 2018)]. However, due to the inherent variation in untargeted metabolomics data such low CV values are rarely reported (t'Kindt *et al.*, 2009). Since this is an untargeted metabolomics

investigation, which ideally aims to detect and quantify as many metabolites in a sample as possible, in an unbiased manner, a hypothetical cut-off of 50% was applied here (Schoeman *et al.*, 2012). Figure 3.4 is a visual representation of the distribution of the CV values for the repeatability analyses.

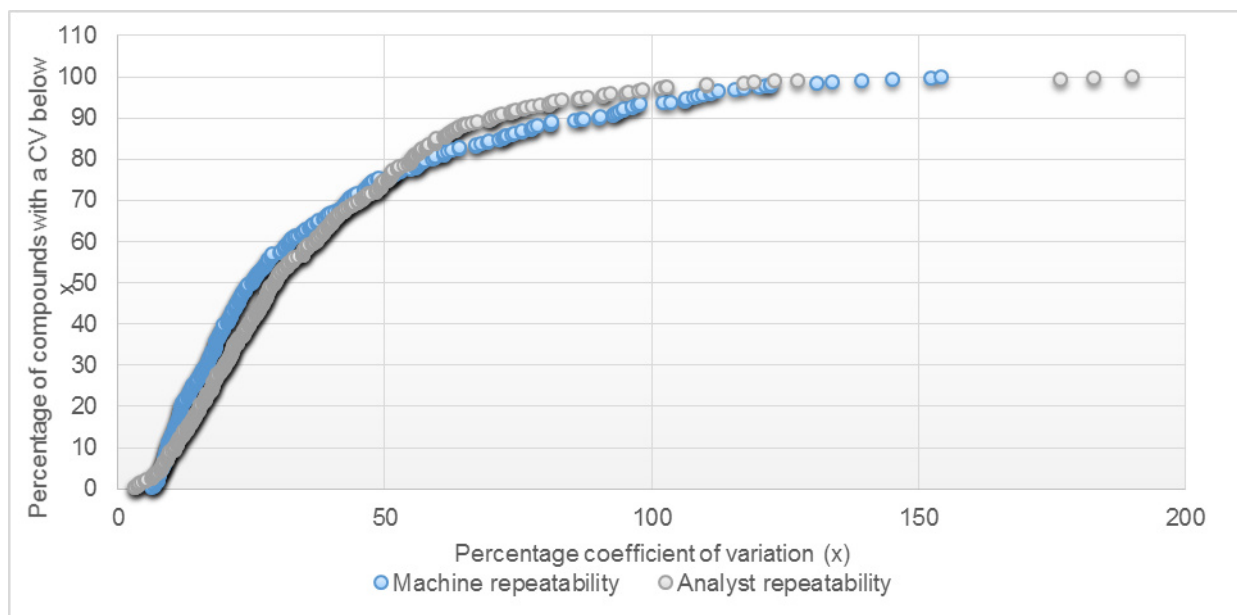


Figure 3.4: QC-CV plot showing the distribution of the coefficient of variation values for the machine (GCxGC-TOFMS) and analyst repeatability.

A total of 529 compounds were detected in this study. A 50% zero filter was applied before repeatability was assessed, as those compounds that were detected in less than 50% of the samples in a group were excluded before statistical analyses. When considering the machine repeatability, 75% of compounds had a CV value below 50%, indicating acceptable repeatability. Similarly, when considering the analyst repeatability, 74% of compounds had a CV value below 50%. A large proportion of compounds with very high CV values (43%, > 70% CV), could not be annotated. This means that 43% of those compounds with a CV above 70% were unannotated. Within the 26% that had CVs above 50%, is a proportion that had CVs above 70%. So, 43% of that proportion could not be annotated. This reflects the ability of the GCxGC-TOFMS to separate and detect novel compounds, although compound libraries have not expanded enough to match the separation capacity.

In light of the limitations relating to the limit of detection for the cytokine analyses, the cytokine data will be used in a non-predictive, explorative sense only and will not be correlated to metabolic data. For the metabolomics data, in the case of both analyst and machine repeatability, the majority of compounds fell within the accepted level of variance for untargeted metabolomics analysis, and therefore the data obtained was deemed reliable for further use to address the aims of this investigation.

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CHAPTER 4: CHARACTERISING THE IMMUNOMETABOLIC PROFILE OF HIV/TB CO-INFECTION IN COHORTS UNTREATED AND TREATED FOR HIV

This chapter has been submitted for publication:

- Liebenberg, C., Luies, L., Loots., D.T. and Williams, A.A. 2021. Characterising the immunometabolic profile of HIV/TB co-infection in cohorts untreated and treated for HIV. Submitted for publication to *eLife* (Manuscript/submission number: 06-12-2021-RA-eLife-76156).

4.1 Abstract

The synergy between HIV and *Mtb* during co-infection is well-established. The immunological deterioration and metabolic mechanisms that drive this synergy are poorly understood. Although ART suppresses viral replication, it gives rise to metabolic disruptions and adaptations beyond that induced by infection. Here, the serum cytokine and metabolic profiles of untreated HIV/TB co-infected, HIV/TB co-infected on ART, and HIV-negative TB-positive patients, as well as healthy controls, were measured and compared. Multiplexed cytokine measurements and untargeted gas chromatography mass spectrometry were used. There was no significant difference between the cytokine levels of the diseased groups when compared to each other, however, the co-infected individuals were characterised by increased IL-6 and IFN- γ when compared to controls. These suggested a failure of immunoregulation, resulting in an increased disease burden. Metabolites indicative of cachexia, intestinal mucosa damage, and microbiome dysbiosis characterised co-infected individuals from the TB-positive population, while co-infected individuals were additionally distinguished from healthy controls by more extensive amino acid changes. Loss of gut integrity and subsequent microbial translocation results in increased inflammation and immune activation, culminating in a reduced appetite and malabsorption, causing exacerbated wasting in co-infected individuals. Treating HIV in the co-infected group revealed a metabolic return to values comparable to the healthy or the TB-positive population. It is unclear whether this represents a return to a more healthy state, as treatment exacerbated other metabolic alterations. These results suggest that HIV partially augments the HIV-*Mtb* synergy through its detrimental effects on gut health, affecting energy availability.

4.2 Introduction

HIV-positive individuals have a 26–31 times greater risk of developing active TB compared to HIV-negative individuals (World Health Organization, 2012) due to the immunosuppressive effects of HIV on

the host. Unsurprisingly, TB is the most common secondary disease among HIV-positive individuals, hence there is a significant global burden of HIV/TB co-infection. The COVID-19 pandemic has increased this burden, with diminished access to health care facilities, and hence poor diagnosis, reporting, and treatment initiation for HIV infection and TB, resulting in increased TB-related deaths during this period (Dorward *et al.*, 2021; Waterfield *et al.*, 2021; World Health Organization, 2020). Approximately 14% of the estimated 1.3 million TB-related deaths in 2020 were among HIV-positive individuals (World Health Organization, 2021). It is well established that HIV and *Mtb* act synergistically during co-infection of a host, amplifying the disease burden. This synergy is centred on immunological deterioration (Pawlowski *et al.*, 2012; Sharan *et al.*, 2020), which in turn is the result of an increased level of immune activation during co-infection (Vanham *et al.*, 1996). The immune and metabolic systems are intricately connected, and changes in the one are reflected in the other. The term “immunometabolism” has mainly been used in the context of the metabolism of immune cells, but the effects of these alterations extend to systemic metabolism also, as the organism’s energy and biosynthetic stores are also directed towards the immune response. The term will be used in a comprehensive sense here, encompassing both immune cell and systemic metabolic alterations and their relation to the immune response. All references to “HIV” imply HIV-1.

Despite being characterised from various perspectives using various comprehensive and sensitive techniques [reviewed by (Bell & Noursadeghi, 2018; Diedrich & Flynn, 2011; Kwan & Ernst, 2011; Sharan *et al.*, 2020; Toossi, 2003)], the immune response of the host during co-infection remains poorly understood. Cytokines have been extensively investigated in the context of HIV infection, TB, and HIV/TB co-infection as biomarkers of immune activation and inflammation (Catalfamo *et al.*, 2012; Domingo-Gonzalez *et al.*, 2016; Siawaya *et al.*, 2007). Previous studies have measured the inherent (i.e. unstimulated) plasma and cellular cytokine profiles of untreated HIV/TB co-infected patients, but this has not been done in serum, and not concurrently with comprehensive metabolic measurements. Representative cytokines from the Th1, Th2 and Th17 cytokine groups, are important in the context of HIV/TB co-infection. In this regard, the importance of IFN- γ (and the chemokines it induces) (Benjamin *et al.*, 2013; Subramanyam *et al.*, 2004), IL-10 (Benjamin *et al.*, 2013), IL-6 (Farr *et al.*, 2018; Rambaran *et al.*, 2021), and IL-17 (Du Bruyn *et al.*, 2021) in the immune response during HIV/TB co-infection has been demonstrated. These cytokines have separately been shown to be involved in the immune response to HIV infection and *Mtb*, respectively (Domingo-Gonzalez *et al.*, 2016; Freeman *et al.*, 2016), indicating that the immune response during HIV/TB co-infection presents aspects of the response to both pathogens. However, it is known that the immune response during HIV/TB co-infection is severely dysregulated and in many ways promote the disease pathology, rather than to subvert it (Bell & Noursadeghi, 2018; Sharan *et al.*, 2020).

The metabolic alterations that occur during co-infection are not well characterised and have mainly been measured using conventional techniques that rely on low-throughput and laborious biochemical assays

(Williams *et al.*, 2011). These techniques have indicated that HIV/TB co-infected patients have an impaired net protein balance in the fed state – while protein balance during HIV infection alone or TB alone, was strongly anabolic (Paton *et al.*, 2003). Co-infected patients, when compared to TB patients, were also shown to suffer from hypoalbuminemia (a further indicator of impaired protein balance) (Van Lettow *et al.*, 2003) but have similar body composition changes (Paton & Ng, 2006). Since these techniques do not provide a comprehensive picture of the metabolic state, metabolomics approaches may supplement this knowledge gap. Although metabolomics has been used to better understand very specific aspects of host metabolism during co-infection (Adu-Gyamfi *et al.*, 2017; Collins *et al.*, 2020), it has not been used in an explorative manner to characterise the metabolic alterations that accompany HIV/TB co-infection. Untargeted metabolomics aims to characterise as much of the metabolome as possible by identifying and quantifying as many metabolites as possible. It is also considered a hypothesis-generating research approach (Dunn, 2008). Therefore, untargeted metabolomics is ideal to provide a preliminary overview of the altered host metabolism during HIV/TB co-infection, and also to highlight areas of interest for future studies. Furthermore, the effects of ART in addition to the effects of HIV infection, on the host have been characterised in various contexts using cytokine measurements (Borges *et al.*, 2015; Kamat *et al.*, 2012; Vanpouille *et al.*, 2016) and metabolomics (Babu *et al.*, 2019; Hewer *et al.*, 2006; Li *et al.*, 2018; Peltenburg *et al.*, 2018; Rodriguez-Gallego *et al.*, 2018). Although ART typically leads to decreased VL, low-level inflammation remains and metabolic complications are common (Jain *et al.*, 2005). A previous investigation of the blood cytokine profile of HIV/TB co-infected patients on ART, showed that the inflammatory network density declines with ART, but that this is dependent on the degree of viral suppression (Du Bruyn *et al.*, 2021). The metabolic effect of ART in the context of HIV/TB co-infection has been investigated using conventional techniques [reviewed by Jain *et al.* (2005)], and with a focus on the IRIS, using metabolomics (Silva *et al.*, 2019). However, metabolomics has as yet not been used to study untreated and treated co-infection in an exploratory manner. Therefore, this investigation aims to better characterise the immunometabolic profile of HIV/TB co-infected cohorts, with and without ART.

4.3 Methods

Refer to Sections 3.3–3.4.

4.4 Results

Figure 4.1 indicates the various group comparisons and their individual aims for the four patient cohorts.

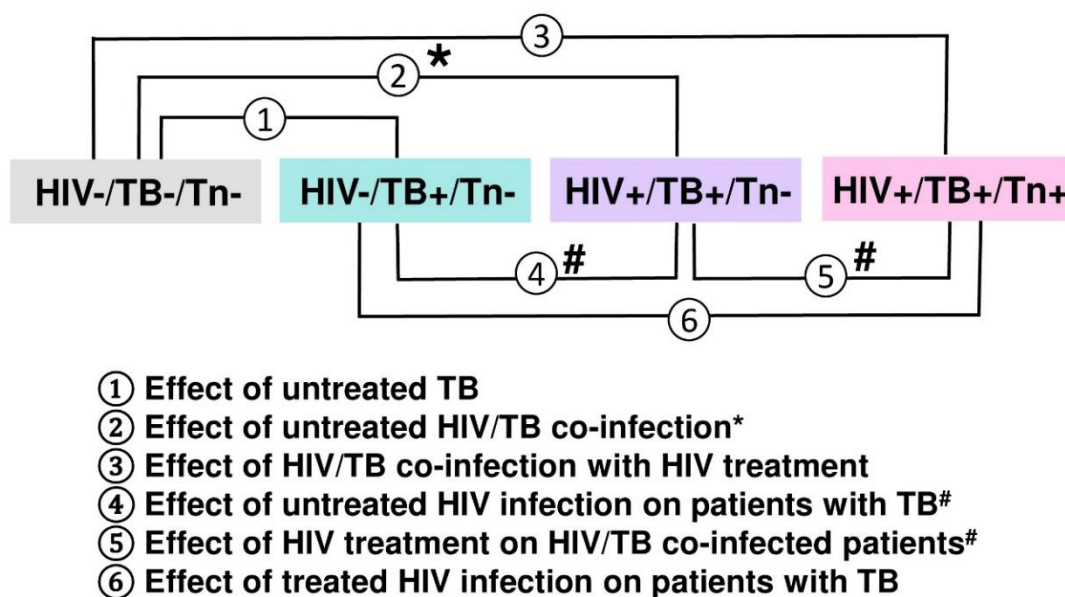


Figure 4.1: The comparisons that are possible with the four patient groups and their purpose/experimental aims. The comparisons that are indicated with an asterisk (comparison 2, Section 4.5.1) and a hashtag (comparisons 4 and 5, Section 4.5.2), respectively, are pertinent to the aims of this investigation. The other comparisons are highlighted where necessary to provide context to the comparisons related to the primary aims. Groups: HIV-/TB-/Tn-: healthy controls; HIV-/TB+/Tn-: untreated HIV-negative patients with active pulmonary TB; HIV+/TB+/Tn-: untreated HIV/TB co-infected patients; HIV+/TB+/Tn+: HIV/TB co-infected patients on ART.

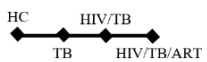
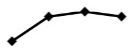
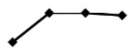
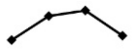
Supplementary demographic data, descriptive statistics, as well as correlation and MSEA data, are provided in Appendix A. There were no significant demographic differences between the aforementioned compared groups for age, sex, smoking, and CD4 (in the HIV-positive groups) using Fisher's exact test (Table S1, Appendix A). Those patients that were on medication or had other medical conditions, which were still included in this study, did not reflect as outliers. Although these factors may have affected the individual cytokine and metabolome profiles of these individuals, and may result in variation, many of the other patients likely had similar conditions unknown to them (especially given the severely ill state of most of the co-infected patients) or which were not disclosed upon sample collection. This may have resulted in those patients who did in fact declare the use of other medication or other diseases, not reflecting as significantly different from the rest of the group. Alternatively, the effect of HIV infection or TB may have overshadowed the effects of other morbidities or treatments.

4.4.1 Cytokines: Trends in relation to disease severity

The results of the cytokine measurements and the corresponding trends and statistics are given in Table 4.1 (also see Tables S3-S5). IL-6 and IFN- γ were significantly increased in all the diseased groups relative to the healthy controls using ANOVA (IL-10 showed the same trend but with borderline significance). The following trends (although not statistically significant, listed in Table 4.1) were also observed in the co-infected group relative to the healthy controls (comparison 2): IL-10 and IL-17A were

increased, IL-4 and IL-2 were decreased, while TNF- α remained largely unchanged. There were no statistically significant differences in cytokine levels between the diseased groups, but the levels of IL-6 were higher in the untreated co-infected group relative to the TB group, IFN- γ similar, and IL-4 reduced (comparison 4). Upon comparing the untreated co-infected group to the co-infected group on ART, IL-6 and IFN- γ remained high (similar levels), while IL-10, IL-17A, TNF- α were decreased, and IL-4 and IL-2 were increased following treatment (comparison 5). It is also noticeable that IL-4 and IL-2 were comparatively the lowest, while IL-6, IL-10, and IL-17A were highest in the untreated co-infected group relative to all other groups.

Table 4.1: The seven cytokines arranged according to disease trends and their statistical significance.

Cytokine	Line graph of means 	ANOVA (BH-corrected p-value)	Post-hoc (comparison where cytokine is significantly different, Figure 4.1)
IL-6		< 0.0001	①②③
IFN- γ		< 0.0001	①②③
IL-10		0.066	②③
IL-4		0.109	-
TNF- α		0.748	-
IL-17A		0.748	-
IL-2		0.975	-

Abbreviations: IL: interleukin; IFN: interferon; TNF: tumour necrosis factor; ANOVA: one-way analysis of variance; BH: Benjamini-Hochberg.

Figure 4.2A shows the PLS-DA scores plot. Cross-validation indicated that the model was overfitted (five-component model: $Q^2 = 0.62$, $R^2 = 0.67$, accuracy = 0.65), likely due to the small sample size and the variation within groups, but permutation testing indicated that the model was not random [p -value = 5×10^{-4} , meaning that the classification of the samples into the designated groups is significantly better than any random classification (Golland *et al.*, 2005)]. Nonetheless, given the explorative nature of the study, the PLS-DA model was included to show the distribution of samples relative to each other. The distribution shows that the majority of HIV/TB co-infected individuals lie furthest away from the healthy controls, whereas co-infected individuals on ART lie anywhere in between in terms of the serum cytokine profile. IL-6 and IFN- γ retained statistical significance in a multivariate approach (PLS-DA VIP > 1), and were significantly positively correlated with each other and with IL-10 and IL-17A (Figure 4.2B and C). Refer to the supplementary information for detailed PLS-DA and correlation results (Tables A3 and A4, Appendix A). CD4 T cell count was significantly negatively correlated with IL-6 and IL-17A (Figure 4.2D).

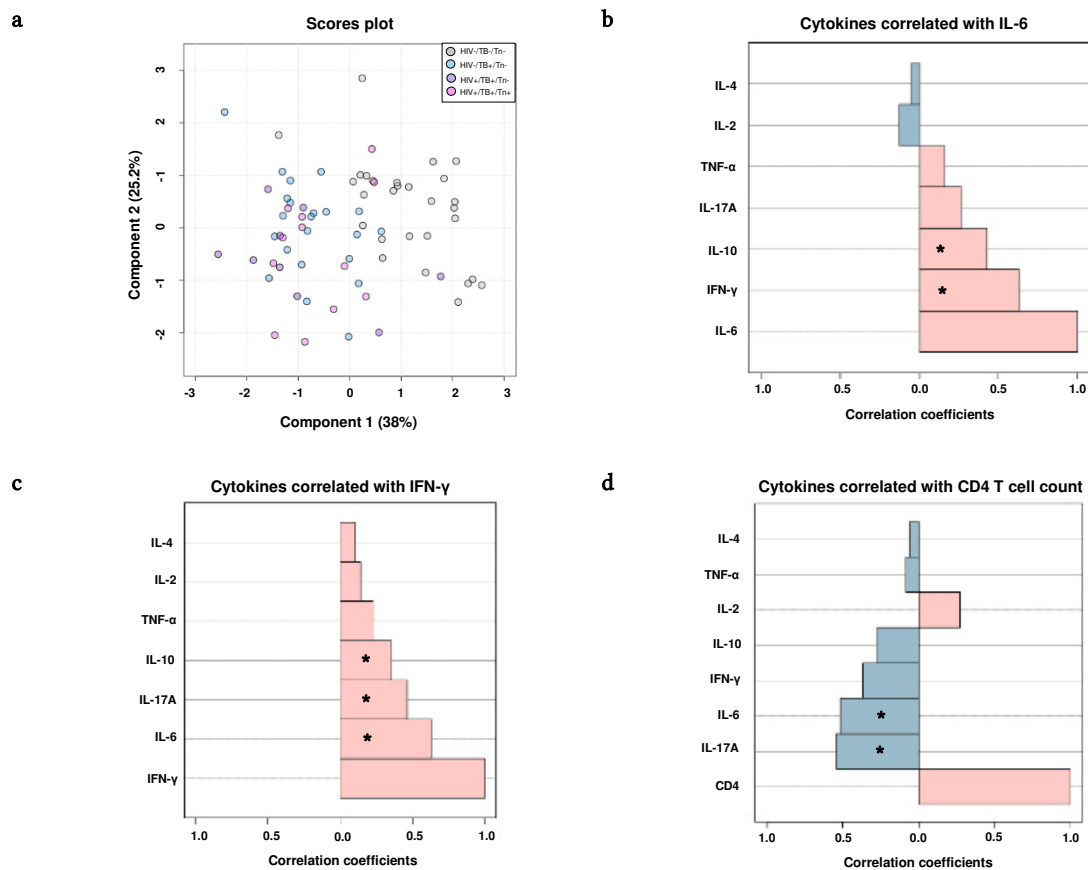


Figure 4.2: Multivariate statistics and correlation histograms for the cytokine data. (a) PLS-DA scores plot for the cytokine data indicates a significant overlap between the cytokine profiles of the diseased groups. (b) and (c): Pearson's r correlations of IFN- γ and IL-6 with all other cytokines measured. (d) Pearson's r correlations of CD4 T cell count and all cytokines measured. Significant correlations (corrected $p < 0.05$) are indicated by an asterisk ().*

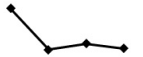
4.4.2 Metabolomics: What did we learn?

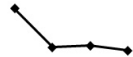
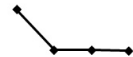
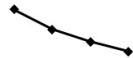
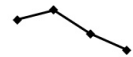
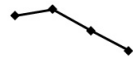
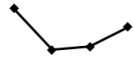
The ANOVA analysis indicated 47 metabolites as significantly different between the groups. These metabolites, their trends, and the corresponding statistics are shown in Table 4.2 (also see Table S5). Four of the statistically significant metabolites could not be correctly annotated, possibly due to high signal-to-noise ratios, unsuccessful deconvolution, or its absence from the libraries used (Tsugawa *et al.*, 2011). Metabolites that could not be related to any biological function are not discussed [exogenous metabolites ($n=11$), Table 4.2], thus their function and presence in human serum require further investigation. Instances where metabolites were annotated with the same name but with different unique masses and where the mass spectra could not be visually matched to each other, were listed separately (Table 4.2).

The data was also subjected to multivariate analysis. The PCA indicated no natural separation between the groups and PLS-DA cross-validation and permutation testing indicated that the model was over-fitted (cross-validation for a one-component model: $Q^2 = 0.56$, $R^2 = 0.80$, accuracy = 0.60; permutation testing p -value = 0.386). As explained, this model was only used in an explorative sense and the scores plot is

displayed in Figure 4.3A to show the distribution of sample groups. Figure 4.3A indicates that the co-infected patients on ART, and not the untreated co-infected patients, lie farthest away from the healthy controls in terms of the serum metabolic profile and that the diseased groups were expectedly more heterogeneous than the healthy controls. All metabolites indicated as significant by the ANOVA also had a $VIP > 1$ considering the first five components of the PLS-DA. Although the metabolites listed in Table 4.2 are not predictive, they can be used to explain the variance between the groups in this cohort, and therefore the metabolic alterations induced during the disease states under investigation. Quantitative MSEA was used to facilitate the biological interpretation of the metabolites in Table 4.2 and indicated that the metabolic pathways of essential amino acids and glycerolipids were most significantly enriched (Figure 4.3B). 3,4-Dihydroxybutyric acid, phenylalanine, lysine, and tryptophan were selected, based on statistical significance and biological relevance, for correlation with other metabolites (Figure 4.4). All metabolites indicated to be “exogenous” (Table 4.2) could not be found in the Human Metabolome Database, and in some cases, no exact match could be found in PubChem, ChemSpider, the National Institute of Standards and Technology Webbook, nor in DrugBank (when the possibility was considered that they may relate to medications/traditional medications not declared by patients). From the context of similar metabolites, the inference was made that these metabolites likely were not endogenous. However, these metabolites do appear to have some importance in the co-infected context since the majority were significantly altered in comparisons 2 and 3 (in which the two co-infected groups were compared to the healthy group).

Table 4.2: The 47 serum metabolite markers that best describe the variation between the comparative groups.

Compound	Line graph of means 	ANOVA (BH-adjusted p-value)	Post-hoc (comparison where metabolite is significantly different. Figure 4.1)
Amino acids and related metabolites (of human and/or microbial origin)			
Phenylalanine (unique derivatised mass = 218)		0.002	①②③④
DL-Phenylalanine (unique derivatised mass = 120)		< 0.0001	①②③
2,6-di-tert-butyl-p-Cresol		0.021	②
Benzoic acid		0.007	②③
L-Norvaline		0.042	①②③
Tryptophan		0.013	②③
3-Indoleacetic acid		0.009	②③⑥
3-Indolepropionic acid		0.018	②③

3-Indolelactic acid		0.007	②③
Methionine		0.002	②③
Threonine		0.002	②③
Lysine		0.001	②③
2-Aminobutyric acid		0.039	②③
Hydroxyproline		0.002	②③
Proline, 1-methyl-, methyl ester		0.036	①②⑤
3-Hydroxyisovaleric acid		0.001	①②③
3-Hydroxypropionic acid		0.033	②③
Cadaverine		0.007	③④⑥
Energy metabolism			
Glycerol-3-phosphate		0.002	②③④
D-Glucuronic acid		0.002	③④⑥
Pyruvate		0.029	①③
Carbohydrates of exogenous or microbial origin			
Rhamnose		0.018	①②③
D-Mannitol		0.036	①②⑤
1-Deoxypentitol		0.001	②③④⑥
Tetrahydrofuran		< 0.0001	②③④⑥
2,4-Di-tert-butyl-phenol		0.001	⑥
D-Arabinose (unique derivatised mass = 204)		0.011	①
D-Arabinose (unique derivatised mass = 217)		0.050	①④
Fatty acid and lipid-related metabolites			
2,4-Dihydroxybutyric acid		0.044	①②
3,4-Dihydroxybutyric acid		< 0.0001	①②③④
alpha-Linolenic acid		0.018	②④⑤
1,E-11,Z-13-Octadecatriene (alpha-linolenic acid, unique derivatised mass = 67)		0.018	①②
Dodecanal		0.021	②④

Linoleyl alcohol		0.040	②
Capric monoethanolamide		0.043	② ⑤
Glycerol (unique derivatised mass = 103)		0.002	① ② ③
Glycerol (unique derivatised mass = 73)		0.009	① ② ③
Unannotated/exogenous metabolites			
Acrylic acid 2-dimethylamino-ethyl ester		0.004	② ③
Carbonic acid, 2-dimethylaminoethyl ethyl ester		0.044	② ③
Carbonic acid, 2-dimethylaminoethyl propyl ester		0.046	② ③
Cyclobutanecarboxylic acid, 2-dimethylaminoethyl ester		0.018	② ③
Tetrahydro-pyran-2,4,5-triol		0.038	② ③
2-Amino-1-methyl-1H-imidazol-4-ol		0.021	③
Analyte 622		0.002	③ ⑥
Analyte 628		0.002	② ③ ④
Analyte 706		0.050	② ③
Analyte 819		0.044	③

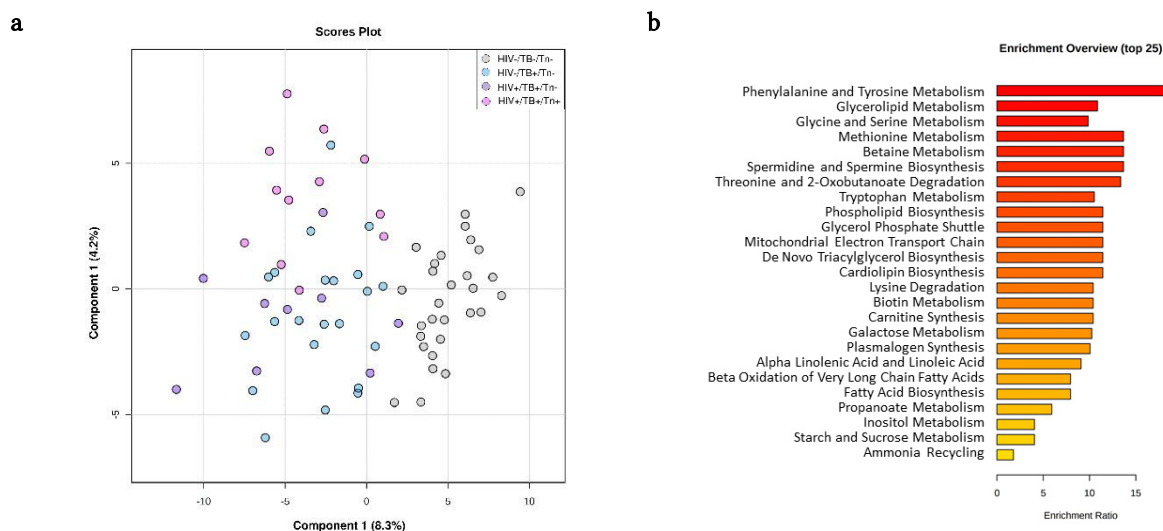


Figure 4.3: Multivariate and functional analyses relating to the metabolomics data. (a) PLS-DA scores plot indicating the metabolic profiles of the diseased groups are similar, but do not overlap completely. This implies that there are areas of uniqueness between the metabolic profiles of TB, untreated HIV/TB co-infected, and treated HIV/TB co-infected patients. (b) Overview of quantitative metabolite set enrichment analysis (for comparison 2, healthy controls versus untreated HIV/TB co-infected patients) identified as significant by ANOVA ($p < 0.05$).

4.5 Discussion

Six of the nine HIV/TB co-infected patients had CD4 T cell counts below 200 cells/mm³, indicating a severe immunodeficiency and (by the immunological definition) the onset of AIDS (World Health Organization, 2007). One patient was close to the immunological definition of AIDS, with a CD4 T count of 248 cells/mm³ and the remaining two patients had CD4 T cell counts above 500 cells/mm³. A CD4 count above 500 cells/mm³ is typical of healthy individuals (Williams *et al.*, 2006) indicating that their disease state is less advanced compared to the others in this group. In the HIV/TB co-infected group on ART, all but two patients (CD4 T cell counts > 300 and > 500 cells/mm³ respectively) met the immunological definition of AIDS. Unfortunately, the cohort cannot be described based on VL since this information was not available for all patients. Given the small sample size, this study is explorative and does not attempt to identify predictive markers. As no comprehensive HIV/TB co-infection metabolomics profile has been published (at the time of submission and to the best of our knowledge), this study will contribute to better understanding the metabolic responses of the host during HIV/TB co-infection and direct future studies on the topic.

4.5.1 The immunometabolic effects of HIV/TB co-infection

The serum cytokine profiles of untreated HIV/TB co-infected patients (Figure 4.1, comparison 2) were mainly characterised by significantly increased pro-inflammatory cytokines of Th1 (IFN- γ) and Th2/immunoregulatory (IL-6) origin. IFN- γ is necessary, but not sufficient, for protection against *Mtb* infection (Kumar *et al.*, 2019; Sudbury *et al.*, 2020) and is essential to enhancing cytokine and other cellular immune responses during HIV infection (Roff *et al.*, 2014). Increased levels of IL-6 is generally considered an indicator of HIV disease progression, as this cytokine positively correlates with VL (Kuller *et al.*, 2008), predicts mortality in HIV patients (Hunt *et al.*, 2014; Kalayjian *et al.*, 2010), is higher in HIV-positive patients who develop cardiovascular disease, and in immunological non-responders (Duprez *et al.*, 2009; Rosado-Sanchez *et al.*, 2019). These cytokines are significantly altered in the unstimulated serum or plasma cytokine profiles of HIV/TB co-infected patients (Benjamin *et al.*, 2013; Farr *et al.*, 2018; Rambaran *et al.*, 2021; Subramanyam *et al.*, 2004). Using IFN- γ and IL-6 in combination best discriminate HIV-patients with and without TB (Farr *et al.*, 2018). Here, the serum metabolic profiles of untreated HIV/TB co-infected patients (comparison 2) were mainly characterised by changes in amino acids, metabolites originating from the gut microbiota, metabolites related to energy production, carbohydrates, and lipids (and related metabolites). This is in line with previous blood-based metabolomics studies done on untreated HIV (Li *et al.*, 2018; Munshi *et al.*, 2013; Peltenburg *et al.*, 2018; Sitole *et al.*, 2019) and TB patients (Collins *et al.*, 2020; Ding *et al.*, 2020; Vrieling *et al.*, 2019; Vrieling *et al.*, 2018). Since there is overlap between the blood metabolic profiles of HIV infection and TB patients (Liebenberg *et al.*, 2021), one can expect that HIV/TB co-infected patients would show similar or related metabolites to those reported in

previous studies done on HIV infection or TB alone, although the trends (increased or decreased) may differ.

Here, IFN- γ was significantly increased in the untreated HIV/TB co-infected group relative to healthy controls, and only marginally compared to the other diseased groups. This pro-inflammatory cytokine induces the enzymes guanosine triphosphate cyclohydrolase (GCH) and IDO1 (Katz, 2008; Neurauter *et al.*, 2008). GCH produces tetrahydrobiopterin (BH₄) and neopterin, which are essential cofactors to enzymes responsible for the production of monoamine neurotransmitters and nitric oxide (Neurauter *et al.*, 2008). Increased phenylalanine concentrations were observed in the untreated TB and untreated HIV/TB co-infected cohorts relative to healthy controls (Table 4.2 and Table S5). Elevated phenylalanine has been reported in biofluids collected from untreated TB (Luies & Loots, 2016; Vrieling *et al.*, 2019; Vrieling *et al.*, 2018; Weiner *et al.*, 2012; Zhou *et al.*, 2013) and untreated HIV (Ghannoum *et al.*, 2013; Sitole *et al.*, 2019; Zangerle *et al.*, 2010) patients. This has been ascribed to a decreased phenylalanine hydroxylase (PAH) activity (Ghannoum *et al.*, 2013; Zangerle *et al.*, 2010), due to a BH₄ deficiency (Zangerle *et al.*, 2010) or compromised insulin secretion (Luies & Loots, 2016). Since the IFN-influenced BH₄ is sensitive to oxidation, such as during high levels of OS, it may be highly labile in co-infected patients (Fuchs *et al.*, 2001; Widner *et al.*, 2001) Awodele *et al.*, 2012; Madebo *et al.*, 2003). Macrophages and dendritic cells produce neopterin at the expense of BH₄. Neopterin is increased during both HIV infection (Munshi *et al.*, 2013) and TB (Isa *et al.*, 2018) and this is even more pronounced during HIV/TB co-infection (Immanuel *et al.*, 2005). Neopterin has also been shown to predict the development of active TB in HIV-positive patients six months before a dramatic increase in VL and reduction in CD4 T cell count. Although neopterin was not detected in this study, the continued immune activation during HIV/TB co-infection (Sharan *et al.*, 2020) likely contributes to BH₄ depletion via neopterin overproduction, decreasing PAH activity. Thus, the increased phenylalanine levels may be ascribed to OS and immune activation.

Microbiota metabolism is an essential component of human metabolism (Gill *et al.*, 2006). Phenylalanine and tryptophan metabolism by gut microbiota yields cresol and indole intermediates (Gryp *et al.*, 2020; Harrison *et al.*, 2020; Vanholder *et al.*, 1999). While previously shown to be elevated during HIV infection (Cassol *et al.*, 2014; Cassol *et al.*, 2013; Ghannoum *et al.*, 2013), p-cresol derivatives (2,4-di-tert-butyl-p-cresol and 2,4-di-tert-butyl-phenol) and benzoic acid (Beloborodova *et al.*, 2012) were reduced in the untreated HIV/TB co-infected group relative to healthy controls here (Table 4.2). The impaired gut mucosal integrity, subsequent microbial translocation, and microbiome dysbiosis (which is also a feature of TB) induced by HIV infection (Epple *et al.*, 2009; Hu *et al.*, 2019; Vujkovic-Cvijin & Somsouk, 2019) are reflected in the pathomorphological changes seen in the small intestine of HIV/TB co-infected patients. These changes include a reduction in the absorptive surface area, a reduced absorption rate due to sclerotic damage, a decreased functional activity of enterocytes, and increased oxidative damage to proteins, relative to healthy controls (Todoriko *et al.*, 2021). Considering this, the decreased

concentrations in the p-cresol derivatives [and of norvaline, another microbial product (Soini *et al.*, 2008)] result from an altered microbiota and increased OS (Todoriko *et al.* (2021), given the antioxidant properties of these metabolites. While 2,4-di-tert-butyl-phenol has not been detected in human blood before it does not exclude the possibility that it could be detected in this biofluid. Given the very high probability of increased gut mucosal permeability, compounds from food and gut microbes could enter the bloodstream. This is especially likely since this compound typically originates from food according to the Human Metabolome Database and has been detected in faeces (Raman *et al.*, 2013). The increased levels of IL-6 in this cohort, which is predictive of anorexia in co-infected patients (Van Lettow *et al.*, 2005), likely results in decreased food intake and therefore decreased levels of antioxidants, which are typically used as food preservatives (Raccach, 1984). The levels of IL-6 correlate with immune activation, the extent of dysbiosis (making extensive dysbiosis very likely in this cohort) and the activity of tryptophan catabolism (through kynurenine) during HIV infection (Vujkovic-Cvijin & Somsouk, 2019).

An altered tryptophan metabolism via the kynurenine pathway as a result of increased IDO1 activity is a well-established consequence of various inflammatory conditions, including HIV (Dagenais-Lussier *et al.*, 2016; Gostner *et al.*, 2015; Jenabian *et al.*, 2013; Li *et al.*, 2018; Peltenburg *et al.*, 2018; Sitole *et al.*, 2019), TB (Almeida *et al.*, 2009; Gautam *et al.*, 2018; Isa *et al.*, 2018; Luies & Loots, 2016; Weiner *et al.*, 2012) and HIV/TB co-infection (Adu-Gyamfi *et al.*, 2017; Collins *et al.*, 2020). The indole products of tryptophan catabolism in the gut microbiota can be produced endogenously in an IL-4 inducible manner in T cells and dendritic cells (Zhang *et al.*, 2020). The importance of tryptophan catabolism by the gut microbiota during co-infection is reflected in the decreased levels of tryptophan, 3-indolepropionic acid (IPA), 3-indoleacetic acid (IAA), and 3-indolelactic acid (ILA) in the untreated HIV/TB co-infected group when compared to the healthy controls. Tryptophan was decreased in all diseased groups relative to healthy controls, and this was most pronounced in the untreated HIV/TB group, although not statistically significant (Table 4.2). There was also a significant positive correlation between IAA, ILA and tryptophan (Figure 4.4). IPA, IAA, and ILA all have roles as antioxidants, ameliorate inflammation (Chimerel *et al.*, 2014; Negatu *et al.*, 2020; Tuomainen *et al.*, 2018; Xiao *et al.*, 2014), and regulate intestinal immunity by activating the aryl-hydrocarbon receptor transcription factor (Gutiérrez-Vázquez & Quintana, 2018; Zhang *et al.*, 2020). Increased IPA has been associated with enhanced intestinal barrier integrity (Jennis *et al.*, 2018) and has mycobacterial-specific antibiotic activity (Negatu *et al.*, 2020), thus its decrease could relate to the loss of gut barrier integrity induced by HIV (Brenchley *et al.*, 2006), intestinal damage observed in co-infected patients (Todoriko *et al.*, 2021), and possibly TB progression in HIV-patients (Negatu *et al.*, 2020). Typically, the catabolic products of tryptophan would increase as tryptophan decreases, however, the advanced disease state (as per CD4 T cell counts in Table 3.1), high levels of OS (Awodele *et al.*, 2012; Madebo *et al.*, 2003), microbiome dysbiosis (Hu *et al.*, 2019; Vujkovic-Cvijin & Somsouk, 2019), and decreased endogenous production of these metabolites due to the decreased levels of IL-4 (Table 4.1, not statistically significant) in the co-infected patients may account for this observation.

These data and previous reports confirm that HIV/TB co-infected patients have a significantly increased tryptophan catabolism (Adu-Gyamfi *et al.*, 2017; Collins *et al.*, 2020) compared to patients with either disease state alone, and suggests that the gut microbiota-derived tryptophan catabolites are important biomarkers for the co-infected state. This could be used in conjunction with the kynurenine/tryptophan ratio, which has been suggested as a biomarker for co-infection (Adu-Gyamfi *et al.*, 2017).

To place these results in context to previous HIV and gut-related findings, HIV infection causes Th17 cell depletion, resulting in an imbalanced Th17:Tregs ratio which has been correlated to the induction of IDO1 and increased levels of microbial products in HIV-patients (Favre *et al.*, 2010). The IDO1 activation initiates a feedback loop that contributes to systemic inflammation and immunosuppression (induced by the higher numbers of Tregs compared to Th17 cells). Seeing that these changes result in irreversible sclerotic damage in the small intestine (Todoriko *et al.*, 2021), targeting IDO1 (Collins *et al.*, 2020) and maintaining gut health should be considered in the development of host-directed therapies.

Apart from its previously mentioned association with anorexia, IL-6 is also an indicator of severe inflammation (Van Lettow *et al.*, 2005), and negatively affects muscle turnover (resulting in net muscle breakdown) (Babu *et al.*, 2019; Van Hall *et al.*, 2008). IL-6 was increased in both untreated patient groups here. The mechanisms underlying the higher risk of malnutrition (Swaminathan *et al.*, 2008) and more severe cachexia (Lucas *et al.*, 1994; Villamor *et al.*, 2006) experienced by HIV/TB co-infected patients remain poorly understood. Prolonged periods of high energy requirements, such as during chronic infection and inflammation, with concomitant infection-associated anorexia, malaise and malabsorption, results in proteolysis for energy production. In TB patients with high inflammation (those with IL-6 and other pro-inflammatory cytokines) the amino acid levels are comparatively lower (Weiner *et al.*, 2012). The use of stored amino acids for energy is a last resort resource and limited since essential proteins and muscle mass needs to be preserved. During the initial stages of starvation, the free amino acids would increase but quickly deplete due to poor feeding and malabsorption. Decreased levels of the essential amino acids methionine, threonine (and their catabolic product, 2-aminobutyric acid), and lysine suggest such a state in the untreated HIV/TB co-infected cohort (Table 4.2). These changes may also be related to insulin resistance, which has previously been associated with HIV infection and TB (Pedro *et al.*, 2018b; Philips *et al.*, 2017). Modulation of insulin levels has far-reaching metabolic consequences, beyond the control of blood glucose levels, as this hormone also affects lipid and protein metabolism (James *et al.*, 2017). The decreased essential amino acids, the decreased 3-hydroxypropionic acid and hydroxyproline, increased levels of a proline derivative (proline, 1-methyl methyl ester) and 3-hydroxyisovaleric acid (a leucine catabolite) (Table 4.2), all support an impaired protein balance in HIV/TB co-infected individuals (Paton *et al.*, 2003; Van Lettow *et al.*, 2003). Decreased energy availability is also evident by the decreased levels of pyruvate (although not significant) and glycerol-3-phosphate detected in this patient group comparatively. Glycerol-3-phosphate is used for the rapid regeneration of reducing equivalents for oxidative phosphorylation (Mugabo *et al.*, 2016) in the brain and skeletal muscle. The

glycerol-3-phosphate shuttle also links fatty acid metabolism, which is typically dysregulated during HIV infection (Williams *et al.*, 2011) and TB (Weiner *et al.*, 2018), to glycolysis and oxidative phosphorylation (Mráček *et al.*, 2013), allowing fatty acids to be preferentially used for energy. These results, and the previously mentioned gut damage in co-infected patients in combination with the correlation of insulin resistance with lipopolysaccharide (a marker of intestinal permeability) in HIV infected individuals (Pedro *et al.*, 2018a), suggest various unexplored avenues of research relating to the HIV/TB co-infected state.

The catabolism of the neurotransmitter 4-aminobutyric acid (GABA) results in the production of 3,4-dihydroxybutyric acid (DHBA) and 2,4-DHBA (Gibson & Nyhan, 1989). These hydroxy fatty acids were increased significantly in the untreated co-infected group relative to healthy controls, and their levels were highest in this group compared to the other diseased groups. 3,4-DHBA induces a feeling of satiety possibly due to its structural similarity to 3-hydroxybutyric acid (Shimizu *et al.*, 1984), which is used as an alternative fuel substrate during fasting. These metabolites are also linked to poor overall health and nutritional status induced by an extremely low energy intake (Hilvo *et al.*, 2016). This further indicates cachexia, which is characteristic of both HIV infection and TB (Semba *et al.*, 2010). Elevated 3,4-DHBA has also been detected in the urine of unsuccessfully treated TB patients (Luies *et al.*, 2017), further confirming its association with a progressive phenotype. However, the exact mechanism of increased 3,4-DHBA and 2,4-DHBA concentrations have yet to be clarified. Lysine, the second most significantly altered essential amino acid, is abundant in muscle tissue and is essential for carnitine synthesis, and therefore for fatty acid metabolism (Cynober, 2002). As such, 3,4-DHBA and lysine were subjected to correlation analysis to determine if this could yield new insight into the mechanisms of cachexia. 3,4-DHBA correlated positively with 2,4-DHBA, phenylalanine, 3-hydroxyisovaleric acid, and various microbial metabolites (Figure 4.4), while negatively correlating with tryptophan, IAA, threonine, 2-ketoisocaproic acid (another intermediate of leucine catabolism), and tetrahydrofuran. Biologically, and in light of how IL-6 and IFN- γ relate to these metabolites, 3,4-DHBA would increase with more pronounced inflammation (as seen in comparisons 4 and 5, see Section 4.5.2) and microbial product translocation. Lysine correlated with other amino acids and the intermediates in their catabolic pathways (Figure 4.4), among which 2-hydroxybutyric acid, 2-ketobutyric acid, and 5-oxoproline associated with alterations to glutathione metabolism, OS, and energy distress (Landaas, 1975; Weiner *et al.*, 2012; Williams *et al.*, 2011). Lysine also correlated with 3,4-DHBA (adjusted p-value = 0.004), although not at the same level of significance as for the other correlations discussed. How this would relate to body composition changes and cachexia is unknown, and further investigation into the origin and effects of 3,4-DHBA in the context of cachexia in HIV infection and TB is needed.

A general decrease in the lipid-related metabolites was observed in the untreated HIV/TB group relative to the healthy controls, reflecting a lack of nutrition. Alpha-linolenic acid is an essential PUFA and is an important structural component of cell membranes (Blondeau *et al.*, 2015). The low CD4 counts found in

HIV-positive patients (Table 3.1 and S2) also result in reduced alpha-linolenic acid, as the phospholipid membranes of CD4 cells are composed of alpha-linolenic acid (Agostoni *et al.*, 1998). An alpha-linolenic acid deficiency is associated with viral inactivation failure during infection, resulting in virus proliferation and immunodeficiency (Das, 1985). PUFAs and glycerophospholipids are also important for distinguishing co-infected patients who develop IRIS from those who do not (Silva *et al.*, 2019). Decreased levels of dodecanal and linoleyl alcohol were observed in the untreated co-infected group (Table 4.2), implicating decanoic and octadecanoic acid metabolism (Rizzo, 2014) as alternative energy substrates (Zhang *et al.*, 2018). These fatty acids are typically produced as intermediates of glycerophospholipid (specifically plasmalogens) metabolism and indicate alterations in membrane turnover. Previously reported decreased plasmalogens during HIV infection (Peltenburg *et al.*, 2018; Scarpellini *et al.*, 2016), is supported by the reduced capric monoethanolamide concentrations in our study, an intermediate of plasmalogen phospholipids (Nagan & Zoeller, 2001). These lipids are essential to membrane structure and function, immunity, and serve as antioxidants to protect against lipid peroxidation (Rizzo, 2014). When these lipids are peroxidised, hydrocarbons may be formed as a penultimate or final product. Thus, the alterations in the lipid-related metabolites could also be ascribed to oxidative damage to membranes (Giammarioli S, 1999; Williams *et al.*, 2017).

The altered carbohydrate metabolites that distinguished the untreated co-infected patients from healthy controls were of microbial and/or exogenous origin, and included increased rhamnose (Harathi *et al.*, 2016), mannitol (Desselberger, 2018), and 1-deoxypentitol (Silhavy *et al.*, 2010), as well as decreased tetrahydrofuran (Imbenotte *et al.*, 2003; Ong *et al.*, 1991) and 2-deoxy-D-pentofuranose. The increased mannitol may additionally indicate subclinical co-infections in the co-infected patients and/or a more virulent microbiota composition (Megson *et al.*, 1996; Niehaus & Flynn, 1994). Gut inflammation also has an impact on mannitol permeability in HIV-patients (Epple *et al.*, 2009), which could explain the increased mannitol observed here. The altered levels of *Mtb*-related metabolites are likely due to a higher bacterial load in the untreated HIV/TB co-infected patients, the majority of which were severely immunosuppressed. It is worth noting that it is difficult to distinguish sugars on GC-MS profiles due to the “chiral-blindness” of mass spectrometry (Kanie *et al.*, 2016) and may represent a future aspect to investigate further on another analytical platform.

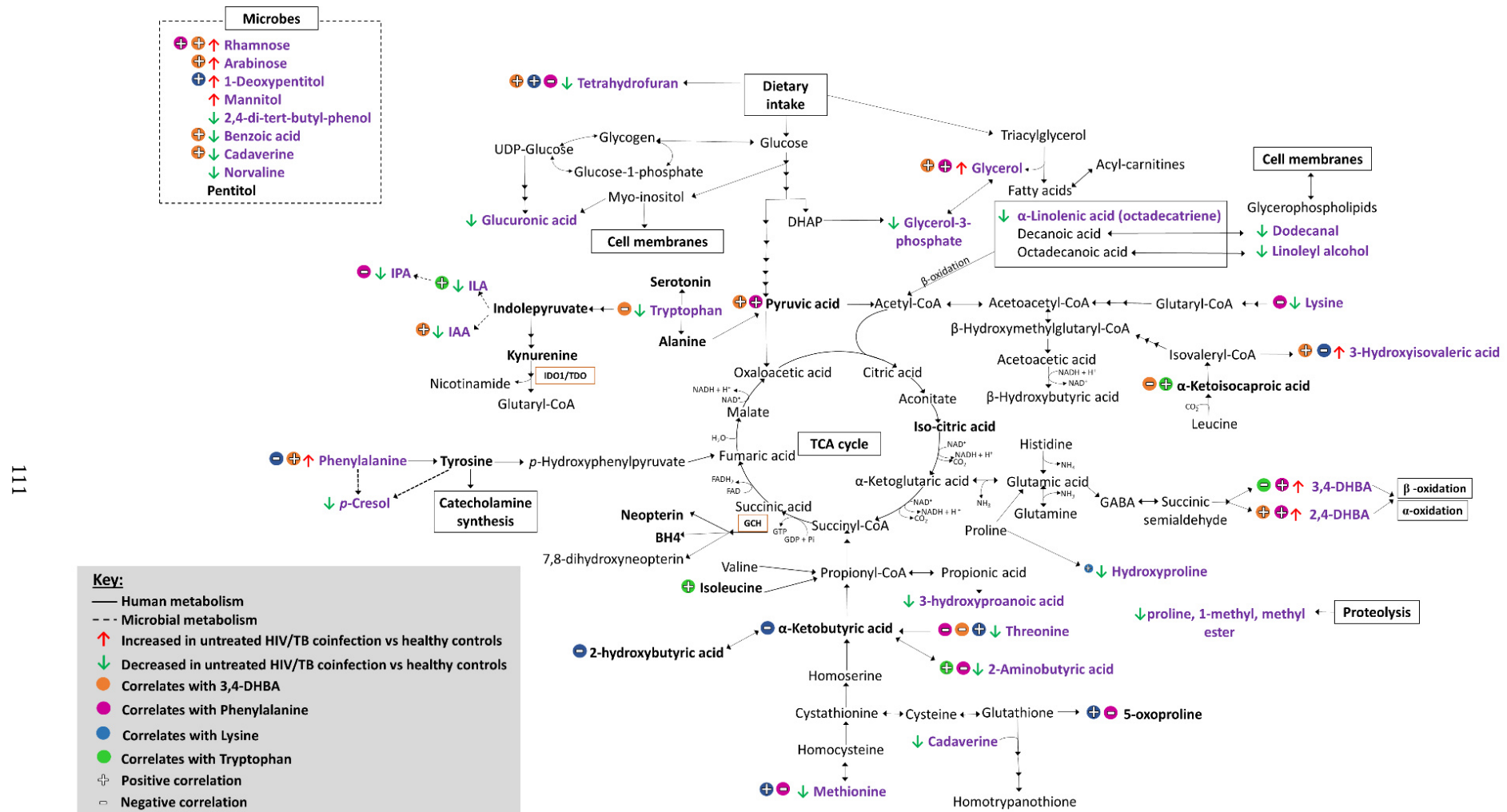


Figure 4.4: A summary of the metabolic effects of HIV/TB co-infection (comparison 2) and the correlations of 3,4-dihydroxybutyric acid, phenylalanine, lysine, and tryptophan with other metabolites. Abbreviations: 3,4-DHBA: 3,4-dihydroxybutyric acid; 2,4-DHBA: 2,4-dihydroxybutyric acid; IDO1: indoleamine-2,3-dioxygenase 1; TDO: tryptophan dioxygenase; IFN- γ : interferon-gamma; GCH: GTP cyclohydrolase; IPA: 3-indolepropionic acid; ILA: 3-indolelactic acid; IAA: 3-indoleacetic acid.

4.5.2 The immunometabolic effects of HIV infection and its treatment on TB patients

Although there were no statistically significant differences between the cytokine profiles of the diseased groups (Figure 4.1, comparisons 4–6), trends could be identified (Table 4.1). HIV infection is primarily associated with altered IL-6 and IL-10, and IL-17 to some extent (Mlambo *et al.*, 2019; Williams *et al.*, 2013), whereas TB induces changes to IFN- γ , TNF- α , IL-6, and IL-17 (Kumar *et al.*, 2019; Rambaran *et al.*, 2021; Verbon *et al.*, 1999). The relatively low IL-2 and IL-4 in the untreated co-infected group, and the increasing trend of these with ART, result from reduced CD4 T cells and ART-induced immune reconstitution, respectively, as these cytokines are predominantly produced by activated CD4 T cells (Kedzierska & Crowe, 2001) and are involved in cell proliferation (Ivanova & Orekhov, 2015; Spolski *et al.*, 2018). However, these cytokines did not correlate with the CD4 T cell count (Figure 4.2D). The source of IL-10 is dynamic as it can be produced by various cell types including Th1, Th2, Th17, Tregs, B cells and macrophages (Redford *et al.*, 2011). The levels of IL-10 and IL-6, which inhibit TNF- α secretion (Aderka *et al.*, 1989; Armstrong *et al.*, 1996), were higher in the untreated co-infected group relative to all other groups, which contributed to the reduced TNF- α in the co-infected patients relative to the TB patients. This could also contribute to TB progression and increased immune activation in co-infected patients, as overexpression of IL-10 has been linked to an inability to control *Mtb* (Turner *et al.*, 2002), and TNF- α is essential to maintaining granuloma integrity (Cavalcanti *et al.*, 2012). Although IL-17A levels were only slightly higher in the untreated HIV/TB co-infected group relative to the TB group, its levels in the HIV/TB co-infected group on ART was comparable to that of healthy individuals and correlated positively with IL-6, IFN- γ and negatively with the CD4 T cell count. IL-17 is important in the success of ART in HIV/TB co-infected patients, showing the highest number of correlation network connections to other cytokines in the untreated co-infected patients, which were reduced upon suppressive ART (Du Bruyn *et al.*, 2021). This strengthens the association between IL-17 and VL, which correlate positively in HIV-patients (Mlambo *et al.*, 2019). The decreased Th17:Treg ratios in HIV-patients described earlier create the expectation that IL-17 levels would decrease in the HIV/TB co-infected patients compared to the TB patients. However, some subsets of Tregs also produce IL-17 and may be partly responsible for its increase in the co-infected group relative to the TB group, especially since these cells were also found to decrease when ART commences (DaFonseca *et al.*, 2015), which is in line with the trend observed here. These trends suggest a failure of immunoregulation, as regulatory and pro-inflammatory cytokines are concomitantly elevated, without showing significant improvement once ART is started. This supports previous reports of improved T cell levels when commencing ART, however their heterogeneity and functionality are not fully restored (Gosselin *et al.*, 2010).

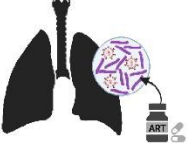
The metabolite changes to tetrahydrofuran, 1-deoxypentitol, glucuronic acid, glycerol-3-phosphate, cadaverine, IAA, alpha-linolenic acid and dodecanal were unique to the untreated HIV/TB co-infected patients when compared to those with TB. These metabolites represent the unique metabolic effects caused by HIV infection and/or the synergistic effects of HIV and *Mtb* during co-infection. 3,4-DHBA and

phenylalanine, which were previously elevated in TB patients relative to healthy controls (comparison 1), were further increased (with statistical significance) in co-infected patients (comparison 4), implying that these are metabolic effects of HIV infection and TB, as supported by literature (Che *et al.*, 2013; Liebenberg *et al.*, 2021; Sitole *et al.*, 2019; Weiner *et al.*, 2012; Zhong *et al.*, 2016). As previously mentioned, increased phenylalanine and neopterin are associated with a progressive phenotype, and neopterin inversely correlates with a CD4 T cell count (Immanuel *et al.*, 2005). This implies that phenylalanine and neopterin could be used in combination as prognostic markers in co-infected patients, or as indicators of TB in HIV-patients. This requires further investigation, given the above-mentioned limitations of this study.

Patients on ART experience inflammation and immune activation, although to a lesser degree than before treatment, as active viral replication is repressed (Hileman & Funderburg, 2017). Although ART did not generally alter the metabolic profile of the HIV/TB co-infected patients (comparison 5), the levels of alpha-linolenic acid, dodecanal and proline, 1-methyl, methyl ester were significantly different and partially recovered (although not to the levels in healthy controls). Also, mannitol levels were comparatively lower than those in the TB group (Table 4.2; similar to the level in healthy controls).

The metabolites identified as significantly different in comparisons 4 and 5, but which did not occur in the untreated co-infection profile, discussed in the previous section, will now be placed into biological context. Figure 4.5 summarises the immunometabolic effect of HIV infection and its treatment on patients with TB.

a

The immunometabolic effect of HIV infection and its treatment on TB patients		Effect of HIV infection on TB (Comparison 4)	
			
		Worsened	
Effect of ART on HIV/TB co-infection (Comparison 5) 	Improved to some extent	 Immune activation and inflammation	
	Minimal improvement/Worsened	 Energy crisis  Gut mucosal damage and microbiota dysbiosis  Oxidative stress	

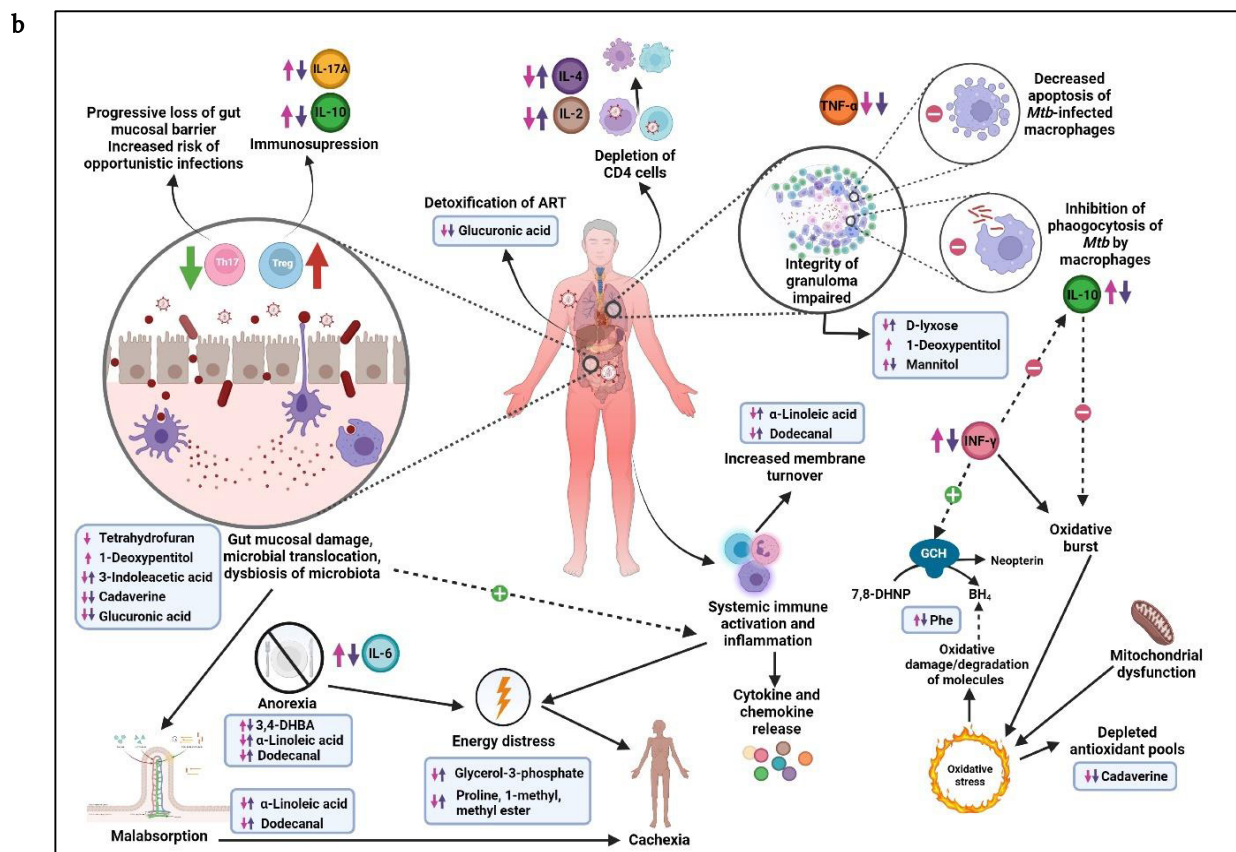


Figure 4.5: The immunometabolic effect of HIV infection and its treatment on TB. (a) A summary of the implications of the immunometabolic data obtained from comparisons 4 and 5. (b) A summary of the immunometabolic effect of HIV infection and its treatment on TB in the context of previous literature. Pink arrows represent the effect of HIV infection on TB (comparison 4) and purple arrows represent the effect of ART on HIV/TB co-infection (comparison 5), as observed in this investigation. Abbreviations: *Mtb*: *Mycobacterium tuberculosis*; ART: antiretroviral therapy; Th: T helper cell; Treg: T regulatory cell; IL: interleukin; TNF: tumour necrosis factor; BH₄: tetrahydrobiopterin; GCH: guanosine triphosphate hydroxylase; 7,8-DHNP: 7,8-dihydroneoterin-phosphate; 3,4-DHBA: 3,4-dihydroxybutyric acid; Phe: phenylalanine.

Cadaverine, a microbially-produced antioxidant polyamine, was increased in the TB patients relative to healthy controls (although not significantly; Table 4.2). This metabolite has been detected in HIV infection (Ghannoum *et al.*, 2013) and *Mtb*-related metabolomics studies (Loots, 2014; Schoeman *et al.*, 2012). Cadaverine was detected in reduced amounts in the untreated co-infected patients relative to the TB patients (comparison 4) and even further reduced in the co-infected patients on ART (although not significantly; comparison 5). Since the gut microbiota produces cadaverine, the reduction observed here is most likely ascribed to the altered gut health and microbiota composition in these groups (Meissner-Roloff *et al.*, 2012). Cadaverine forms part of the glutathione metabolic pathway, and as such, its reduction may also be associated with OS, which is known to persist even during ART (Williams *et al.*, 2017). Furthermore, decreased levels of glutathione with concomitantly increased indicators of lipid peroxidation has been reported in co-infected patients relative to patients with HIV infection only (with

and without ART) (Awodele *et al.*, 2012), which further supports exacerbated OS during HIV/TB co-infection.

Glucuronic acid, a metabolite of glucose involved in xenobiotic detoxification (Wiederschain, 2009), was reduced in the untreated and treated HIV/TB co-infected patients relative to the TB group (comparisons 4 and 6). Various members of the gut microbiota can cleave glucuronic acid from its conjugated xenobiotic or steroid during detoxification. The activity of the beta-glucuronidase responsible for this cleavage is variable among species (Dabek *et al.*, 2008; Wallace *et al.*, 2015). Thus, if the composition or functionality of the microbiome was altered in such a way as to decrease the number of species able to perform this cleavage (Ho *et al.*, 2019), it will alter the levels of unconjugated glucuronic acid. Increased and chronic xenobiotic detoxification due to the leaky gut caused by HIV, as well as the continual detoxification of ART in treated patients (Andrade *et al.*, 2011) contributes to the reduced levels of this metabolite.

Interestingly, D-arabinose, an essential component of the *Mtb* cell wall (Khoo *et al.*, 1996), was elevated in the TB patients relative to healthy controls (comparison 1) but reduced in HIV/TB co-infected patients (comparison 4). TB patients and HIV/TB co-infected patients on ART better phagocytose *Mtb*, releasing their cell wall components into the bloodstream, compared to the severely immunocompromised untreated HIV/TB. The absence of HIV (in TB patients) or reduced viral activity (in patients on ART) contributes to this, as the Nef protein of HIV inhibits macrophage phagocytosis (Mazzolini *et al.*, 2010) and clearance of the phagocytosed bacteria via autophagy (Bell & Noursadeghi, 2018), which also explains the trends in the concentrations of D-arabinose observed here.

When the co-infected patients on ART were compared to the healthy controls (comparison 3), the qualitative metabolic profiles were comparatively similar to that of untreated co-infected patients, with some quantitative differences and additional alterations to 2-amino-1-methyl-imidazol-4-ol (decreased, biological function unknown) and pyruvate (increased). Pyruvate was elevated in all diseased groups relative to healthy controls but decreased in the untreated co-infected patients relative to those with TB only (comparison 4). This trend in a metabolite central to the regulation of energy metabolism further stresses the significant effect of HIV infection on energy metabolism. In the HIV/TB group on ART, the levels of phenylalanine increased relative to healthy controls but were similar to that of TB patients. This indicates that ART relieved some of the immune activation induced by uncontrolled active HIV replication, but that the effects of TB, residual effects of HIV (of those treated co-infected patients for whom VL was available, only one was virally suppressed), and of ART itself (as the majority of patients had been on ART for more than one year) are still evident.

When comparing the co-infected patients on ART to the TB patients (comparison 6), significantly reduced tetrahydrofuran, glucuronic acid, cadaverine, IAA and elevated 1-deoxypentitol and 2,4-di-tert-butyl-phenol were detected. This implies reduced IAA in the co-infected group on ART relative to the TB group,

but it trended back to TB levels, i.e. was less significantly decreased than in the untreated co-infected group (see comparison 4 above). Although not statistically significant, IPA also trended back to a level comparable with that of TB patients, while ILA was comparable between the untreated and treated co-infected groups. Thus, the alterations to ILA, IPA, and ILA are more pronounced in combination with HIV infection than during TB alone, with only ILA not being altered when commencing ART.

4.6 Conclusions

The cytokine profile of untreated HIV/TB co-infected individuals was mainly characterised by elevated IL-6 and IFN- γ , cytokines that are typically increased during HIV infection, TB, and HIV/TB co-infection. There were no significant differences between the cytokine levels of the diseased groups, although the observed trends suggest a failure of immunoregulation. The roles of IL-10 and IL-17 during co-infection warrant further investigation in light of the trends observed here and those reported in previous literature. The metabolic profile of the HIV/TB co-infected individuals shows significant overlap with profiles previously described for HIV infection and TB, with major areas affected including amino acids (involved in immune activation and antioxidant functions), lipids, and their related intermediates (as it relates to immune cell activation and phagocytic processes), and metabolites related to central energy metabolism and gut dysbiosis. These alterations suggest considerable energy distress, which is reflected in those metabolites associated with increased amino acid catabolism and partially explained by high levels of the satiety-inducing metabolites 3,4-DHBA and 2-DHBA. These metabolites also suggest a mechanism by which cachexia is mediated in HIV infection, TB, and HIV/TB co-infection. The large proportion of the altered microbial metabolites, suggests that gut microbiome dysbiosis, damage of the intestinal mucosa and potential microbial translocation may be more severe during HIV/TB co-infection. This may be mediated by, and contribute to, increased immune activation, inflammation, and subsequent OS, due to concurrent HIV and *Mtb* infection. As some of these metabolites could be related to *Mtb* directly, these results also reflect the increased success of *Mtb* during co-infection. The energy distress also contributes to the lipid alterations observed, but as these are mainly related to phospholipids, it most likely is associated with the chronic immune activation in these patients, as immune cells are perpetually being produced and remodelled for activation. This study provides a preliminary overview of the immunometabolic profile of HIV/TB co-infected patients, untreated and treated for HIV, and as such, these findings need to be investigated in greater detail. Further investigation on this very important global health issue could benefit using (i) a larger cohort, (ii) with VL and CD4 T cell counts for all patients (including HIV-negative TB patients), (iii) with other clinical information (such as body composition measurements, chest X-rays, bacterial load, etc.), (iv) in a longitudinal context, and (v) with confirmatory experiments, such as enzyme assays.

4.7 References

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CHAPTER 5: FINAL CONCLUSIONS

This chapter summarises the main findings of this investigation with a focus on the implications thereof for future studies with similar designs. Further research prospects will also be discussed.

5.1 Concluding summary

There is a considerable history of TB research given its long-prevailing resilience, despite arguably effective treatment approaches. The emergence of HIV/AIDS in the 1980s, and the consequent spread to pandemic proportions, sparked massive research efforts into its management and eradication. The importance of investigating HIV/TB co-infection was quickly realised, although the inherent complexity of such a state has left research in this area comparatively sparse, despite being a major force driving the progression of both these pandemics. Recently, interest in HIV/TB co-infection has increased, and with the availability of new, more sensitive and higher-throughput techniques, the complexity of the problem can be better matched to the available research techniques.

The immune and metabolic alterations experienced by the co-infected host have been extensively investigated and applied to the development of better diagnostic, prognostic, and management techniques, although various aspects of HIV infection, TB and the improved treatment thereof, remains to be elucidated. Although there is a considerable amount of research into how the immune response is altered during HIV/TB co-infection, the metabolic alterations have as yet not received as much attention, with such investigations mostly hindered due to the limited scope of prior research methodologies. Metabolomics has overcome much of these in the context of HIV infection and TB separately, but has not been systematically applied to the co-infected state. This investigation provided a preliminary overview of the immune and metabolic changes induced by HIV/TB co-infection, using untargeted metabolomics.

The systemic nature of blood, and the abundance of immune cells it contains, make it the ideal sample type for the investigation of immune function and the metabolic state of an individual. Once the serum samples had been collected and analysed, the repeatability of the methodologies and apparatuses had to be assessed, and the data generated validated for use to answer the aims of this investigation. Due to cytokines occurring at levels below the detection limit of the CBA kit, the cytokine dataset was only used to report on trends and was not correlated to the metabolomics data directly. After a batch effect in the metabolomics data had been corrected for based on the QC samples, the data was deemed reliable for use in the identification of important metabolites as well as the biological interpretation thereof. Given the small size of the cohorts, the purpose of this investigation was not to identify cytokine and metabolic markers that are predictive of HIV/TB co-infection, but rather to explore the immunometabolic alterations in the HIV/TB co-infected host holistically, providing the groundwork for future studies.

HIV/TB co-infection was characterised by increased amounts of the pro-inflammatory cytokines IFN- γ and IL-6, in conjunction with other trends that were not statistically significant, but which support previous literature suggesting a failure of immunoregulation in these patients, which worsens the associated pathology. The altered metabolic profiles, in turn are ascribed to the high levels of immune activation, OS, and most prominently damage to the gut mucosa and dysbiosis of the microbiota, all of which contribute to a cachexic state, previously associated with HIV/TB co-infection. Although there were no statistically significant differences between the cytokine levels in the diseased groups, the trends observed could be linked to known effects of HIV on *Mtb* during HIV/TB co-infection, which contribute to the dysfunction of immunoregulation observed in co-infected individuals. ART reversed these trends to some extent in all cytokines, except for TNF- α , which was further decreased in those patients on ART. By comparing the metabolic profiles of TB patients to those with HIV/TB co-infection, it was determined that HIV infection primarily affected metabolites associated with the microbiota and energy metabolism, contributing to the increased disease burden during co-infection, primarily through its detrimental effects on gut health. Some of the metabolic trends were reversed by ART to some extent, although typically not to levels comparable to healthy controls.

HIV infection and TB are heterogeneous conditions, and the outcome for a particular patient depends, to a great extent, on that patient's overall state of health, genetic and environmental factors, lifestyle, amongst others. This becomes even more prominent in HIV/TB co-infection, as the trajectory of the infection occurring first (typically HIV infection) and the timing of the development of the second disease (which is TB in this case), could greatly affect the immunometabolic state of a patient at a particular point in time. As such, the first step in a systematic approach to the investigation of HIV/TB co-infection should be characterisation studies in an explorative context. Furthermore, it should also be considered that the immunometabolic state of a co-infected individual in a sub-Saharan or other developing countries, might inherently be different from that of a patient in a developed country. As the burden of HIV infection and TB is by far the greatest in the developing world, obtaining samples to study from such countries should take priority. However, for true control of the HIV/AIDS and TB pandemics, a study in all populations affected by these diseases is necessary, and by comparison, may provide additional insights.

5.2 Future prospects

Administration of supportive treatments in conjunction with ART and anti-TB treatment, such as antioxidants, essential or highly labile nutrients (such as BH₄), and pre- and probiotics (given the significant proportion of microbial metabolites and other metabolites relating to gut health identified in this study), may be beneficial to patients with HIV infection, TB or both. The importance of early diagnosis has been previously proposed on a number of occasions, and is supported by literature showing that if the intestinal damage caused by HIV can be ameliorated and VL controlled early in the course of infection, much of the effects of HIV during HIV/TB co-infection may be significantly reduced. Studies

characterising the composition and functionality of the gut microbiome of HIV/TB patients relative to those with HIV or TB alone, would be highly complementary and allow for better mechanistic insight into the interplay of the gut microbial and human metabolism during HIV/TB co-infection. Measuring specific markers of intestinal damage, such as assessing microbial translocation by measurement of lipopolysaccharides, may also provide better context to metabolic interpretations.

This study could be repeated using a larger patient cohort, and adding other metabolomics platforms, in order to get a more comprehensive coverage of the metabolome. Based on these results, a semi-targeted metabolomics approach specific for amino acids and/or lipids would serve well in addition to the untargeted approach used here.

The ultimate design for a study such as this would be that the same patients, from a large cohort (at least 30 individuals in a group) would be followed before and after the development of TB or of HIV infection, as well as before and after the initiation of anti-TB treatment and ART. In this way, the observed changes could be ascribed with a higher level of certainty to a specific infection and/or treatment, allowing the areas of synergy to be elucidated. This is, however, an ideal and would be very difficult to achieve in practice. Other considerations for future study designs that would at least fulfil some of the requirements for the ideal are the following:

- Including untreated HIV-positive patients in addition to untreated TB patients as diseased controls in HIV/TB co-infection studies.
- Longitudinal studies in any configuration, including patients with HIV and/or TB alone, HIV/TB co-infection, and all of the aforementioned with and without HIV and/or TB treatment.
- Collecting different biofluids (such as sputum, saliva, blood, urine, and faeces) and cells such as peripheral blood mononuclear cells from the same patients.
- Measuring clinical parameters as part of the study design, rather than passively relying on clinics to provide such information. Parameters such as CD4 T cell count (even for HIV-negative TB patients), VL, bacterial load, chest X-rays and other typical clinical parameters (such as insulin levels) would greatly improve the level of certainty that can be ascribed to interpretations of the immunometabolic data.

Furthermore, assessing a wider range of cytokines and chemokines in conjunction with metabolic measurements, and a parallel analysis of which cell types produce which cytokines, would allow for a more extensive interpretation of the origin of metabolic and phenotypical changes observed. Specifically, literature has demonstrated the importance of IL-12 and interferon-induced chemokines such as IP-10 (or CXLC-10) during HIV infection, TB, and HIV/TB co-infection, thus further investigation into this is warranted. Combining metabolomics with enzyme assays for amino acid catabolising enzymes would allow for more definite mechanistic conclusions.

APPENDIX A: SUPPLEMENT TO CHAPTER 4

Table S1: Fisher's exact tests for categorical variables.

Count

Sex

Group Crosstabulation

		Group				
		HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB+/Tn+	Total
Gender	Female	16	9	4	8	37
	Male	13	13	5	4	35
Total		29	22	9	12	72

Sex

Count

Age

Group Crosstabulation

		Group				
		HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB+/Tn+	Total
Age	18-30	12	10	4	3	29
	30-50	15	9	5	8	37
	50-70	2	3	0	1	6
Total		29	22	9	12	72

Age

Count

Other diseases/conditions

Group Crosstabulation

		Group				
		HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB+/Tn+	Total
OtherCondition	No	23	20	8	11	62
	Yes	6	2	1	1	10
Total		29	22	9	12	72

Other diseases/conditions

Count

Smoking status

Group Crosstabulation

		Group				
		HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB+/Tn+	Total
Smoking	No	20	11	7	9	47
	Yes	9	11	2	3	25
Total		29	22	9	12	72

Smoking status

Count

CD4 Tcell count [advanced disease (< 200 cells/mm³) versus not advanced (> 200 cells/mm³)]

Group Crosstabulation

		CD4		
		Advanced	Not advanced	Total
Group	HIV+/TB+/Tn-	7	2	9
	HIV+/TB+/Tn+	11	1	12
Total		18	3	21

CD4 Tcell count [advanced disease (< 200 cells/mm³) versus not advanced (> 200 cells/mm³)]

Count

Other diseases/conditions

Group Crosstabulation

		Group				
		HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB+/Tn+	Total
OtherCondition	No	23	20	8	11	62
	Yes	6	2	1	1	10
Total		29	22	9	12	72

Other diseases/conditions

Count

Smoking status

Group Crosstabulation

		Group				
		HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB+/Tn+	Total
Smoking	No	20	11	7	9	47
	Yes	9	11	2	3	25
Total		29	22	9	12	72

Smoking status

Count

CD4 Tcell count [advanced disease (< 200 cells/mm³) versus not advanced (> 200 cells/mm³)]

Group Crosstabulation

		CD4		
		Advanced	Not advanced	Total
Group	HIV+/TB+/Tn-	7	2	9
	HIV+/TB+/Tn+	11	1	12
Total		18	3	21

CD4 Tcell count [advanced disease (< 200 cells/mm³) versus not advanced (> 200 cells/mm³)]

Count

Other diseases/conditions

Group Crosstabulation

		Group				
		HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB+/Tn+	Total
OtherCondition	No	23	20	8	11	62
	Yes	6	2	1	1	10
Total		29	22	9	12	72

Other diseases/conditions

Count

Smoking status

Group Crosstabulation

		Group				
		HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB+/Tn+	Total
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		CD4		
		Advanced	Not advanced	Total
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Other diseases/conditions

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		Group				
		HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB+/Tn+	Total
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Other diseases/conditions

Group Crosstabulation

		Group				
		HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-		

Table S2: Detailed clinical data for HIV-positive cohorts.

Participant name	Group	Sex	CD4 count (cells/mm ³)	Viral load (HIV-1 RNA/mm ³)	ART combination	Duration of ART (months)
010_VM	HIV+/TB+/Tn-	Female	115	Unavailable	N/A	-
030_SP	HIV+/TB+/Tn-	Female	603	Unavailable	N/A	-
092_BM	HIV+/TB+/Tn-	Female	248	Unavailable	N/A	-
103_CT	HIV+/TB+/Tn-	Female	15	124	N/A	-
006_VC	HIV+/TB+/Tn-	Male	621	< 20	N/A	-
015_MR	HIV+/TB+/Tn-	Male	< 1	Unavailable	N/A	-
021_BC	HIV+/TB+/Tn-	Male	99	Unavailable	N/A	-
026_JM	HIV+/TB+/Tn-	Male	63	Unavailable	N/A	-
100_MN	HIV+/TB+/Tn-	Male	7	Unavailable	N/A	-
002_NS	HIV+/TB+/Tn+	Female	20	513509	FDC: Tenofovir 300mg; Emtricitabine 200mg; Efavirenz 600mg	79.8
005_CB	HIV+/TB+/Tn+	Female	602	Unavailable	FDC: Tenofovir 300mg; Emtricitabine 200mg; Efavirenz 600mg	18.8
008_NM	HIV+/TB+/Tn+	Female	39	Unavailable	FDC: Tenofovir 300mg; Emtricitabine 200mg; Efavirenz 600mg	10.0
017_SS	HIV+/TB+/Tn+	Female	211	Unavailable	FDC: Tenofovir 300mg; Emtricitabine 200mg; Efavirenz 600mg	81.9
084_YV	HIV+/TB+/Tn+	Female	192	Unavailable	FDC: Tenofovir 300mg; Emtricitabine 200mg; Efavirenz 600mg	84.8
094_PM	HIV+/TB+/Tn+	Female	186	76386	Lopinavir/Ritonavir; Abacavir; Lamivudine	135.9
095_KJ	HIV+/TB+/Tn+	Female	344	< detectable	FDC: Tenofovir 300mg; Emtricitabine 200mg; Efavirenz 600mg	74.2
101_VJ	HIV+/TB+/Tn+	Female	217	85	FDC: Tenofovir 300mg; Emtricitabine 200mg; Efavirenz 600mg	29.5
011_MM	HIV+/TB+/Tn+	Male	128	Unavailable	FDC: Tenofovir 300mg; Emtricitabine 200mg; Efavirenz 600mg	0.4
016_JU	HIV+/TB+/Tn+	Male	148	20	FDC: Tenofovir 300mg; Emtricitabine 200mg; Efavirenz 600mg	8.4
018_MG	HIV+/TB+/Tn+	Male	66	Unavailable	Combivir; Alluvia	Unavailable
096_SM	HIV+/TB+/Tn+	Male	39	216208	FDC: Tenofovir 300mg; Emtricitabine 200mg; Efavirenz 600mg	39.9

Abbreviations: FDC: fixed-dose combination.

Table S3: Normalised mean concentrations and standard deviations of cytokines.

Label	Normalised concentration (pg/ml)* [mean (standard deviation)]			
	HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB-/Tn+
IL-6	0.337 (0.278)	1.491 (0.504)	1.715 (0.651)	1.472 (0.528)
IFN- γ	-1.319 (1.108)	0.163 (0.586)	0.152 (1.115)	0.04 (0.672)
IL-10	0.186 (0.242)	0.326 (0.403)	0.523 (0.316)	0.407 (0.281)
IL-4	-0.156 (0.741)	-0.048 (0.561)	-0.745 (0.847)	-0.425 (0.688)
TNF- α	-0.871 (1.005)	-0.526 (0.943)	-0.753 (0.966)	-0.959 (1.08)
IL-17A	0.621 (0.762)	0.849 (0.833)	0.904 (0.626)	0.659 (0.687)
IL-2	-0.924 (0.821)	-0.995 (1.001)	-1.059 (0.822)	-1.021 (0.896)

*The colour gradient represents the range of values for each cytokine, with red being the highest and green the lowest.

Table S4: ANOVA results and PLS-DA variable influence on projection scores for the cytokine data.

Cytokine	ANOVA		PLS-DA				
	p-value	BH-corrected p-value	C1	C2	C3	C4	C5
IL-6	0.00000	0.00000	1.63950	1.57040	1.56470	1.55950	1.48440
IFN- γ	0.00000	0.00000	2.01210	1.87530	1.86460	1.85900	1.77180
IL-10	0.02833	0.06610	0.26033	0.24167	0.24475	0.25818	0.26166
IL-4	0.06207	0.10862	0.10989	0.48275	0.50944	0.52173	0.50855
TNF- α	0.56651	0.74828	0.30692	0.57264	0.58265	0.59374	0.64445
IL-17A	0.64138	0.74828	0.27415	0.59061	0.60617	0.60535	0.58429
IL-2	0.97530	0.97530	0.1197	0.22093	0.22061	0.23307	0.75723

Abbreviations: IL: interleukin; IFN: interferon; TNF: tumour necrosis factor; ANOVA: one-way analysis of variance; BH: Benjamini-Hochberg; PLS-DA: partial least squares-discriminant analysis; C1-5: principal components 1-5.

Table S5: Pearson's correlations between cytokines and between cytokines and CD4 T cell count.

	Pearson's r	p-value	BH-corrected p-value
Correlation with IL-6			
IFN- γ	0.632	<0.001	< 0.001
IL-10	0.428	< 0.001	< 0.001
IL-17A	0.266	0.025	0.043
TNF- α	0.162	0.176	0.246
IL-2	-0.125	0.297	0.347
IL-4	-0.051	0.673	0.673
Correlation with IFN- γ			
IL-6	0.632	< 0.001	< 0.001
IL-17A	0.463	< 0.001	< 0.001
IL-10	0.353	< 0.001	< 0.001
TNF- α	0.228	0.056	0.078

IL-2	0.141	0.240	0.280
IL-4	0.103	0.392	0.392
Correlation with IL-10			
IL-6	0.428	< 0.001	< 0.001
IL-17A	0.427	< 0.001	< 0.001
IFN- γ	0.353	< 0.001	0.004
TNF- α	0.257	0.030	0.042
IL-2	0.229	0.054	0.063
IL-4	0.007	0.951	0.951
Correlation with IL-4			
TNF	0.563	< 0.001	< 0.001
IL2	0.254	0.032	0.075
IL17A	0.235	0.049	0.085
INF	0.103	0.392	0.549
IL6	-0.051	0.673	0.786
IL10	0.007	0.952	0.951
Correlation with TNF-α			
IL4	0.563	< 0.001	< 0.001
IL17A	0.488	< 0.001	< 0.001
IL10	0.257	0.030	0.053
INF	0.228	0.056	0.078
IL6	0.162	0.176	0.205
IL2	0.111	0.357	0.357
Correlation with IL-17A			
TNF- α	0.488	< 0.001	< 0.001
IFN- γ	0.463	< 0.001	< 0.001
IL-10	0.427	< 0.001	< 0.001
IL-6	0.266	0.025	0.035
IL-4	0.235	0.049	0.057
IL-2	0.079	0.512	0.512
Correlation with IL-2			
IL4	0.254	0.032	0.113
IL10	0.229	0.054	0.127
INF	0.141	0.240	0.416
IL6	-0.125	0.297	0.416
TNF	0.111	0.357	0.417
IL17A	0.079	0.512	0.512
Correlation with CD4 T cell count			
IL-17A	-0.543	0.011	0.044
IL-6	-0.515	0.0168	0.045
IFN- γ	-0.368	0.101	0.201
IL-10	-0.273	0.231	0.310
IL-2	0.272	0.233	0.310
TNF- α	-0.084	0.717	0.807
IL-4	-0.057	0.807	0.807

Abbreviations: IL: interleukin; IFN: interferon; TNF: tumour necrosis factor; BH: Benjamini-Hochberg.

Table S6: The 47 serum metabolite markers that best describe the variation between the untreated HIV/TB co-infected group and the healthy controls.

Compound	Mean peak intensity (standard deviation)				ANOVA	
	HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB-/Tn-	HIV+/TB+/Tn+	p-value	BH-adjusted p-value
3,4-Dihydroxybutyric acid	0.093 (0.018)	0.164 (0.056)	0.239 (0.149)	0.178 (0.051)	< 0.0001	< 0.0001
Tetrahydrofuran	0.127 (0.021)	0.099 (0.018)	0.082 (0.021)	0.085 (0.023)	< 0.0001	< 0.0001
DL-Phenylalanine (unique derivatised mass = 120)	1.735 (0.298)	2.437 (0.683)	2.748 (0.692)	2.534 (0.746)	< 0.0001	< 0.0001
2,4-Di-tert-butyl-phenol	0.067 (0.014)	0.050 (0.009)	0.052 (0.014)	0.058 (0.01)	< 0.0001	< 0.001
Lysine	0.358 (0.125)	0.218 (0.105)	0.208 (0.097)	0.197 (0.065)	< 0.0001	< 0.001
1-Deoxypentitol	0.014 (0.009)	0.015 (0.007)	0.037 (0.032)	0.029 (0.011)	< 0.0001	< 0.001
3-Hydroxyisovaleric acid	0.077 (0.02)	0.141 (0.078)	0.184 (0.127)	0.127 (0.059)	< 0.0001	< 0.001
Analyte 622	0.018 (0.005)	0.014 (0.005)	0.012 (0.003)	0.011 (0.005)	< 0.0001	0.002
Analyte 628	0.361 (0.064)	0.311 (0.056)	0.254 (0.082)	0.272 (0.067)	< 0.0001	0.002
D-Glucuronic acid	0.161 (0.174)	0.207 (0.184)	0.087 (0.063)	0.072 (0.05)	< 0.0001	0.002
Hydroxyproline	0.283 (0.128)	0.176 (0.089)	0.158 (0.051)	0.155 (0.095)	< 0.0001	0.002
Glycerol-3-phosphate	0.030 (0.009)	0.024 (0.005)	0.020 (0.008)	0.021 (0.006)	< 0.0001	0.002
Threonine	0.821 (0.279)	0.573 (0.187)	0.463 (0.082)	0.600 (0.23)	< 0.0001	0.002
Phenylalanine (unique derivatised mass = 218)	0.438 (0.176)	0.705 (0.409)	1.036 (0.419)	0.776 (0.65)	< 0.0001	0.002
Glycerol (unique derivatised mass = 103)	0.009 (0.008)	0.018 (0.014)	0.030 (0.024)	0.022 (0.016)	< 0.001	0.002
Methionine	0.117 (0.031)	0.075 (0.029)	0.071 (0.026)	0.067 (0.026)	< 0.001	0.002
Acrylic acid 2-dimethylamino-ethyl ester	2.133 (0.427)	1.739 (0.507)	1.589 (0.331)	1.682 (0.204)	< 0.001	0.004
Benzoic Acid	0.142 (0.033)	0.102 (0.038)	0.088 (0.027)	0.101 (0.055)	< 0.001	0.007
Cadaverine	0.036 (0.011)	0.053 (0.033)	0.027 (0.013)	0.028 (0.022)	< 0.001	0.007
3-Indolelactic acid	0.030 (0.019)	0.019 (0.01)	0.018 (0.016)	0.012 (0.009)	< 0.001	0.007
3-Indoleacetic acid	0.067 (0.066)	0.072 (0.087)	0.027 (0.027)	0.031 (0.029)	< 0.001	0.009
Glycerol (unique derivatised mass = 73)	0.012 (0.015)	0.025 (0.026)	0.050 (0.046)	0.030 (0.026)	< 0.001	0.009
D-Arabinose (unique derivatised mass = 204)	0.020 (0.008)	0.044 (0.046)	0.023 (0.008)	0.035 (0.013)	< 0.001	0.011
Tryptophan	0.304 (0.131)	0.200 (0.103)	0.157 (0.103)	0.184 (0.123)	< 0.001	0.013
3-Indolepropionic acid	0.029 (0.04)	0.011 (0.014)	0.006 (0.008)	0.010 (0.01)	0.002	0.018
Linoleyl alcohol	0.062 (0.015)	0.047 (0.018)	0.040 (0.018)	0.049 (0.013)	0.002	0.018
alpha-Linolenic acid	0.209 (0.063)	0.173 (0.026)	0.148 (0.044)	0.183 (0.045)	0.002	0.018
Cyclobutanecarboxylic acid, 2-dimethylaminoethyl ester	0.029 (0.012)	0.022 (0.011)	0.017 (0.008)	0.018 (0.008)	0.001	0.018
Rhamnose	0.091 (0.03)	0.113 (0.035)	0.145 (0.064)	0.139 (0.046)	0.002	0.018
2-Amino-1-methyl-1H-imidazol-4-ol	1.363 (0.279)	1.045 (0.347)	1.285 (0.709)	0.995 (0.299)	0.002	0.021
2,6-di-tert-butyl-p-Cresol	0.064 (0.009)	0.054 (0.01)	0.056 (0.009)	0.058 (0.011)	0.002	0.021
Dodecanal	0.119 (0.019)	0.109 (0.011)	0.096 (0.028)	0.109 (0.007)	0.002	0.021
Pyruvate	0.615 (0.235)	1.034 (0.578)	0.723 (0.216)	0.865 (0.423)	0.003	0.029
3-Hydroxypropionic acid	0.029 (0.008)	0.025 (0.007)	0.022 (0.007)	0.021 (0.008)	0.004	0.033
D-Mannitol	0.123 (0.047)	0.175 (0.127)	0.230 (0.124)	0.117 (0.034)	0.004	0.036
Proline, 1-methyl-, methyl ester	0.022 (0.029)	0.071 (0.144)	0.220 (0.364)	0.032 (0.058)	0.004	0.036
Tetrahydro-pyran-2,4,5-triol	0.142 (0.037)	0.106 (0.033)	0.112 (0.044)	0.110 (0.03)	0.004	0.038
2-Aminobutyric acid	0.31 (0.147)	0.203 (0.091)	0.198 (0.079)	0.204 (0.104)	0.005	0.039
alpha-Linolenic acid	0.376 (0.088)	0.305 (0.087)	0.300 (0.068)	0.306 (0.061)	0.005	0.040
L-Norvaline	7.833 (1.242)	6.618 (1.177)	6.809 (1.394)	6.652 (1.208)	0.005	0.042

Capric monoethanolamide	0.144 (0.031)	0.12 (0.045)	0.109 (0.033)	0.123 (0.016)	0.006	0.044
Analyte 819	0.398 (0.086)	0.344 (0.096)	0.302 (0.058)	0.322 (0.071)	0.006	0.044
Carbonic acid, 2-dimethylaminoethyl ethyl ester	0.113 (0.026)	0.091 (0.033)	0.078 (0.032)	0.080 (0.029)	0.006	0.044
2,4-Dihydroxybutyric acid	0.039 (0.011)	0.055 (0.023)	0.084 (0.068)	0.057 (0.035)	0.006	0.044
Carbonic acid, 2-dimethylaminoethyl propyl ester	0.835 (0.161)	0.736 (0.171)	0.635 (0.114)	0.700 (0.138)	0.006	0.050
Analyte 706	0.037 (0.021)	0.023 (0.015)	0.021 (0.013)	0.020 (0.013)	0.007	0.050
D-Arabinose (unique derivatised mass = 217)	0.063 (0.056)	0.104 (0.067)	0.057 (0.049)	0.060 (0.019)	0.007	0.050

Table S7: PLS-DA VIP scores for metabolites identified as statistically significant using ANOVA.

Compound	PLS-DA VIP				
	C1	C2	C3	C4	C5
3,4-Dihydroxybutyric acid	2.954	2.870	2.834	2.809	2.755
DL-Phenylalanine (unique derivatised mass = 120)	2.578	2.504	2.472	2.447	2.410
Tetrahydrofuran	2.446	2.406	2.376	2.353	2.317
Lysine	2.418	2.349	2.321	2.296	2.255
2,4-Di-tert-butyl-phenol	2.302	2.279	2.263	2.241	2.207
3-Hydroxyisovaleric acid	2.253	2.188	2.163	2.140	2.100
Methionine	2.202	2.142	2.123	2.107	2.067
Hydroxyproline	2.199	2.136	2.112	2.089	2.051
Threonine	2.141	2.079	2.052	2.038	2.001
Acrylic acid 2-dimethylamino-ethyl ester	2.096	2.036	2.020	2.009	1.971
Glycerol (unique derivatised mass = 103)	2.009	1.967	1.941	1.921	1.890
Benzoic Acid	2.005	1.947	1.921	1.903	1.903
Glycerol-3-phosphate	1.993	1.951	1.925	1.907	1.878
Analyte 622	1.992	1.949	1.948	1.927	1.890
Phenylalanine (unique derivatised mass = 218)	1.913	1.872	1.849	1.832	1.814
D-Arabinose (unique derivatised mass = 204)	1.907	1.870	1.871	1.851	1.834
Glycerol (unique derivatised mass = 73)	1.832	1.795	1.773	1.754	1.724
2,6-di-tert-butyl-p-Cresol	1.827	1.808	1.793	1.774	1.751
Analyte 628	1.816	1.820	1.796	1.778	1.759
Tetrahydro-pyran-2,4,5-triol	1.809	1.761	1.738	1.720	1.706
2-Aminobutyric acid	1.796	1.746	1.728	1.713	1.692
alpha-Linolenic acid	1.793	1.742	1.734	1.745	1.714
L-Norvaline	1.784	1.744	1.728	1.718	1.708
2-Amino-1-methyl-1H-imidazol-4-ol	1.740	1.708	1.709	1.691	1.665
Tryptophan	1.736	1.694	1.677	1.687	1.658
Pyruvate	1.731	1.719	1.697	1.691	1.664
3-Indolepropionic acid	1.728	1.681	1.668	1.662	1.638
Cyclobutanecarboxylic acid, 2-dimethylaminoethyl ester	1.716	1.693	1.673	1.663	1.651
Linoleyl alcohol	1.702	1.654	1.660	1.642	1.613
3-Indolelactic acid	1.697	1.673	1.670	1.662	1.635
Rhamnose	1.686	1.662	1.648	1.631	1.622

Analyte 706	1.642	1.598	1.579	1.563	1.534
Capric monoethanolamide	1.635	1.588	1.603	1.586	1.556
Analyte 819	1.626	1.592	1.583	1.591	1.562
Carbonic acid, 2-dimethylaminoethyl ethyl ester	1.607	1.575	1.562	1.555	1.528
alpha-Linolenic acid	1.568	1.530	1.535	1.523	1.507
3-Hydroxypropionic acid	1.562	1.533	1.523	1.507	1.489
2,4-Dihydroxybutyric acid	1.537	1.497	1.480	1.469	1.449
Carbonic acid, 2-dimethylaminoethyl propyl ester	1.534	1.509	1.509	1.508	1.479
Proline, 1-methyl-, methyl ester	1.440	1.406	1.419	1.422	1.405
1-Deoxypentitol	1.432	1.541	1.539	1.524	1.496
Dodecanal	1.309	1.297	1.286	1.325	1.335
3-Indoleacetic acid	1.101	1.209	1.195	1.218	1.208
D-Mannitol	1.096	1.068	1.103	1.157	1.144
D-Arabinose (unique derivatised mass = 217)	1.031	1.201	1.208	1.195	1.207
D-Glucuronic acid	0.590	0.948	1.022	1.049	1.037
Cadaverine	0.305	0.817	0.923	0.913	0.902

Table S8: Pearson's correlations between selected metabolites and between metabolites and CD4 T cell count.

	Pearson's r	p-value	BH-corrected p-value
Metabolites correlating with 3,4-dihydroxybutyric acid			
DL-Phenylalanine	0.690	< 0.0001	< 0.0001
2,4-Dihydroxy-butyric acid	0.686	< 0.0001	< 0.0001
Phenylalanine (218)	0.653	< 0.0001	< 0.0001
Glycerol (103)	0.595	< 0.0001	< 0.0001
3-Hydroxyisovaleric acid	0.581	< 0.0001	< 0.0001
1-Deoxypentitol	0.541	< 0.0001	< 0.0001
Glycerol (73)	0.506	< 0.0001	< 0.001
Arabinose	0.497	< 0.0001	< 0.001
Rhamnose	0.492	< 0.0001	< 0.001
Tryptophan	-0.477	< 0.0001	< 0.001
Tetrahydrofuran	-0.470	< 0.0001	< 0.001
Analyte 826	0.447	< 0.0001	0.002
Analyte 555	0.441	< 0.001	0.002
Pentitol	0.439	< 0.001	0.002
Indole-3-lactic acid	-0.436	< 0.001	0.002
2-ketoisocaproic acid	-0.433	< 0.001	0.003
Pyruvate	0.419	< 0.001	0.004
L-Threonine	-0.418	< 0.001	0.004
Metabolites correlating with Phenylalanine (120)			
Phenylalanine (218)	0.730	< 0.0001	< 0.0001
3,4-Dihydroxy-butyric acid	0.690	< 0.0001	< 0.0001
Glycerol(103)	0.539	< 0.0001	< 0.0001
Cyclobutanecarboxylic acid, 2-dimethylaminoethyl ester	-0.525	< 0.0001	< 0.0001
3-Hydroxyisovaleric acid	0.518	< 0.0001	< 0.001
Pyruvate	0.480	< 0.0001	< 0.001

Tetrahydrofuran	-0.477	< 0.0001	< 0.001
Glycerol(73)	0.471	< 0.0001	< 0.001
2,4-Dihydroxy-butyric acid	0.464	< 0.0001	0.001
Acrylic acid 2-dimethylamino-ethyl ester	-0.455	< 0.0001	0.001
L-Threonine	-0.452	< 0.0001	0.001
L-5-Oxoproline	-0.449	< 0.0001	0.001
Rhamnose	0.437	< 0.001	0.002
2-Hydroxyethyl palmitate	-0.431	< 0.001	0.003
2-Aminobutanoic acid	-0.431	< 0.001	0.003
Carbonic acid, 2-dimethylaminoethyl ethyl ester	-0.429	< 0.001	0.003
Lysine	-0.423	< 0.001	0.003
Analyte 628	-0.422	< 0.001	0.003
Methionine	-0.414	< 0.001	0.004
Analyte 622	-0.411	< 0.001	0.004
3-Indolepropionic acid	-0.410	< 0.001	0.004
Metabolites correlating with lysine			
Analyte 706	0.687	< 0.0001	< 0.0001
5-Oxoproline	0.564	< 0.0001	< 0.0001
Hydroxyproline	0.537	< 0.0001	< 0.0001
Analyte 622	0.534	< 0.0001	< 0.0001
2,4-Di-tert-butyl-phenol	0.494	< 0.0001	< 0.0001
Threonine	0.494	< 0.0001	< 0.0001
Tetrahydrofuran	0.493	< 0.0001	< 0.0001
Methionine	0.489	< 0.0001	< 0.0001
2,3,4-Trihydroxy-butyraldehyde	0.458	< 0.0001	0.002
Glycerol (103)	-0.453	< 0.0001	0.002
2-Hydroxybutyric acid	-0.451	< 0.0001	0.002
2-Ketobutyric acid	-0.425	< 0.001	0.004
Analyte 628	0.423	< 0.001	0.004
DL-Phenylalanine	-0.423	< 0.001	0.004
3-Hydroxyisovaleric acid	-0.418	< 0.001	0.005
Mannonic acid, ϵ -lactone	0.413	< 0.001	0.005
Metabolites correlating with tryptophan			
3-Indolelactic acid	0.675	< 0.0001	< 0.0001
L-Norvaline	0.510	< 0.0001	< 0.001
3-Indoleacetic acid	0.485	< 0.0001	0.001
3,4-Dihydroxybutyric acid	-0.477	< 0.0001	0.001
2-Aminobutyric acid	0.458	< 0.0001	0.003
2-Ketoisocaproic acid	0.448	< 0.0001	0.003
3-Methyl-2-oxo-pentanoic acid	0.444	< 0.0001	0.003
Top ten metabolites correlating with CD T cell count			
2-Ketobutyric acid	-0.600	0.004	0.495
Analyte 1076	0.591	0.005	0.495
11,14-Eicosadienoic acid	0.577	0.006	0.495
Analyte 738	0.558	0.009	0.554
Glycerol (103)	-0.529	0.014	0.726
Lysine	0.517	0.017	0.726
Dopamine	0.503	0.020	0.726
Glycerol (73)	-0.485	0.026	0.726

[illegible]

Table S10: Quantitative metabolite set enrichment analysis results.

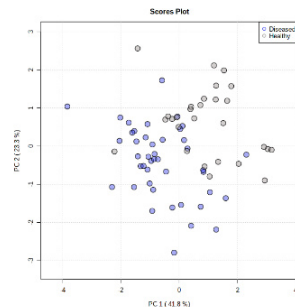
	Total Compounds	Hits	Statistic Q	Expected Q	Raw p	FDR	Hit identity
Phenylalaninetyrosine and Tyrosine Metabolism	28	1	49.399	2.7027	< 0.0001	< 0.0001	Phenylalanine
Glycerolipid Metabolism	25	2	29.367	2.7027	< 0.0001	< 0.001	Glycerol-3-phosphate
Glycine and Serine Metabolism	59	3	26.617	2.7027	< 0.0001	< 0.001	Threonine, Methionine, pyruvate
Methionine Metabolism	43	1	36.96	2.7027	< 0.0001	< 0.001	Methionine
Betaine Metabolism	21	1	36.96	2.7027	< 0.0001	< 0.001	Methionine
Spermidine and Spermine Biosynthesis	18	1	36.96	2.7027	< 0.0001	< 0.001	Methionine
Threonine and 2-Oxobutanoate Degradation	20	1	36.103	2.7027	< 0.0001	< 0.001	Methionine
Tryptophan Metabolism	60	2	28.439	2.7027	< 0.0001	< 0.001	Tryptophan, 3-Indoleacetic acid
Phospholipid Biosynthesis	29	1	30.906	2.7027	< 0.001	< 0.001	Glycerol-3-phosphate
Glycerol Phosphate Shuttle	11	1	30.906	2.7027	< 0.001	< 0.001	Glycerol-3-phosphate
Mitochondrial Electron Transport Chain	19	1	30.906	2.7027	< 0.001	< 0.001	Glycerol-3-phosphate
De Novo Triacylglycerol Biosynthesis	9	1	30.906	2.7027	< 0.001	< 0.001	Glycerol-3-phosphate
Cardiolipin Biosynthesis	11	1	30.906	2.7027	< 0.001	< 0.001	Glycerol-3-phosphate
Lysine Degradation	30	1	28.085	2.7027	< 0.001	0.001	Lysine
Biotin Metabolism	8	1	28.085	2.7027	< 0.001	0.001	Lysine
Carnitine Synthesis	22	1	28.085	2.7027	< 0.001	0.001	Lysine
Galactose Metabolism	38	1	27.692	2.7027	< 0.001	0.002	Glycerol
Plasmalogen Synthesis	26	1	27.216	2.7027	< 0.001	0.002	Octadecanol
alpha-Linolenic acid and Linoleic Acid Metabolism	19	1	24.535	2.7027	0.002	0.003	alpha-Linolenic acid
Beta Oxidation of Very Long Chain Fatty Acids	17	1	21.512	2.7027	0.003	0.006	Capric acid
Fatty Acid Biosynthesis	35	1	21.512	2.7027	0.003	0.006	Capric acid
Propanoate Metabolism	42	1	16.009	2.7027	0.013	0.022	3-hydroxypropionate
Inositol Metabolism	33	1	10.945	2.7027	0.042	0.067	D-Glucuronic acid
Starch and Sucrose Metabolism	31	1	10.945	2.7027	0.042	0.067	D-Glucuronic acid
Ammonia Recycling	32	1	4.7919	2.7027	0.187	0.187	Pyruvate
Cysteine Metabolism	26	1	4.7919	2.7027	0.187	0.187	Pyruvate
Glycolysis	25	1	4.7919	2.7027	0.187	0.187	Pyruvate
Amino Sugar Metabolism	33	1	4.7919	2.7027	0.187	0.187	Pyruvate
Alanine Metabolism	17	1	4.7919	2.7027	0.187	0.187	Pyruvate
Citric Acid Cycle	32	1	4.7919	2.7027	0.187	0.187	Pyruvate
Urea Cycle	29	1	4.7919	2.7027	0.187	0.187	Pyruvate
Pyruvate Metabolism	48	1	4.7919	2.7027	0.187	0.187	Pyruvate
Glutamate Metabolism	49	1	4.7919	2.7027	0.1867	0.1867	Pyruvate
Glucose-Alanine Cycle	13	1	4.7919	2.7027	0.1867	0.1867	Pyruvate
Gluconeogenesis	35	1	4.7919	2.7027	0.1867	0.1867	Pyruvate
Pyruvaldehyde Degradation	10	1	4.7919	2.7027	0.1867	0.1867	Pyruvate
Transfer of Acetyl Groups into Mitochondria	22	1	4.7919	2.7027	0.1867	0.1867	Pyruvate
Warburg Effect	58	1	4.7919	2.7027	0.1867	0.1867	Pyruvate

Healthy controls vs patients

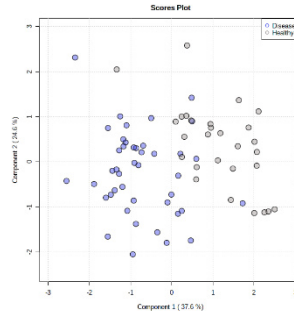
Healthy controls vs patients
(stratified based on HIV, TB and Tn status)

Cytokine analyses

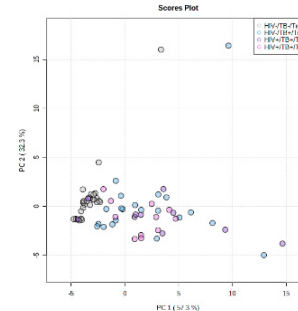
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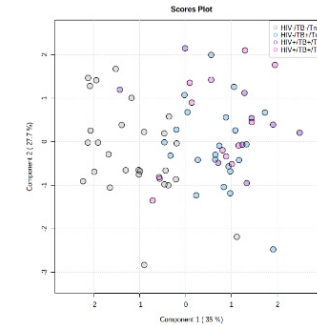
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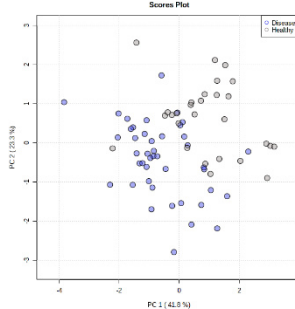


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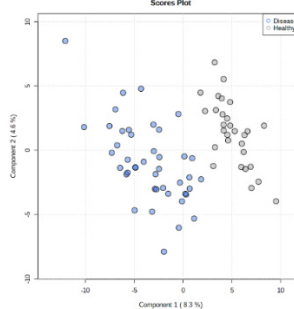


Metabolomics

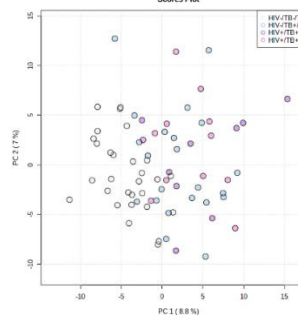
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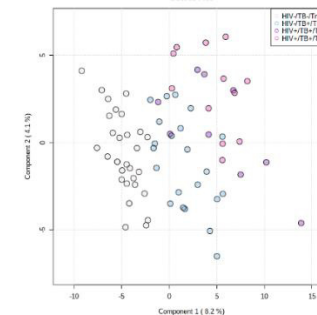


Figure S1: PCA and PLS-DA scores plots. A-D represents the cytokine data, while E-H represents the metabolomics data. (a) and (e) PCA scores plots when healthy controls are compared to all patients (all have active pulmonary TB, with and without HIV infection and ART), indicating no clear natural separation between the groups; (b) and (f) PLS-DA scores plot indicating separation between the healthy controls and all the patients. (c) and (g) PCA scores plot when healthy controls are compared to patients stratified based on HIV infection, TB and ART status [HIV-/TB+/Tn- (blue); HIV+/TB+/Tn- (purple); HIV+/TB+/Tn+ (pink)]; (d) and (h): PLS-DA scores plot indicating that separation the separation is less clear when diseased groups are defined [healthy controls (grey) and the patients (blue, purple, and pink) stratified based on HIV infection, TB and ART status].

APPENDIX B: PUBLICATIONS

The following review paper resulted from this investigation and has been published:

- Liebenberg, C., Luies, L., and Williams, A.A. 2021. Metabolomics as a tool to investigate HIV/TB co-infection. *Frontiers in Molecular Biosciences* 8, 1009.

The following data paper has been submitted for publication:

- Liebenberg, C., Luies, L., Loots, D.T. and Williams, A.A. Characterising the immunometabolic profiles of HIV/TB co-infection in cohorts untreated and treated for HIV. *Submitted on 1 December 2021 to eLife* (Manuscript/submission number: 06-12-2021-RA-eLife-76156).



Metabolomics as a Tool to Investigate HIV/TB Co-Infection

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The HIV/AIDS (human immunodeficiency virus/acquired immunodeficiency syndrome) and tuberculosis (TB) pandemics are perpetuated by a significant global burden of HIV/TB co-infection. The synergy between HIV and *Mycobacterium tuberculosis* (*Mtb*) during co-infection of a host is well established. While this synergy is known to be driven by immunological deterioration, the metabolic mechanisms thereof remain poorly understood. Metabolomics has been applied to study various aspects of HIV and *Mtb* infection separately, yielding insights into infection- and treatment-induced metabolic adaptations experienced by the host. Despite the contributions that metabolomics has made to the field, this approach has not yet been systematically applied to characterize the HIV/TB co-infected state. Considering that limited HIV/TB co-infection metabolomics studies have been published to date, this review briefly summarizes what is known regarding the HIV/TB co-infection synergism from a conventional and metabolomics perspective. It then explores metabolomics as a tool for the improved characterization of HIV/TB co-infection in the context of previously published human-related HIV infection and TB investigations, respectively as well as for addressing the gaps in existing knowledge based on the similarities and deviating trends reported in these HIV infection and TB studies.

Keywords: HIV/AIDS, tuberculosis, HIV/TB co-infection, metabolomics, metabolism, treatment

INTRODUCTION

Tuberculosis (TB) ranks as the top cause of death due to a single infectious pathogen, ranking above the human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) (World Health Organization, 2019). These diseases are pandemics with high morbidity and mortality rates (World Health Organization, 2020). Although HIV-positive individuals suffer from many serious secondary infections, TB is the most common disease, especially in sub-Saharan Africa. Due to the immunosuppressive effects of HIV on the host, HIV-positive individuals have a 26–31 times greater risk of developing active TB compared to HIV-negative individuals (Wood et al., 2000; World Health Organization, 2012). This increased risk, along with various other factors, contributes to the significant global burden of HIV/TB co-infection. Although pulmonary TB is the most common form of active TB in HIV/TB co-infected individuals, this population is also more prone to the development of independent or concurrent extrapulmonary TB, especially when CD4 T cell counts are low (Chaisson et al., 1987; Elliott et al., 1995; Schutz et al., 2010). When co-infection with these pathogens is established, they act synergistically to increase the disease burden in the host (Kwan and Ernst, 2011). As expected, the pathophysiology of *Mycobacterium tuberculosis*

(*Mtb*) infection differs in HIV-positive individuals and even more so in those who have progressed to severe immunosuppression, i.e., AIDS, typically showing an accelerated clinical course (Badri et al., 2001; Schutz et al., 2010). In general, HIV/TB co-infected patients present with characteristic symptoms of active pulmonary TB (Burman and Jones, 2003; Luies and du Preez, 2020) but often have more severe systemic symptoms including fevers and weight loss. A mucus-producing cough is less common, which is linked to HIV-induced immunosuppression, as a less vigorous immune response results in fewer pulmonary complications (Selwyn et al., 1998; Garcia et al., 2007; Schutz et al., 2010). In 2019, 208,000 of the approximately 1.4 million TB-related deaths were among HIV-positive people (World Health Organization, 2020).

The incidence of smear-negative (culture-negative, symptomatic) and subclinical (culture-positive, asymptomatic active) TB is also higher among co-infected patients. This leads to decreased sensitivity in screening tests and subsequent diagnostic delays, as the most widely applied TB diagnostic tests are based on sputum examination (Schutz et al., 2010; Tiberi et al., 2017). This manifests as higher mortality rates for smear-negative HIV-positive TB patients, the discovery of subclinical TB only after the initiation of antiretroviral therapy (ART) (Sterling et al., 2010), and poor treatment outcomes for these patients. Therefore, it is recommended that all HIV-positive individuals are screened for *Mtb* infection, latent or otherwise (Tiberi et al., 2017). TB can be cured in these individuals, although treatment is complicated by various factors (Tiberi et al., 2017). Co-infected individuals also have an increased rate of recurrence, mainly due to reinfection as opposed to relapse (Sonnenberg et al., 2001; Charalambous et al., 2008).

Various aspects of the pathology of HIV infection, TB, and HIV/TB co-infection remain incompletely understood, which hampers the development of improved or novel diagnostic and management strategies. There is a need to better understand and characterize the complex metabolic changes induced by co-infection since many deaths can be prevented with timely diagnosis and treatment. Metabolomics is a promising tool in this regard and refers to the analysis of small molecules (i.e. metabolites) present in a biological system, to infer biological functions or changes in response to specific internal or external stimuli (Dunn et al., 2005).

Metabolomics has yielded new insights into HIV and *Mtb* infection- and treatment-induced metabolic alterations experienced by the host. Despite the contributions that metabolomics has made to date, this approach has not yet been systematically applied to characterize the HIV/TB co-infected state. Considering this, we briefly review the HIV/TB co-infection synergism from a conventional and metabolomics perspective. We then evaluate the role of metabolomics as a tool to investigate HIV/TB co-infection in the context of previously published human-related HIV infection (specifically relating to HIV-1) and TB investigations, respectively. This will inform the design of future experiments and allow for a better understanding of the

contributions that HIV and *Mtb* make toward host metabolic adaptations during co-infection. Due to the complexity of the synergism between HIV and *Mtb* these metabolic changes might be similar, exacerbated, or altered, compared to HIV infection or TB alone.

HUMAN IMMUNODEFICIENCY VIRUS/ TUBERCULOSIS CO-INFECTION: WHAT DO WE KNOW AND WHAT ARE THE CURRENT GAPS?

HIV and *Mtb* display the same outcome in their interactions with the host: effective replication without killing the host for as long as possible, to improve the chances of transmission to a new host and perpetuation of this process. During co-infection, the core of the synergism, which amplifies the disease burden, lies in the deterioration of immunological functions (Pawlowski et al., 2012; Sharan et al., 2020). The overall immune activation is worse in patients with HIV/TB co-infection relative to those with TB only (Vanham et al., 1996). A detailed discussion of the immunology concerned is outside the scope of this review, however, several publications relating to this topic are available (Toossi, 2003; Diedrich and Flynn, 2011; Kwan and Ernst, 2011; Bell and Noursadeghi, 2018; Sharan et al., 2020).

Considering that immunological deterioration has profound effects on the host metabolism (Cheng et al., 2014; Li et al., 2017), the field of immunometabolism has emerged to better characterize these changes. Immunometabolism refers to the convergence of bioenergetic pathways and the specific functions of immune cells (Shi et al., 2016). To explain briefly, during HIV and *Mtb* infections, the collective actions of the innate and adaptive immune responses result in a concentrated effort to eliminate the pathogenic threats (Kwan and Ernst, 2011). A state of chronic inflammation is established (Semba et al., 2010; Aounallah et al., 2016) and the balance between the immune system and metabolism disrupted. This is mainly due to the significant energy demands and biosynthesis required to mount and sustain an immune response (Aounallah et al., 2016). As such, the infected host experiences anorexia via effects on the hypothalamus (Gautron and Laye, 2010), which collectively results in a negative energy balance, and the consequent utilization of stored fat and eventually protein (from muscle tissue) (Powanda and Beisel, 2003) for energy. This often culminates in malnutrition and the condition known as cachexia (Moldawer and Sattler, 1998), an established feature of both HIV infection and TB. Consequently, there is an inability to control infection, resulting in increased mortality (Zachariah et al., 2002; Paton et al., 2006).

To date, metabolic changes during HIV infection, TB, and HIV/TB co-infection have typically been measured by conventional assays [such as resting energy expenditure measurement by indirect calorimetry (Melchior et al., 1991; Schwenk et al., 2003), body composition measurement by dual-energy X-ray absorptiometry (Meininger et al., 2002; Schwenk et al., 2003) and nitrogen flux by the administration

of, for example, a glycine isotope (Paton et al., 2003)]. Using such techniques, HIV infection was characterized by alterations in energy, glucose, lipid, and protein metabolism (Salas-Salvado and Garcia-Lorda, 2001), while active TB was characterized by altered protein metabolism (MacAllan et al., 1998), low serum cholesterol (Taylor and Bamgboye, 1979), and glucose intolerance (Oluboyo and Erasmus, 1990). In HIV/TB co-infected patients, protein metabolism was mainly affected. The net protein balance in the fed state was shown to be impaired, compared to a strongly anabolic protein balance in those with HIV infection or TB only (Paton et al., 2003). Serum albumin levels serve as indicators of protein status. HIV/TB co-infected patients were shown to suffer from hypoalbuminemia relative to TB patients (Van Lettow et al., 2003). The distribution and magnitude of body composition changes were not altered in HIV/TB co-infection relative to that of TB patients (Paton and Ng, 2006).

Although informative, conventional techniques allow for only one specific aspect of metabolism to be investigated at a time (Sitole et al., 2013). This complicates the process of forming a holistic, coherent view of infection- and treatment-induced metabolic alterations. Since metabolomics allows for the measurement of multiple metabolites at a time, it has aided in better understanding HIV infection and TB, respectively. In the case of HIV infection, additional changes in other carbohydrates, amino acids (as well as proteins), lipids, and nucleotides (and/or DNA), has been established by untargeted (Munshi et al., 2013; Williams et al., 2014; Sitole et al., 2015; Li et al., 2018; Sitole et al., 2019) and targeted (Riddler et al., 2008; Williams et al., 2011; Scarpellini et al., 2016; Peltenburg et al., 2018; Sitole et al., 2019) metabolomics studies in an untreated, treated, treatment over time, treatment response and/or comorbidity context (Rodriguez-Gallego et al., 2018; Babu et al., 2019; Rosado-Sanchez et al., 2019). Metabolomics has also been extensively applied to various aspects of TB research, i.e. metabolic mechanisms associated with latent *Mtb* infection and conversion to active disease, *Mtb* infection at the cellular level, active TB, comorbidities, as well as TB treatment (Luies et al., 2017; Vrieling et al., 2019; Albers-Vaquer et al., 2020; Vrieling et al., 2020). The approach has thus contributed, among others, to a better understanding of the pathogen, its interaction with the host, and its effect on the physiology of the host. Many studies have focused on the discovery of biomarkers for specific TB-related applications, but the results are often not extensively interpreted in a biological context. Metabolomics, however, has not yet been systematically applied to characterize the co-infected state.

It is worth mentioning that the metabolic characteristics of HIV/TB co-infection derived from conventional methods mainly focused on the synergistic effects of HIV infection, TB, and malnutrition, which together have been termed “triple trouble” with reference to the public health term “double trouble” when referring to HIV/TB co-infection (Van Lettow et al., 2003). The risk of malnutrition (Swaminathan et al., 2008) and the severity of wasting (Lucas et al., 1994; Villamor et al., 2006) are exacerbated during HIV/TB co-infection relative to either disease state alone, although the mechanisms underlying this are not fully

understood. HIV infection is associated with the loss of gut mucosal integrity. The CD4 T cells of the gut mucosa are preferentially depleted, leading to impaired immune function in the gut (Ibarrondo et al., 2013; Sharan et al., 2020). This leads to a “leaky gut” and the translocation of gut microbial products into the blood, which exacerbates systemic immune activation (Brenchley et al., 2006; Sharan et al., 2020). Furthermore, it is well established that HIV induces changes in the gut microbiota composition (Vujkovic-Cvijin and Somsouk, 2019). How these processes are affected by *Mtb* during HIV/TB co-infection are not yet understood (Waters et al., 2020), although it is known that patients with TB also have an altered gut microbiota composition (Winglee et al., 2014; Luo et al., 2017; Hu et al., 2019).

The effects of HIV infection on the gut and the high energy requirement during chronic infection culminates in micronutrient deficiencies [reviewed in detail by Semba et al. (2010)] and alterations to the macronutrient status (e.g., increased utilization of protein and fat for energy). Micronutrients have major roles in maintaining homeostasis. Deficiencies have far-reaching complications for the immune system and metabolism (Katona and Katona-Apte, 2008), of which tracing the source might be extremely difficult due to the confounding effect of the inflammatory state and the inherent interconnectedness of metabolism.

Although aspects of metabolism and nutrition improve by treating HIV-positive or TB patients with ART and anti-TB drugs, respectively (Kennedy et al., 1996; Drain et al., 2007), these treatments do not restore patients to a healthy state. HIV-positive individuals often still suffer from wasting even after the initiation of ART (Tang et al., 2002). ART also induces metabolic alterations separate from HIV infection (van der Valk et al., 2002; Jain et al., 2005), while anti-TB treatment fails to completely recover protein reserves (Onwubalili, 1988; Schwenk et al., 2004) and induces oxidative stress (OS) (Sodhi et al., 1997). These effects are reflected in the metabolomics literature for HIV infection (Hewer et al., 2006; Li et al., 2018; Babu et al., 2019) and TB (Luies et al., 2017; Combrink et al., 2019; Opperman et al., 2021). The causes of malnutrition and wasting in HIV-positive and TB patients are multifactorial and are influenced by disease progression. It is known that the development and degree of HIV-induced wasting is influenced by factors such as CD4 T cell count (Forrester et al., 2001), viral load (Lyles et al., 1999; Zackin et al., 1999), inflammatory markers [reviewed by Chang et al. (1998)], and nutrition [reviewed by Koethe and Heimbürger. (2010)]. The role of these factors and the HIV-induced gut complications mentioned above, in the co-infected individual is not well understood.

The signaling pathways involved in pathogenic control are closely involved in the regulation of nutrients and metabolic homeostasis (Aounallah et al., 2016) and are mediated by cytokines, chemokines, and various reactive oxygen species (ROS). Besides directly damaging the invading microbe, ROS stimulates the production of pro-inflammatory cytokines, which exacerbates the inflammatory state and perpetuates immunometabolic alterations (Zhang et al., 2018). Increased

TABLE 1 | Metabolomics studies published to date investigating a specific aspect of HIV/TB co-infection.

Research Model	Aim	Metabolome fraction targeted	Analytical apparatus	Groups compared	Statistical approaches	References
Human: plasma	To investigate whether IDO activity (measured as Trp/Kyn) could be used as a diagnostic/predictive marker of TB in HIV-positive individuals	Tryptophan and kynurenine	UPLC-MS	*HIV+/TB+/ART+ *HIV+/TB+/ART+/ATT+ HIV+/TB-/pneumonia+	<i>t</i> -test, Mann-Whitney U, Kruskal-Wallis, AUC of ROC, Spearman correlations	Adu-Gyamfi et al. (2017)
Human: plasma	To investigate whether individuals with and without TB-IRIS could be distinguished based on metabolic differences and if these differences are detectable before the onset of TB-IRIS	Total metabolome	LC-MS	HIV+/TB+/ART+/non-IRIS HIV+/TB+/ART+/IRIS+	<i>t</i> -test with Bayesian adjustment, FC, PCA, pathway analysis in MetaboAnalyst, Lasso regression models	Silva et al. (2019)
Human: plasma	To determine whether tryptophan metabolism is among the highest regulated metabolic pathways in the response of the host to <i>Mtb</i> infection, TB, and treatment	Total metabolome	LC-MS, MS/MS	HIV-/LTBI- (healthy controls) *HIV-/LTBI+/TB-/DOTS+ *HIV-/TB+/DOTS+ *HIV-/MDR-TB+/MDR-Tn+ *HIV+/MDR-TB+/MDR-Tn+	Wilcoxon rank, AUC of ROC, pathway enrichment analysis	Collins et al. (2020)

Aim: IDO: indoleamine 2,3-dioxygenase; Kyn: kynurenine; Trp: tryptophan; TB: tuberculosis; IRIS: immune reconstitution inflammatory syndrome.

Analytical apparatus: (UP)LC-MS: (ultra-performance) liquid chromatography mass spectrometry; MS/MS: tandem mass spectrometry.

Groups: HIV: human immunodeficiency virus; TB: tuberculosis; ART: antiretroviral treatment; ATT: anti-TB treatment; IRIS: immune reconstitution inflammatory syndrome; +: positive; -: negative; *: longitudinal study; LTBI: latent mycobacterium tuberculosis infection; DOTS: directly observed treatment, short-course; MDR: multidrug-resistant tuberculosis; MDR-Tn: treatment for MDR-TB.

Statistical approaches: AUC of ROC: area under the curve of the receiver operating characteristic; FC: fold change; PCA: principal component analysis.

levels of ROS and pro-inflammatory cytokines are the hallmarks of chronic inflammation (Awodele et al., 2012), and have been studied in HIV infection (Williams et al., 2013), TB (Yao et al., 2017), and co-infection (Verma et al., 2018). Although these interactions have been linked to various metabolic changes and are more pronounced during co-infection (Madebo et al., 2003; Awodele et al., 2012), these are complex and not yet fully understood (Zhang et al., 2018).

No single factor can be attributed to the cause of the co-infected phenotype as the interactions between the host and the two pathogens are multidirectional: either oppositional or synergistic and to various degrees. Given that conventional assays are less sensitive, less specific, and laborious to perform (Williams et al., 2011), researchers have turned to newer, high-throughput omics techniques to better match the dimensionality of the problem to the data obtained and thus allow for a more comprehensive profile of host metabolism, as it relates to HIV/TB co-infection.

HUMAN IMMUNODEFICIENCY VIRUS/TUBERCULOSIS CO-INFECTION AND METABOLOMICS

Only three metabolomics investigations that study a very specific aspect of HIV/TB co-infection have been published to date (Table 1). To the best of our knowledge, no metabolomics study has hitherto characterized the HIV/TB co-infected

metabolic profile relative to healthy individuals or either HIV-positive or TB-positive individuals, or in terms of a treatment-associated profile.

The optimal physiological function is reliant on a balanced tryptophan metabolism (Wang et al., 2021), as the immune, central nervous, and reproductive systems respond to alterations in its catabolic flux (Ball et al., 2014). The enzymes tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase 1 (IDO1) catabolize tryptophan via the kynurenine pathway, producing a range of immunomodulatory and neuroactive catabolites. IDO1 activity in particular has been widely investigated for its role in immune tolerance and suppression (Wang et al., 2021). Increased IDO1 activity, induced by interferon- γ (Katz et al., 2008), and the resulting alterations to the kynurenine/tryptophan (K/T) ratio have been investigated as biomarkers for various disease states (Sucher et al., 2010; Mancuso et al., 2015; Pett et al., 2017; Li et al., 2019), including HIV infection and TB measured conventionally (Almeida et al., 2009; Jenabian et al., 2013; Gostner et al., 2015; Dagenais-Lussier et al., 2016; Gautam et al., 2018; van Laarhoven et al., 2018) and via metabolomics (Weiner et al., 2012; Luies and Loots, 2016; Isa et al., 2018; Li et al., 2018; Peltenburg et al., 2018; Sitole et al., 2019).

Of the three studies mentioned, two investigated the effects of IDO1 activity on host metabolism during HIV/TB co-infection. Adu-Gyamfi et al. (2017) monitored HIV-positive individuals longitudinally for the development of active TB. At the time of

diagnosis, IDO1 activity was higher in those individuals who developed TB compared to those who did not, an effect that was reversed after 6 months of anti-TB treatment. The IDO1 activity of HIV/TB co-infected individuals was also four-fold higher when compared to that of HIV-positive individuals with confirmed pneumonia. Collins et al. (2020) confirmed that individuals with HIV/TB co-infection have higher IDO1 activity compared to those with a single disease. Collins et al. (2020) further found that HIV-positive individuals with multidrug-resistant (MDR) TB also had an increased K/T ratio relative to those with TB alone. After receiving treatment for MDR-TB for 2 years, there was no significant change in the K/T ratio, which is in concurrence with a slower bacterial clearance rate in MDR-TB patients (Collins et al., 2020). Indoleamine 2,3-dioxygenase 2 (IDO2) plays a smaller role in tryptophan catabolism and has been suggested to promote pro-inflammatory B cell responses, as opposed to the immunosuppressive effects of IDO1, through the induction of T regulatory cell responses (Manches et al., 2008) and other mechanisms (Munn et al., 1999; Mellor et al., 2002; Merlo et al., 2020). The levels of IDO2 transcripts is higher during co-infection compared to individuals with TB alone, suggesting that HIV infection or the co-infected state causes increased production of IDO2 in addition to IDO1 (Collins et al., 2020). These studies indicate that increased IDO activity is significantly more pronounced in the HIV/TB co-infected state and that the K/T ratio may be a suitable biomarker for TB in HIV-positive patients (Adu-Gyamfi et al., 2017) and a possible target for host-directed therapies (Collins et al., 2020). Studies are needed to evaluate whether the significantly increased IDO1 activity contributes to the increased TB progression risk in HIV-positive individuals (Pawlowski et al., 2012; Collins et al., 2020) and those studies comparing HIV-positive individuals with other secondary (specifically respiratory) diseases as diseased controls to validate this are also warranted. Diagnostically, it would be ideal to include both HIV-negative TB-positive patients and HIV-positive TB-negative patients as controls in a single study.

Within the context of co-infection, ART is typically initiated based on a patient's immunological status. Patients with CD4 <50 cells/mm³ or significantly advanced disease are prescribed to start ART within 2 weeks of starting anti-TB treatment, while those with an improved immunological profile (CD4 >50 cells/mm³) start ART two to 8 weeks after anti-TB treatment initiation. These guidelines have been proposed in response to the development of an aberrant immune response (immune reconstitution inflammatory syndrome; IRIS) in HIV/TB co-infected patients when ART is initiated early in the course of anti-TB treatment (Török et al., 2011; Manosuthi et al., 2012; World Health Organization, 2016b). IRIS is characterized by a severe inflammatory response that is triggered against a pre-existing condition in HIV-positive individuals shortly after the initiation of ART. When this reaction is directed against *Mtb*, it is referred to as TB-IRIS. It may be transient and resolve of its own, but it may also be fatal. Since there are currently no diagnostic tests for TB-IRIS, its diagnosis is based on clinical presentation and medical history (Pozniak et al., 2011; Tiberi et al., 2017). Considering this, Silva et al. (2019) collected samples from

HIV/TB co-infected individuals after the initiation of ART and as close as possible to the onset of IRIS (at a time point compared to this for those who did not develop IRIS). Those individuals who developed IRIS experienced increased arachidonic acid (most notably increased hydroxyeicosatetraenoic acids) and glycerophospholipid metabolism relative to the non-IRIS group at the start of the study, whereas altered sphingolipid and linolenic acid metabolism distinguished the groups during the IRIS window. This study showed that altered polyunsaturated fatty acid metabolism distinguished patients who develop IRIS from those who do not. Since IRIS involves an acute inflammatory response these results are expected, as polyunsaturated fatty acids give rise to various inflammatory mediators. This study again underscores the reflection of immunological changes in the metabolism and its potential prognostic application.

The studies reviewed here stress the many gaps that exist in our knowledge of HIV/TB co-infection, including an incomplete understanding of the mechanisms underlying diagnostic problems, the effects of ART and anti-TB treatment on the host, recurrent TB, and the effect of *Mtb* on the gut mucosal damage induced by HIV. These studies also highlight the potential of metabolomics to address such gaps. Comparing the existing HIV infection and TB metabolomics profiles (Tables 2–4) in the absence of a comprehensive HIV/TB profile may yield insight into the mechanisms of what is known about host metabolism during HIV/TB co-infection and inform the design of future metabolomics studies.

COMPARING HUMAN IMMUNODEFICIENCY VIRUS INFECTION AND TUBERCULOSIS METABOLIC PROFILES

To date, metabolomics has been used to investigate various aspects of HIV infection and TB, using various sample matrices, which can be used as a guide in terms of what to expect during co-infection. HIV infection metabolomics studies have often been concerned with characterizing the disease state, prognosis, and the effect of ART, using blood-based (serum or plasma), cerebrospinal fluid (CSF), cell culture, urine, bronchoalveolar lavage fluid (BALF), oral wash/saliva, and tissue samples (Williams et al., 2017). Within the TB metabolomics literature, blood-based, urine, sputum, tissue, pleural effusion, and cell culture samples have been used, with a focus on biomarker discovery for diagnosis and treatment outcome. As *Mtb* has its own metabolism, unlike HIV, bacterial culture metabolomics has also been extensively used to characterize the *mycobacterium*'s metabolic adaptations (Du Preez et al., 2019).

The normal course of the interactions between the metabolic, immune and endocrine systems is distorted due to the chronic nature of immune activation and inflammation during HIV and *Mtb* infection. At the time of diagnosis, when treatment-naïve samples are typically collected, overlapping biochemical

TABLE 2 | A comparison of the findings of HIV infection and TB serum metabolomics investigations.

Similarities					
Metabolic alteration		Proposed origin(s) of metabolic alterations ^a		References	
Decreased tryptophan and increased levels of its catabolic products (e.g. kynurenine)	Increased IDO activity ^b			HIV: Sitole et al. (2019)	
Altered indole-3-acetic acid	Uremic cytotoxic activity, gut microbes			TB: Weiner et al. (2012)	
				HIV: Williams et al. (2011)	
Increased glutamate	<i>Mtb</i> nitrogen balance, redox homeostasis, and survival mechanisms.			TB: Weiner et al. (2018)	
Increased aspartate	Immune activation and oxidative stress of the host			HIV: Sitole et al. (2019)	
	Immune activation and oxidative stress			TB: Zhou et al. (2013); Cho et al. (2020)	
Altered phenylalanine and tyrosine	Decreased activity of phenylalanine hydroxylase			HIV: Sitole et al. (2019)	
				TB: Weiner et al. (2012); Cho et al. (2020)	
Decreased alanine	Energy production			HIV: Sitole et al. (2019)	
				TB: Zhou et al. (2013)	
Altered pyroglutamic acid/5-oxoproline	Mitochondrial dysfunction, glutathione deficiency (i.e. oxidative stress), or reduced glutathione cycling			HIV: Williams et al. (2011)	
Altered phospholipids	<i>Mtb</i> -induced inhibition of phospholipase A2, lipolysis in the granuloma, inflammation, and immune activation			TB: Weiner et al. (2012); Che et al. (2013)	
Increased fatty acids and/or their catabolic products	Inflammation and immune activation, mitochondrial dysfunction			HIV: Williams et al. (2011)	
Decreased creatine				TB: Weiner et al. (2018)	
				HIV: Sitole et al. (2019)	
				TB: Weiner et al. (2018)	
Differences					
HIV infection	Proposed origin(s) of metabolic alterations	References	TB	Proposed origin(s) of metabolic alterations	References
			Altered urea cycle intermediates		Weiner et al. (2012); Che et al. (2013)
			Decreased methionine, increased oxidative products of methionine	Oxidative stress	Weiner et al. (2012); Cho et al. (2020)
			Increased fibrinopeptides	Tuberculous granulomas	Weiner et al. (2012)

^aObserved trends are not always attributed to a specific mechanism by the investigators. Thus, metabolite changes may overlap between the HIV infection and TB metabolic profiles, but the proposed mechanism given in the table may represent only that given in either the HIV or TB literature.

^bIDO: indoleamine 2,3-dioxygenase.

processes have already been set in motion and become so interconnected that no single measure of causality can be attributed. This makes it difficult to interpret metabolomics data. Despite this, and although the trends are not always consistent, an overlapping pattern of metabolites frequently altered during HIV infection and TB can be identified and linked to known effects of these infections. **Tables 2–4** compare the serum, plasma and urine metabolomics profiles of untreated HIV-positive and TB patients (where these patients were compared to healthy controls), respectively. Other sample matrices were not compared in this way as there is very little overlap in the use of matrices other than blood and urine between the two disease states. In cases where a metabolite was found to be significantly altered during HIV infection and/or TB, but where the trend was inconsistent, the metabolite is marked as “altered,” instead of “increased” or “decreased.”

Despite the potential for metabolic differences between serum and plasma (Liu et al., 2018), the blood-based metabolomics investigations reviewed here show that there is significant overlap between the metabolic alterations, as induced by HIV infection and TB. For example, alterations to tryptophan and its catabolic products, glutamine and/or glutamate, aspartate, as well as alanine are commonly reported for HIV infection and TB in both serum and plasma (see “similarities” in **Tables 2** and **3**). Alterations to the following metabolites are similar (being frequently cited as statistically significant and, in some cases, trending in the same direction) between HIV infection and TB but occur only in either serum or plasma: indole-3-acetic acid, phenylalanine and tyrosine, pyroglutamic acid (also called 5-oxoproline), phospholipids, fatty acids, and creatine in serum (**Table 2**), as well as urea cycle intermediates (other than aspartate), histidine, choline, and sarcosine in plasma (**Table 3**).

Amino acids are central to metabolism and the function of the immune system, especially during infection. As an infection becomes chronic, these alterations become more complex as various other areas of metabolism become impaired, and amino acids may antagonize each other's effects (Li et al., 2007). Besides the specific functions of amino acids in the immune system, free amino acids, as well as stored amino acids released from muscle tissue, are used as a last resort to produce energy via the tricarboxylic acid cycle (TCA) cycle during extended periods of high energy expenditure, such as chronic infection. Glutaminolysis has been postulated as a mechanism by which the TCA cycle is replenished during viral infection (Plaza et al., 2016). Continual oxidation of amino acids combined with malnutrition and HIV-associated malabsorption (Grinspoon and Mulligan, 2003) further contributes to alterations in amino acid metabolism. Cachexia is associated with progressive HIV infection and TB and is characterized by the loss of muscle mass. As such, the mobilization of amino acids for energy production may contribute to the observed amino acid changes.

The interconversion of glutamine and glutamate (**Tables 2** and **3**) is important in metabolic reactions essential to cellular function (Newsholme et al., 2003). Although alterations to both these metabolic partners are not always statistically significant (likely due to the untargeted nature of studies), one

or both of these metabolites are a common feature of HIV infection (Munshi et al., 2013; Sitole et al., 2019; Tarancon-Diez et al., 2019) and TB metabolic profiles (Zhou et al., 2013; Vrieling et al., 2018; Vrieling et al., 2019; Cho et al., 2020). Where these partners are found together, increased levels of glutamate with a concurrent decrease in glutamine results in a decreased glutamine/glutamate ratio (Cho et al., 2020; Ding et al., 2020). The alterations to glutamine and glutamate have been attributed to nitrogen balance, redox homeostasis and survival mechanisms of *Mtb* (Frediani et al., 2014; Cho et al., 2020; Thandi et al., 2020). It has also been associated with immune activation, stress on the host, and has been indicated as an early metabolic alteration during HIV (Tarancon-Diez et al., 2019) and *Mtb* (Albors-Vaquer et al., 2020) infection.

The balance of glutamine and glutamate, as well as asparagine and aspartate, have various roles in the immune system. An imbalance in these ratios affects the immune-competence of T cells and macrophages (Yoneda et al., 2009). Increased serum and plasma aspartate was reported for HIV (Munshi et al., 2013; Scarpellini et al., 2016; Sitole et al., 2019). An increased aspartate/asparagine ratio was also observed in the serum (**Table 2**) and plasma (**Table 3**) of TB patients (Cho et al., 2020; Ding et al., 2020), confirming the finding of increased aspartate by Weiner et al. (2012).

Aspartate forms part of the urea cycle, where it is combined with citrulline to form argininosuccinate by argininosuccinate synthase. In the liver mitochondria, aspartate is also produced from oxaloacetate by aspartate aminotransferase, with the concurrent conversion of glutamate to α -ketoglutarate (Watford, 2003). This highlights the interconnected nature of the observed alterations to glutamine/glutamate and aspartate in HIV infection and TB metabolomics. Since part of the urea cycle occurs in mitochondria, these alterations relate to mitochondrial dysfunction that has previously been associated with HIV infection (Pinti et al., 2010) and TB (Luies et al., 2017; Ramond et al., 2019). Alterations to the urea cycle were only reported for TB in serum (**Table 2**) but not for HIV infection. Similar urea cycle alterations were reported for both HIV infection and TB in plasma (**Table 3**). Decreased urea (Weiner et al., 2012), ornithine (Vrieling et al., 2019), and citrulline, with concurrently increased aspartate (Weiner et al., 2012; Vrieling et al., 2019; Ding et al., 2020) are indicative of urea cycle dysregulation in TB patients. Although the exact mechanisms underlying these alterations are still unclear, they may be related to antimicrobial mechanisms of immune cells. For example, Thandi et al. (2020) showed that ornithine inhibits *Mtb* growth and enhances autophagy in mouse alveolar macrophages. Reduced levels of ornithine and putrescine suggest a decreased urea cycle and reduced ammonia clearance in HIV-positive individuals (Peltenburg et al., 2018).

Alterations to plasma phenylalanine are more commonly reported for TB than for HIV infection [see “similarities” (**Table 2**) and “differences” (**Table 3**)], with only one HIV serum investigation reporting on this metabolite (**Table 2**). These changes have been postulated to be related to decreased activity of phenylalanine hydroxylase, which converts phenylalanine to tyrosine (Ghannoum et al., 2013), as a

TABLE 3 | A comparison of the findings of HIV infection and TB plasma metabolomics investigations.**Similarities**

Metabolic alteration	Proposed origin(s) of metabolic alterations ^a	References
Decreased tryptophan and increased levels of its catabolic products (e.g. kynurenine)	Increased IDO activity ^b	HIV: Li et al. (2018); Peltenburg et al. (2018) TB: Vrieling et al. (2018); Vrieling et al. (2019); Collins et al. (2020)
Altered glutamine and/or glutamate	Involved in multiple overlapping metabolic pathways	HIV: Munshi et al. (2013); Tarancon-Diez et al. (2019) TB: Vrieling et al. (2018); Vrieling et al. (2019); Ding et al. (2020)
Increased aspartate	Dysregulated urea cycle, involved in many other metabolic processes	HIV: Munshi et al. (2013); Scarpellini et al. (2016) TB: Vrieling et al. (2019); Ding et al. (2020)
Dysregulation of the urea cycle (decreased ornithine and related metabolites which do not correspond exactly)	Dysregulation of amino acid metabolism by various mechanisms	HIV: Peltenburg et al. (2018) TB: Vrieling et al. (2019)
Decreased alanine	Wasting/cachexia	HIV: Peltenburg et al. (2018) TB: Vrieling et al. (2018); Vrieling et al. (2019)
Decreased histidine	Oxidative stress and/or inflammation	HIV: Li et al. (2018) TB: Vrieling et al. (2018); Vrieling et al. (2019); Ding et al. (2020)
Altered choline		HIV: Munshi et al. (2013) TB: Vrieling et al. (2019)
Increased sarcosine	Muscle wasting and folate deficiency during HIV	HIV: Munshi et al. (2013), Peltenburg et al. (2018) TB: Vrieling et al. (2019)

Differences

HIV infection	Proposed origin(s) of metabolic alterations	References	TB	Proposed origin(s) of metabolic alterations	References
Altered BCAAs and their catabolic intermediates (↑ during early stages of infection, ↓ during later stages)	Immune activation, muscle catabolism (wasting)	Li et al. (2018); Peltenburg et al. (2018); Tarancon-Diez et al. (2019)	Altered phenylalanine		Vrieling et al. (2018); Vrieling et al. (2019); Ding et al. (2020)

^aObserved trends are not always attributed to a specific mechanism by the investigators. Thus, metabolite changes may overlap between the HIV infection and TB metabolic profiles, but the proposed mechanism given in the table may represent only that given in either the HIV or TB literature. BCAA: branched-chain amino acid; ↑: increased; ↓: decreased.

^bIDO: indoleamine 2,3-dioxygenase.

consequence of compromised insulin secretion (Luies and Loots, 2016). Inflammatory diseases (such as TB) are often associated with insulin resistance, hence diabetes mellitus (DM) is a known risk factor for TB. Evidence for insulin resistance has also been found in TB patients without a clinical diagnosis of DM, indicating that the condition is caused by the diseased state (Philips et al., 2017). Two recent metabolomics studies by Vrieling et al. (2018); Vrieling et al. (2019) characterized the metabolic alterations underlying TB and DM, as well as how these change when DM presents as a comorbidity in TB patients. These studies confirmed previous reports of increased serum (Weiner et al., 2012; Zhou et al., 2013) and urinary (Luies and Loots, 2016) phenylalanine levels in TB patients. One serum-based study (Che et al., 2013), however, reported decreased phenylalanine. Altered energy regulation through the endocrine system may also contribute to the other metabolic alterations discussed here, as various enzymes are influenced by insulin (Dashty, 2013). Furthermore, mitochondrial metabolism, which is modulated by HIV and *Mtb*, is central to coupling amino acids (such as glutamine, glutamate, and alanine) to insulin secretion (Newsholme et al., 2007).

During gluconeogenesis, non-carbohydrate molecules are converted to glucose, an essential energy substrate for leukocytes (Newsholme and Newsholme, 1989) during times of decreased energy availability. In the liver, the glucose-alanine cycle functions as a gluconeogenesis pathway, using alanine as a substrate. The amino group of alanine is transaminated to a keto acid, and the resulting carbon skeleton used in gluconeogenesis. This process contributes to the decreased levels of alanine (see “similarities” in **Tables 2** and **3**) that have been reported in both HIV infection (Peltenburg et al., 2018; Sitole et al., 2019) and TB (Zhou et al., 2013; Vrieling et al., 2018; Vrieling et al., 2019). The decrease in alanine relates to cachexia in HIV-positive and TB patients, as a study that included lung cancer patients related aberrations in glucose and alanine metabolism to the degree of weight loss, rather than the presence of cancer (Leij-Halfwerk et al., 2000). In other tissues, the carbon skeletons of amino acids (except for leucine and lysine) are also used for gluconeogenesis. Such oxidation of amino acids results in the need for increased ammonia secretion through the urea cycle (Dashty, 2013), previously mentioned to be dysfunctional (due to mitochondrial dysfunction or other mechanisms), as per the urea cycle alterations discussed above.

Histidine is associated with anti-inflammatory and anti-oxidative processes (Li et al., 2018), mainly through the actions of or effects on cytokines (Son et al., 2005; Hasegawa et al., 2012). As such, decreased levels of this essential amino acid have been associated with inflammatory diseases (Hisamatsu et al., 2012; Yu et al., 2015) including HIV infection (Li et al., 2018) and TB (Weiner et al., 2012; Vrieling et al., 2018; Vrieling et al., 2019) (see “similarities” in **Table 3**). The levels of histidine were found to be significantly correlated with CD4 T cell count, which implies a role for this amino acid in CD4 T cell regeneration (Li et al., 2018).

A metabolite directly associated with OS, which was common amongst HIV infection (Williams et al., 2011) and TB (Weiner

et al., 2012; Che et al., 2013) metabolomics studies, was pyroglutamic acid (also referred to as 5-oxoproline) (see “similarities” in **Table 2**). The gamma-glutamyl cycle (γ -GC) forms an integral part of glutathione recycling. Gamma-glutamyl dipeptides, by-products of glutathione synthesis, have been reported to be lower in the serum of TB patients compared to healthy controls (Weiner et al., 2012; Vrieling et al., 2019). Similarly, 5-oxoproline, a metabolite directly downstream from the gamma-glutamyl amino acid in the γ -GC, shows the same trend (Che et al., 2013; Combrink et al., 2019) and correlates with pathological lung damage (Che et al., 2013). The imbalance of the γ -GC during active TB suggests decreased antioxidant capacity because of the prolonged OS. The alterations observed for other amino acids in HIV infection, such as glutamate and aspartate, have also been associated with OS (Sitole et al., 2019). In serum, decreased methionine and increased oxidative products of this amino acid (sulfoxymethionine) have been reported for TB (Suzuki et al., 2016; Ding et al., 2020), but not HIV infection (“differences,” **Table 2**). Decreased methionine has been reported for HIV infection in plasma (Peltenburg et al., 2018) but this is an inconsistent finding. Based on previous reports (using conventional techniques), OS-associated alterations are worse during the co-infected state (Madebo et al., 2003; Awodele et al., 2012). However, the mechanisms that give rise to these alterations may interact in more complex ways than that which would result in simple compounding of effects.

Immune cells remodel energetically and structurally upon immune activation to accomplish the appropriate differentiation. This results in metabolic changes associated with increased energetic and biosynthetic demands as the immune system responds to the increased viral load (Plaza et al., 2016). Mitochondrial dysfunction affects multiple systems (Nunnari and Suomalainen, 2012) contributing to the loss of lipid homeostasis, as this organelle plays key roles in the biosynthesis of phospholipids for membranes, as well as the catabolism of fatty acids (Tilokani et al., 2018). Immune cell function is thus impacted, as phospholipids are essential in the structural remodeling [especially glycerophospholipids and phosphatidylcholines (Sonnino et al., 2018)], differentiation, and cell signaling of immune cells, all of which are crucial to mounting an effective immune response (O'Donnell et al., 2018). As such, it is not surprising that alterations to phospholipids are a common finding in HIV infection and TB metabolic profiles (“similarities,” **Table 2**). Increased cholesterol esters and decreased phospholipids in TB patients have also been associated with the utilization of host lipids by *Mtb* as a carbon source and in various immune evasion mechanisms. Furthermore, the combination of two phosphatidylcholines, a cholesterol ester, and sphingomyelin, was able to distinguish TB patients from those with other lung conditions (Han et al., 2020).

Phosphatidylcholines (phospholipids, **Table 2**) have also been implicated as markers of a more progressive/worsened outcome in HIV infection and TB. In a cohort of HIV-positive individuals on ART, a decrease in phosphatidylcholines predicted poor immunological recovery (Rosado-Sanchez et al., 2019). Interestingly, these lipids were restored after 1 year of ART (Peltenburg et al., 2018). Huang et al. (2020) searched for

serum metabolic biomarkers of MDR-TB, as alternative diagnostic options in future. Decreased NADH metabolites, phosphatidylcholines, caprylic acid, and increased D-xylulose in serum distinguished MDR-TB patients from drug-susceptible TB patients and healthy controls. The authors speculated that these alterations may represent mechanisms of drug resistance, especially relating to cell membrane remodeling in resistant strains of *Mtb*.

Alterations to fatty acids were common between HIV infection and TB in serum (see “similarities” in **Table 2**). Viral infections are associated with fatty acid synthesis (Plaza et al., 2016), which, in addition to various other factors such as mitochondrial dysfunction, contribute to a profile of increased unsaturated triglycerides and decreased sphingomyelin species, as observed by Peltenburg et al. (2018). Alterations to acylcarnitine metabolism, which is linked to fatty acid metabolism, during HIV infection are suggestive of mitochondrial dysfunction (Li et al., 2018). The importance of fatty acids as indicators of TB is exemplified by Che et al. (2018) and Cao et al. (2021). These studies showed that increased levels of tryptophan catabolic products, decreased fatty acids, as well as increased acylcarnitines and bile acids could distinguish TB pleural effusion (TPE) from pleural effusion caused by lung cancer (malignant pleural effusion; MPE), a condition which is also characterized by inflammation and altered energy metabolism. A combination of four acylcarnitines and two fatty acids discriminated TPE from MPE with the highest sensitivity and specificity (Cao et al., 2021). Fatty acids have already been proven to be important in the context of HIV/TB co-infection, as polyunsaturated fatty acids, glycerophospholipids, and sphingomyelins distinguished individuals who developed IRIS from those who did not (Silva et al., 2019).

The metabolism of choline, sarcosine and creatinine is closely linked, as all three these metabolites are intermediates in pathways related to glycine metabolism. While the plasma levels of choline were increased during HIV infection (Munshi et al., 2013), they were lower for TB patients and even more so in TB patients with DM (Vrieling et al., 2019) (see “similarities” in **Table 3**). Choline is a major component of phospholipids, and as such alterations in the levels of this metabolite is associated with membrane turnover (Munshi et al., 2013), which is high during immune activation. Furthermore, choline is also a precursor for acetylcholine, a neurotransmitter, and has been suggested as a biomarker for the AIDS dementia complex (Munshi et al., 2013). A gut microbe-related choline metabolite has also been related to carotid atherosclerosis progression in HIV-positive individuals (Shan et al., 2018). Decreased levels of choline have been associated with non-alcoholic fatty liver disease due to decreased intake of choline or gut microbe dysbiosis (Leermakers et al., 2015; Sharpton et al., 2019). This trend had not been reported in TB patients before the study of Vrieling et al. (2019), who advised caution in the interpretation of this finding. Sarcosine is a downstream metabolite of choline and is converted to glycine. It is an important intermediate in one-carbon metabolism, which is involved in many physiological processes, including redox defense (notably through glutathione). Increased levels of sarcosine (see “similarities” in

Table 3) have been associated with folate deficiencies and muscle wasting during HIV infection (Munshi et al., 2013), but this metabolic alteration has not been interpreted in the context of TB. However, as sarcosine is a product of muscle degradation and cachexia is a characteristic feature of TB (Semba et al., 2010), there may be a similar mechanism driving the increased levels of sarcosine. Glycine and arginine are precursors for creatine. The majority of all creatine in the body is in muscle, where it serves as the primary fuel to maintain ATP availability for cellular metabolism, especially during periods of high energy demand (Kreider and Stout, 2021). As such, the observed serum creatine in HIV infection (Sitole et al., 2019) and TB (Weiner et al., 2018) relates to the prolonged period of high energy expenditure and cachexia associated with these diseases (see “similarities” in **Table 2**). Another contributor to this alteration may be mitochondrial dysfunction, as creatine crosses the mitochondrial membrane as part of the creatine phosphate shuttle, which facilitates the replenishment of ATP (Kreider and Stout, 2021).

Considering the comparisons (**Tables 2 and 3**), the HIV/TB co-infected metabolic profile obtained from blood is likely to be characterized primarily by exacerbated amino acid alterations (especially the essential amino acids and those non-essential amino acids involved in immune function and redox balance such as glutamate and glutamine). There may also be lipid alterations (deduced from similar metabolic changes highlighted in **Tables 2 and 3**). **Figure 1** represents the overlapping metabolic alterations and maps these changes and their interpretations, which have been identified by HIV infection and TB metabolomics, separately, onto what is known about host metabolism during the co-infected state (dark purple arrows). It shows that HIV infection and TB (to the left and right of the Venn diagram, respectively) is associated with constant immune activation and inflammation as well as alterations to the gut microbiome (metabolites 1 and 2), which increases the host energy expenditure as immune cells differentiate (reflected by metabolites 3–14) and pathogens carry out biosynthetic processes (metabolites 3 and 10). This adds to the oxidative profile (metabolites 2–4, 6, 7 and 14), impacting mitochondrial structure and function (metabolites 5, 9, 11 and 14). Part of the urea cycle occurs in mitochondria, and since this organelle's structure and function is impacted, there is reduced clearance of ammonia, which translates to urea cycle alterations. In this chronic state of immune activation and inflammation, the individual experiences malaise and anorexia, leaving a malnourished individual who eventually suffers from cachexia (metabolites 7, 13 and 14). Dysfunctional mitochondria impact lipid metabolism and is reflected as changes in phospholipids, choline, sarcosine and fatty acids—all with interrelated and sometimes compounding effects on the immune system, inflammation and biosynthetic processes. Given that these changes occur during HIV infection and TB respectively, it is highly likely that they will be detected in co-infected samples (represented by the overlapping Venn diagram) and that more quantitative versus qualitative differences will reflect.

Although the lipids did not overlap between the studies compared, the classes affected were similar, and their

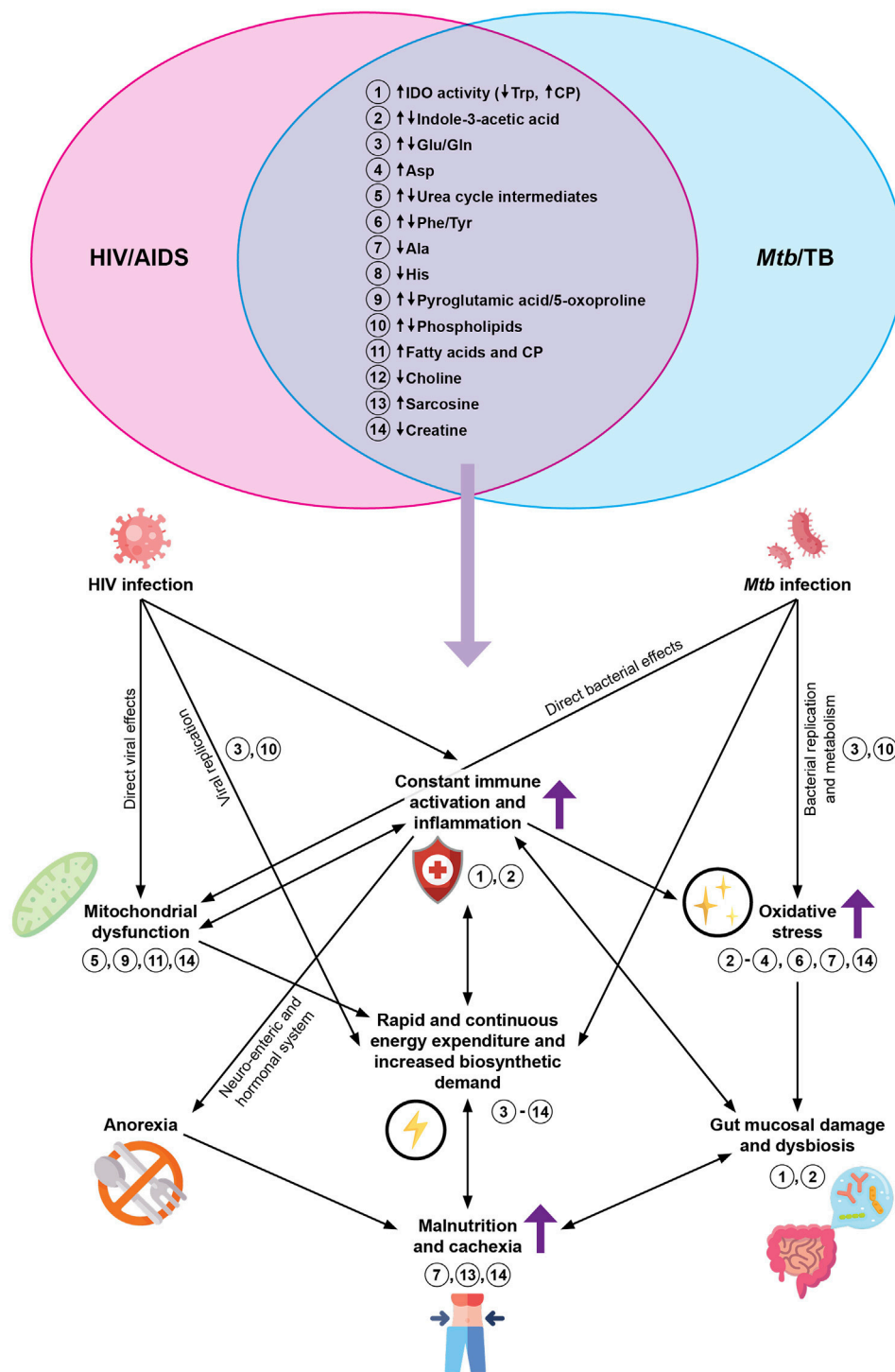


FIGURE 1 | The proposed metabolic effects of HIV/TB co-infection, based on the overlapping findings of prior blood-based HIV infection and TB metabolomics studies (as indicated by the Venn diagram at the top). The dark purple arrows represent effects that are known to be exacerbated during HIV/TB co-infection. The light purple arrow represents metabolites hypothesized to be altered during HIV/TB co-infection, based on published literature. The numbers indicate the overlapping metabolites and where they play a role in what is known about host metabolism during HIV/TB co-infection. Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; *Mtb*, *Mycobacterium tuberculosis*; TB, tuberculosis; IDO, indoleamine-2,3-dioxygenase; Trp, tryptophan; CP, catabolic products; Glu, glutamate; Gln, glutamine; Phe, phenylalanine; Tyr, tyrosine; Asp, aspartate; Ala, alanine; His, histidine.

TABLE 4 | A comparison of the findings of HIV infection and TB urinary metabolomics investigations^a.

Similarities					
Metabolic alteration		Proposed origin(s) of metabolic alterations			References
Increased neopterin		Product of the catabolism of guanosine triphosphate in immune cells in response to interferon-gamma — indicative of a pro-inflammatory immune status			HIV: Munshi et al. (2013) TB: Isa et al. (2018)
Increased catecholamines (e.g. epinephrine, norepinephrine)		Various roles in the immune system and as neurotransmitters and hormones			HIV: Munshi et al. (2013) TB: Das et al. (2015)
Differences					
HIV infection	Proposed origin(s) of metabolic alterations	References	TB	Proposed origin(s) of metabolic alterations	References
Increased isoleucine and methionine, decreased alanine	Immune activation, increased amino acid oxidation for energy	Munshi et al. (2013)*	Increased tryptophan catabolic products (e.g. kynurenine)	Increased IDO activity	Luies and Loots (2016), Isa et al. (2018)
Increased formic acid and decreased 2-methylglutaric acid	Altered central energy metabolism; pyruvate and TCA cycle metabolism		Increased intermediates of phenylalanine and tyrosine metabolism	Compromised insulin production and resulting in decreased PAH activity, altered gut microbial activity	Luies and Loots (2016); Das et al. (2015)
			Increased fatty acids and their catabolic products	Compromised insulin production, various bacterial mechanisms	Luies and Loots (2016)
			<i>Mtb</i> -specific metabolites	Cell wall components, intermediates of bacteria-specific metabolic pathways	Luies and Loots (2016)

^aThere is only one HIV metabolomics study on urine that focused on the characterization of the disease state. IDO: indoleamine 2,3-dioxygenase 1; PAH: phenylalanine hydroxylase; TCA: tricarboxylic acid cycle.

numerous roles in HIV infection, TB, and the immune response are already established. The exacerbated immune dysfunction during co-infection (Sharan et al., 2020) is likely to be reflected in the metabolic profile in terms of the overlapping HIV infection and TB metabolites (**Figure 1** and “similarities” in **Tables 2–4**). Furthermore, those altered metabolites which have to date only been associated with either HIV infection or TB may also be significant during co-infection (“differences” in **Tables 2–4**) and metabolites that have not previously been associated with HIV infection or TB may also be significant in the co-infection metabolic profile.

Features unique to TB pathology have also been reported, such as fibrinopeptides, proposed to originate from the granuloma (Weiner et al., 2012) (see “differences,” **Table 2**). Within plasma, alterations to the branched-chain amino acids were more commonly reported for HIV infection than for TB, although alterations to these amino acids are commonly attributed to immune activation and wasting, which are also features of TB (see “differences,” **Table 3**). Given the limited number of metabolomics studies that focused on HIV/TB co-infection to date, the metabolic mechanisms underlying HIV/TB co-infection remains incompletely characterized. The comparison made here yields insight into how the metabolic changes during HIV infection and TB may interact during the co-infected state and could serve to inform future study design. Compared to blood, urine is used less frequently for characterization or diagnostic purposes of either HIV infection or TB, although it has been popular for TB drug metabolism elucidation (see Du Preez et al., 2019). As such, the extensive comparison made for blood could not be made for urine

(and other sample matrices), and hence a large piece of the puzzle is still missing.

Given the advantages of urine as a clinical specimen (Du Preez et al., 2019), it has been considered an excellent alternative to sputum for TB diagnostics. Characterizing the urine metabolic profile of HIV-positive individuals will be a necessary step if urine were to be developed as a robust TB diagnostic matrix. The HIV infection and TB urine metabolic profiles overlap to a smaller degree than the blood-based profiles. While the urinary metabolic profile of HIV-positive patients indicates altered energy metabolism and the utilization of amino acids for energy, the urinary TB metabolic profile indicates increased inflammation and compromised insulin production in addition to reflecting *Mtb*-related alterations (see “differences,” **Table 4**). This relatively smaller degree of overlap may be due to the limited number of studies eligible for the comparison (in which participants were untreated for either condition; see **Table 4**). Nevertheless, increased levels of neopterin (which has been detected using conventional assays for both diseases) and catecholamines were points of similarity in the urine metabolic profiles of HIV infection and TB (**Table 4**).

The study of Munshi et al. (2013) provides unique insights into HIV infection since it relied on the use of various sample types, namely plasma, urine, and saliva. The only other urinary HIV-related metabolomics study is that of Chetwynd et al. (2017), which does not concur with Munshi et al. (2013) on all results. For instance, the authors’ observations regarding the extent to which immunosuppression is reflected in urine

differs. Chetwynd et al. (2017) found that no separation could be achieved within the HIV-positive treatment-naïve group upon stratification by CD4 T cell count (point of division at 250 cells/mm³), which led them to suggest that metabolic markers of immunosuppression are not visible in urine. However, conventional methodologies established that urinary neopterin has an inverse correlation with CD4 T cell count (Immanuel et al., 2005). Increased levels of urinary neopterin have been recognized as a marker of immune activation, a trend which has been observed in urinary HIV infection (Munshi et al., 2013) and TB metabolomics studies, as well as in a small group of co-infected patients included in the study of Isa et al. (2018), in which the elevation was more pronounced. These results confirm previous findings [reviewed by Siawaya et al. (2007)] and suggest that urinary neopterin may be useful as part of the HIV/TB co-infection biosignature. There have been observations where urinary neopterin decreased in response to ART (Munshi et al., 2013) as well as in serum upon anti-TB treatment. This suggests the additional usefulness of neopterin as a prognostic marker. In co-infected patients, consistently high neopterin levels have been associated with HIV disease progression and poor outcomes (Immanuel et al., 2005). As co-infection metabolic profiles have not yet been systematically studied, this remains a viable avenue for future investigation.

There are a few studies not relevant to the comparisons in **Tables 2–4**, but which are important to mention in the context of the co-infected state. Alterations to the human microbiome have been associated with both HIV infection and TB, but it remains unclear exactly how the microbiome is affected during co-infection. The composition of the microbiome of the upper respiratory tract of HIV-positive treatment-naïve individuals was similar to those of HIV/TB co-infected patients except for a lower abundance of species belonging to the *Veillonellaceae* family of bacteria. This family was also less abundant in the treatment-naïve TB group relative to the treated TB group. This suggests such an alteration to the microbiome of HIV-positive patients to be indicative of TB, but this requires further investigation (Htun et al., 2018).

Two metabolomics studies focused on lung health in HIV-positive individuals, which provides the opportunity to evaluate the state of the lungs before *Mtb* infection in HIV-positive individuals from a metabolomics perspective. Cribbs et al. (2014) studied the BALF metabolic profiles of HIV-positive individuals compared to healthy controls and found that the primary differences reflected in metabolites have their origin in the lung microbiota, and could further indicate a subclinical infection in the HIV-positive lung. This study established a specific metabolic profile of the HIV-positive lung environment, which gives an indication of lung immune function and could contribute to improving diagnostic and prognostic techniques. In another study, HIV-positive individuals could not be distinguished from healthy controls based on lung microbial population composition (determined by 16S rRNA gene sequencing); however, a distinction could be made based on the BALF metabolic profile (Cribbs et al., 2016). These studies indicate that the interaction of the host and the microbiota contribute to a functional difference in the HIV-positive lung, rather

than the species present. Given that HIV-induced metabolic alterations have their origin in immune dysfunction, it is the most likely cause for the alterations observed by the authors. Such an altered environment may change the way that the HIV-positive host interacts with *Mtb*, and vice versa, contributing to the altered clinical course and phenotype of co-infected individuals.

METABOLOMICS AS A TOOL TO INVESTIGATE HUMAN IMMUNODEFICIENCY VIRUS/TUBERCULOSIS CO-INFECTION

There are several aspects in the HIV/TB co-infection field that remain unanswered. These gaps were reinforced/emphasized in our analysis of the HIV infection and TB metabolomics literature on informing potential changes during co-infection. The overlap in metabolites from the respective HIV infection and TB studies which confirm what is already known about co-infection indicates the potential use of metabolomics in further characterizing the co-infected state.

The diagnostic problems experienced in HIV/TB co-infected individuals are related to the diagnosis of TB rather than the diagnosis of HIV infection, as mentioned above, due to the limitations of sputum smear and culture-based tests [reviewed by Schutz et al. (2010); Tiberi et al. (2017)]. Co-infected individuals are also often unable to provide a sufficient sputum sample for diagnostic testing, especially when severely immunocompromised (World Health Organization, 2015). Molecular tests for TB diagnosis (GeneXpert MTB/RIF assay and the improved version, GeneXpert MTB/RIF Ultra) are highly effective in diagnosing TB in HIV-negative individuals, has a better turn-around time, and can give an early indication of drug resistance. However, access to such tests in high burden areas is limited by cost and there are concerns about the specificity (false-positives) of this test in HIV-negative individuals (World Health Organization, 2019). Similarly, a urinary point-of-care diagnostic assay based on the detection of an *Mtb* cell wall component, lipoarabinomannan (LAM), has not found widespread application partly due to its inapplicability to HIV-negative samples. It is recommended that this test not be used for screening or diagnosis in HIV-negative individuals nor HIV-positive individuals with relatively high CD4 T cell counts (World Health Organization, 2015). Given the low sensitivity of this test, an improved version (Fujifilm SILVAMP TB LAM) has been developed which retained specificity with improved diagnostic sensitivity (Broger et al., 2019). Despite high efficacy when this test is applied to immunocompromised individuals, a difficulty remains in assigning diagnostic categories [such as definite TB, possibly TB, or not TB, as used by Broger et al. (2019)] based on the results of various indicators of TB. For example, several patients could not be diagnostically classified, but tested positive using Fuji-LAM. Consequently, these patients were not started on anti-TB drugs and died within 3 months of follow-up (Broger et al., 2019).

Considering the sputum diagnostic limitations, the focus for the development of new TB diagnostic techniques should be on the identification of non-sputum biomarkers. Metabolomics can

aid in identifying markers with diagnostic application or be combined with existing tests to increase sensitivity and specificity. Urine is an ideal sample in this sense as the procedures for collection and storage are simple and require minimal biosafety considerations (World Health Organization, 2015). This makes identifying markers to be used in conjunction with the LAM assay an attractive avenue of research to improve the early detection of TB and timely intervention in HIV-positive individuals. However, the criteria by which such a marker is selected must be carefully considered, particularly amenability to accurate and sensitive detection by an inexpensive point-of-care platform (Walzl et al., 2018) and specificity to TB (as opposed to a general inflammatory marker). One must also consider the increased incidence of extrapulmonary TB in this regard. Often, extrapulmonary TB is not included in the TB diagnostic testing procedure, and even if it was, sample collection for diagnosis remains difficult (Denkinger et al., 2014). If biomarkers specific to extrapulmonary disease are identified, including these in the standard TB diagnostic repertoire may significantly improve the early diagnosis of TB in HIV-positive individuals.

Metabolomics has been applied to identify metabolic determinants that indicate conversion of latent *Mtb* infection to clinical disease in the studies of Weiner et al. (2018) and Albors-Vaquer et al. (2020). The efficacy of such a profile has not yet been tested in HIV-positive individuals, for whom the risk of conversion to clinical disease is much greater than for HIV-negative individuals. Untreated profiles aid in understanding the underlying mechanisms that drive conversion of latent *Mtb* infection to active TB in HIV-positive individuals and provide early prognostic information, especially regarding TB-IRIS and treatment response. However, this might prove difficult considering sample availability, given the recommendation for early treatment of latent *Mtb* infection (World Health Organization, 2016a) as well as the “Test and Treat” policy for HIV infection, recommended by the World Health Organization (World Health Organization, 2016b). Nonetheless, such basic understanding is necessary for the development of robust and sustainable diagnostic and prognostic procedures.

Even if a highly effective diagnostic technique for TB in HIV-positive persons is developed, its success will depend on the integration of HIV infection and TB services on the ground, especially in high-burden areas, which are unfortunately also most often resource-limited. Once the difficult diagnosis is made, various factors complicating the management of HIV/TB co-infection come into play. The management of HIV/TB co-infection is challenging and is usually associated with unfavorable treatment outcomes due to various factors, reviewed by Tiberi et al. (2017) and Letang et al. (2020). Pharmacometabolomics is a branch of metabolomics wherein the effects of a drug or combination of drugs underlie the observed metabolic alterations (Combrink et al., 2019) and have been applied to HIV infection (Chetwynd et al., 2017; Li et al., 2018; Babu et al., 2019) and TB (reviewed by Du Preez and Loots, (2018), see Table 3). The interactions between ART and anti-TB drugs, and their interactions with enzymes, have been characterized by high-performance LC-MS (Wenning et al.,

2009) and LC-MS/MS (Varshneya et al., 2019), demonstrating how metabolomics aids the understanding of *in vivo* drug metabolism and efficacy, which is essential to the development of better treatment strategies for co-infected patients.

Besides the interactions of the drugs themselves, variations on an individual level, identified as one of the factors contributing to TB treatment failure (De Villiers and Loots, 2013) will also affect the efficacy of drug regimens. The rate of recurrent TB (relapse and reinfection) is greater among HIV-positive compared to HIV-negative individuals (Sonnenberg et al., 2001; Charalambous et al., 2008). The only way to prevent reinfection would be an effective vaccine. Also, by better understanding the mechanisms underlying treatment failure in co-infected patients, the cases of relapse will be minimized by optimizing treatment regimens. Metabolomics has been used to identify the host factors that contribute to TB treatment failure in HIV-negative TB patients (Luies et al., 2017). Such an approach could provide valuable insights, as co-infection is associated with poor treatment outcomes and susceptibility to infection in general, due to the additional immunosuppressive effects of HIV (Guerra-Assuncao et al., 2015). Another aspect of interest from a metabolomics perspective is how the effect of the second *Mtb* co-infection event on the HIV-positive host would compare to that of the first.

Given that much of the HIV and *Mtb* infection-induced metabolic changes are driven by immune activation, inflammation and OS with subsequent impact on the mitochondria and the gut (Figure 1), metabolomics could aid in elucidating the effect that *Mtb* has on the gut mucosal damage as induced by HIV. The metabolic markers obtained are not unique and have been reported in the metabolomics investigations of other inflammatory conditions (Leermakers et al., 2015; Pett et al., 2017). As such, more longitudinal studies, especially in the treated context, with the inclusion of more disease controls to get more specific markers associated with each condition are thus needed.

CONCLUDING REMARKS

For the rational design of novel or improved diagnostic, treatment, and monitoring techniques, the research must first focus on better characterizing a disease state so that the origin of identified markers is understood, as opposed to unqualifiedly advocating statistically significant markers that have no true specificity for the disease state. It is of extreme importance to develop and validate highly specific biomarkers for HIV/TB co-infection—although biosignatures may be more practical considering the overlapping pathologies of HIV infection and TB. Furthermore, when developing these metabolic signatures, it should be kept in mind that the ultimate goal is point-of-care, individualized assessments that should be accessible even in resource-limited settings, as these are commonly the areas of high incidence and spread. This further requires the complete integration of HIV infection and TB services and for this, a greater awareness of co-infection should be brought among clinicians and built into infrastructure to ensure that even those with minimal education could apply it.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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1 **Characterising the immunometabolic profile of HIV/TB co-infection in cohorts**
2 **untreated and treated for HIV**

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1 Abstract

The immunological deterioration and metabolic mechanisms driving the HIV-*Mtb* synergy during co-infection are poorly understood. Although ART suppresses viral replication, it causes metabolic alterations beyond that induced by infection. The serum cytokine and metabolic profiles of untreated HIV-negative TB-positive patients, HIV/TB co-infected, and HIV/TB co-infected on ART, as well as healthy controls, were measured using multiplexed flow cytometric cytokine measurements and untargeted gas chromatography mass spectrometry. The cytokine levels of the diseased groups did not differ significantly when compared to each other, however, the co-infected individuals were characterised by increased IL-6 and IFN- γ when compared to controls. These suggested a failure of immunoregulation, resulting in an increased disease burden. Metabolites indicative of cachexia, intestinal mucosa damage, and microbiome dysbiosis characterised co-infected individuals from the TB-positive population, while co-infected individuals were additionally distinguished from healthy controls by more extensive amino acid changes. Treating HIV in the co-infected group revealed a general metabolic return to values comparable to the healthy or the TB-positive population, although some metabolic alterations associated with gut mucosal damage and oxidative stress were exacerbated. These results suggest that HIV partially augments the HIV-*Mtb* synergy by increasing oxidative stress and through its detrimental effects on gut health, affecting energy availability in turn.

Keywords: HIV/AIDS₁, tuberculosis₂, HIV/TB co-infection₃, metabolomics₄, cytokines₅ immunometabolism₆

2 Introduction

Human immunodeficiency virus (HIV)-positive individuals have a 26–31 times greater risk of developing active tuberculosis (TB) compared to HIV-negative individuals (1) due to the immunosuppressive effects of HIV on the host. Unsurprisingly, TB is the most common secondary disease among HIV-positive individuals, hence there is a significant global burden of HIV/TB co-infection. The coronavirus disease 2019 (COVID-19) pandemic has increased this burden, with diminished access to health care facilities, and hence poor diagnosis, reporting, and treatment

initiation for HIV infection and TB, resulting in increased TB-related deaths during this period (2-4). Approximately 14% of the estimated 1.3 million TB-related deaths in 2020 were among HIV-positive individuals (5). It is well established that HIV and *Mycobacterium tuberculosis* (*Mtb*) act synergistically during co-infection of a host, amplifying the disease burden. This synergy is centred on immunological deterioration (6, 7), which in turn is the result of an increased level of immune activation during co-infection (8). The immune and metabolic systems are intricately connected, and changes in the one are reflected in the other. The term “immunometabolism” has mainly been used in the context of the metabolism of immune cells, but the effects of these alterations extend to systemic metabolism also, as the organism’s energy and biosynthetic stores are also directed towards the immune response. The term will be used in a comprehensive sense here, encompassing both immune cell and systemic metabolic alterations and their relation to the immune response. All references to “HIV” imply HIV-1.

Despite being characterised from various perspectives using comprehensive and sensitive techniques [reviewed by (7, 9-12)], the immune response of the host during co-infection remains poorly understood. Cytokines have been extensively investigated in the context of HIV infection, TB, and HIV/TB co-infection as biomarkers of immune activation and inflammation (13-15). Previous studies have measured the inherent (i.e. unstimulated) plasma and cellular cytokine profiles of untreated HIV/TB co-infected patients, but this has not been done in serum, and not concurrently with comprehensive metabolic measurements. Representative cytokines from the T helper cell type (Th) 1, Th2 and Th17 cytokine groups, are important in the context of HIV/TB co-infection. In this regard, the importance of interferon-gamma (IFN- γ ; and the chemokines it induces) (16, 17), interleukin (IL)-10 (17), IL-6 (18, 19), and IL-17 (20) in the immune response during HIV/TB co-infection has been demonstrated. These cytokines have separately been shown to be involved in the immune response to HIV infection and *Mtb*, respectively (15, 21), indicating that the immune response during HIV/TB co-infection presents aspects of the response to both pathogens. However, it is known that the

immune response during HIV/TB co-infection is severely dysregulated and in many ways promote the disease pathology, rather than to subvert it (7, 12).

The metabolic alterations that occur during co-infection are not well characterised and have mainly been measured using conventional techniques that rely on low-throughput and laborious biochemical assays (22). These techniques have indicated that HIV/TB co-infected patients have an impaired net protein balance in the fed state – while protein balance during HIV infection alone or TB alone, was strongly anabolic (23). Co-infected patients, when compared to TB patients, were also shown to suffer from hypoalbuminemia (a further indicator of impaired protein balance) (24) but have similar body composition changes (25). Since these techniques do not provide a comprehensive picture of the metabolic state, metabolomics approaches may supplement this knowledge gap. Although metabolomics has been used to better understand very specific aspects of host metabolism during infection (26, 27), it has not been used in an explorative manner to characterise the metabolic alterations that accompany HIV/TB co-infection. Untargeted metabolomics aims to characterise as much of the metabolome as possible by identifying and quantifying as many metabolites as possible. It is also considered a hypothesis-generating research approach (28). Therefore, untargeted metabolomics is ideal to provide a preliminary overview of the altered host metabolism during HIV/TB co-infection, and also to highlight areas of interest for future studies. Furthermore, the effects of ART in addition to the effects of HIV infection, on the host have been characterised in various contexts using cytokine measurements (29-31) and metabolomics (32-36). Although ART typically leads to decreased viral load (VL), low-level inflammation remains and metabolic complications are common (37). A previous investigation of the blood cytokine profile of HIV/TB co-infected patients on ART showed that the inflammatory network density declines with ART, but that this is dependent on the degree of viral suppression (20). The metabolic effect of ART in the context of HIV/TB co-infection has been investigated using conventional techniques [reviewed by Jain, Furfine (37)], and with a focus on the immune reconstitution inflammatory syndrome, using metabolomics (38). However, metabolomics has as yet not been used to study untreated and treated co-infection in an

exploratory manner. Therefore, this investigation aims to better characterise the immunometabolic profile of HIV/TB co-infected cohorts, with and without ART.

3 Results

Figure 1 indicates the various group comparisons and their individual aims for the four patient cohorts. The demographical and clinical summary of the cohort is given in Table 1, while detailed demographic data, descriptive statistics, as well as correlation and quantitative metabolite set enrichment analysis (MSEA) data, are provided as supplementary information. There were no significant differences between the aforementioned compared groups for age, sex, smoking, and CD4 T cell count (in the HIV-positive groups) using Fisher's exact test (Table S1, supplementary information). Those patients that were on medication or had other medical conditions, which were still included in this study, did not reflect as outliers. Although these factors may have affected the individual cytokine and metabolome profiles of these individuals, and may result in variation, many of the other patients likely had similar conditions unknown to them (especially given the severely ill state of most of the co-infected patients) or which were not disclosed upon sample collection. This may have resulted in those patients who did in fact declare the use of other medication or other diseases, not reflecting as significantly different from the rest of the group.

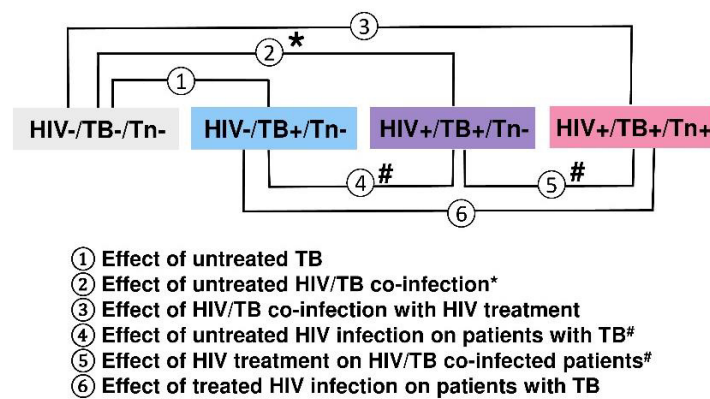


Figure 1: The comparisons that are possible with the four patient groups and their purpose/experimental aims. The comparisons that are indicated with an asterisk (comparison 2) and a hashtag (comparisons 4 and 5), respectively, are pertinent to the aims of this investigation. The other comparisons are highlighted where necessary to provide context to the comparisons related to the primary aims. Groups: HIV-/TB-/Tn-: healthy

controls; HIV-/TB+/Tn-: untreated HIV-negative patients with active pulmonary TB; HIV+/TB+/Tn-: untreated HIV/TB co-infected patients; HIV+/TB+/Tn+: HIV/TB co-infected patients on ART.

Table 1: Cohort demographics and clinical information.

	HIV-/TB-/Tn-	HIV-/TB+/Tn-	HIV+/TB+/Tn-	HIV+/TB+/Tn+
No. of patients (%)	29 (40.3)	22 (30.6)	9 (12.5)	12 (16.7)
Age (years), mean $\pm$ SD (range)	33.4 $\pm$ 10.3	34.4 $\pm$ 12.8	31.9 $\pm$ 6.2	40.9 $\pm$ 10.6
Sex, male:female ratio (%)	13:16 (45:55)	13:9 (59:41)	5:4 (56:44)	4:8 (33:67)
Smokers in the group (%)	9 (31.0)	11 (50.0)	2 (22.2)	3 (25.0)
CD4 T cell count (cells/mm ³ blood), mean $\pm$ SD	N/A	N/A	221.4 $\pm$ 252.4	182.7 $\pm$ 162.2
Viral load (copies/mm ³), range*	N/A	N/A	<20–124	<detectable–513 509
Duration of ART (years), mean $\pm$ SD	N/A	N/A	N/A	4.3 $\pm$ 3.6
Patients on FDC ART combination (%)	N/A	N/A	N/A	10 (83.3)
Patients on different ART combinations(%)	N/A	N/A	N/A	2 (16.7)

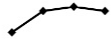
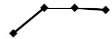
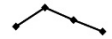
ART: antiretroviral therapy; SD: standard deviation; FDC: fixed-dose combination. Groups: HIV-/TB-/Tn-: healthy controls; HIV-/TB+/Tn-: untreated HIV-negative patients with active pulmonary TB; HIV+/TB+/Tn-:untreated HIV/TB co-infected patients; HIV+/TB+/Tn+: HIV/TB co-infected patients on ART. *Viral load was not available for all patients, hence the data shown here are not representative of the entire group. Viral load also varied due to differences in ART duration.

3.1 Cytokines

The results of the cytokine measurements and the corresponding trends and statistics are given in Table 2. IL-6 and IFN- γ were significantly increased in all the diseased groups relative to the healthy controls using one-way analysis of variance (ANOVA; IL-10 showed the same trend but with borderline significance). The following trends (although not statistically significant, listed in Table 2) were also observed in the co-infected group relative to the healthy controls (comparison 2): IL-10 and IL-17A were increased, IL-4 and IL-2 were decreased, while TNF- α remained largely unchanged. There were no statistically significant differences in cytokine levels between the diseased groups, but the levels of IL-6 were higher in the untreated co-infected group relative to the TB group, IFN- γ similar, and IL-4 reduced (comparison 4). Upon comparing the untreated co-infected group to the co-infected group on ART, IL-6 and IFN- γ remained high (similar levels), while IL-10, IL-17A, TNF- α were decreased, and IL-4 and IL-2 were increased following treatment (comparison 5). It is also noticeable

that IL-4 and IL-2 were comparatively the lowest, while IL-6, IL-10, and IL-17A were highest in the untreated co-infected group relative to all other groups.

Table 2: The seven cytokines arranged according to trend and statistical significance.

Cytokine	Line graph of means 	ANOVA (BH-corrected p-value)	Post-hoc (comparison where cytokine is significantly different, Figure 1)
IL-6		< 0.0001	①②③
IFN- γ		< 0.0001	①②③
IL-10		0.066	②③
IL-4		0.109	-
TNF- α		0.748	-
IL-17A		0.748	-
IL-2		0.975	-

Abbreviations: IL: interleukin; IFN: interferon; TNF: tumour necrosis factor; ANOVA: one-way analysis of variance; BH: Benjamini-Hochberg.

Figure 2a shows the partial least squares-discriminant analysis (PLS-DA) scores plot. Cross-validation indicated that the model was overfitted (five-component model: $Q^2 = 0.62$, $R^2 = 0.67$, accuracy = 0.65), likely due to the small sample size, but permutation testing indicated that the model was not random [p-value = 5×10^{-4} , meaning that the classification of the samples into the designated groups is significantly better than any random classification (39)]. Nonetheless, given the explorative nature of the study, the PLS-DA model was included to show the distribution of samples relative to each other. The distribution shows that the majority of HIV/TB co-infected individuals lie furthest away from the healthy controls, whereas co-infected individuals on ART lie anywhere in between in terms of the serum cytokine profile. IL-6 and IFN- γ retained statistical significance in a multivariate approach [PLS-DA variable influence on projection (VIP) > 1] and were significantly positively correlated with each other and with IL-10 and IL-17A (Figure 2b and c). Refer to the supplementary information for detailed PLS-DA and correlation results (Tables S3 and S4, supplementary information). CD4 T cell count was significantly negatively correlated with IL-6 and IL-17A (Figure 2d).

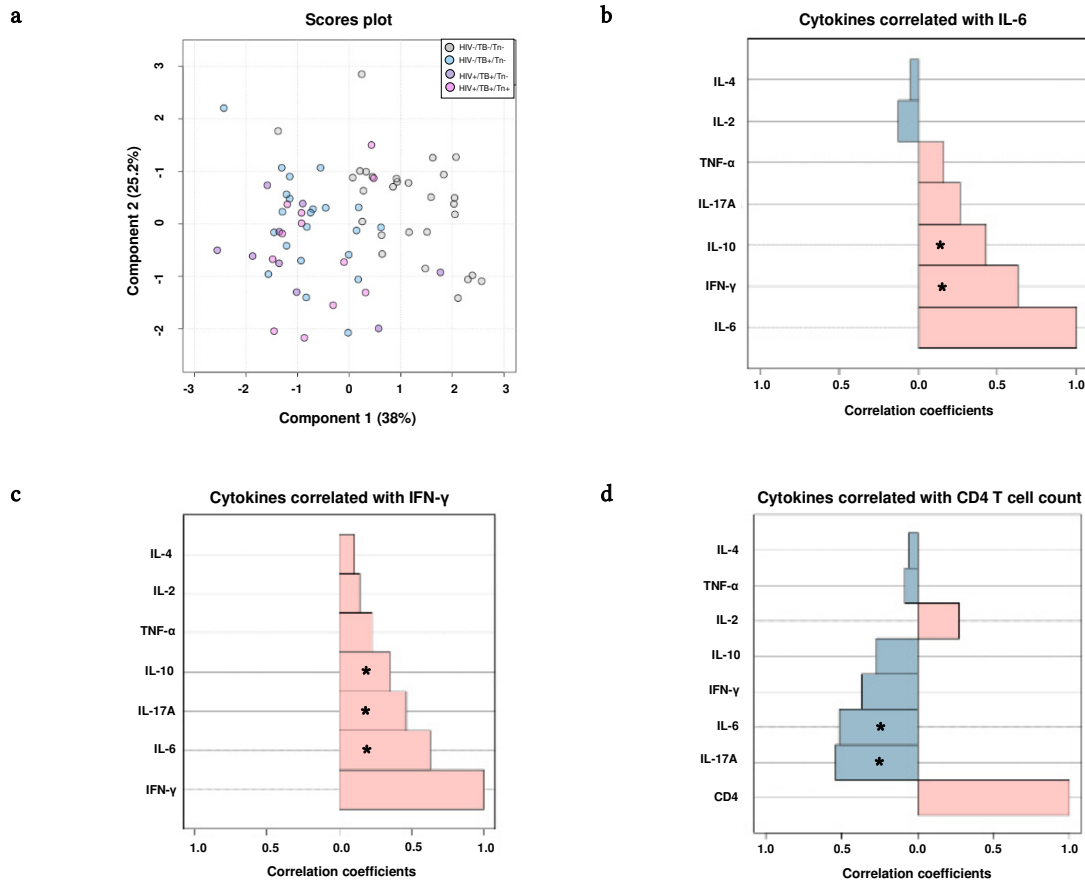


Figure 2: Multivariate statistics and correlation histograms for the cytokine data. (a) PLS-DA scores plot for the cytokine data indicates a significant overlap between the cytokine profiles of the diseased groups. (b) and (c): Pearson's r correlations of IFN- γ and IL-6 with all other cytokines measured. (d) Pearson's r correlations of CD4 T cell count and all cytokines measured. Significant correlations (corrected $p < 0.05$) are indicated by an asterisk (*).

3.2 Metabolomics

The ANOVA analysis indicated 47 metabolites as significantly different between the groups. These metabolites, their trends, and the corresponding statistics are shown in Table 3 (also see Table S5). Four of the statistically significant metabolites could not be correctly annotated, possibly due to high signal-to-noise ratios, unsuccessful deconvolution, or its absence from the libraries used (40). Metabolites that could not be related to any biological function are not discussed [exogenous metabolites (n=11), Table 3], thus their function and presence in human serum require further investigation. Instances where metabolites were annotated with the same name but with different

unique masses and where the mass spectra could not be visually matched, were listed separately (Table 3).

The data was also subjected to multivariate analysis. Principal component analysis (PCA) indicated no natural separation between the groups and PLS-DA cross-validation and permutation testing indicated that the model was over-fitted (cross-validation for a one-component model: $Q^2 = 0.56$, $R^2 = 0.80$, accuracy = 0.60; permutation testing p-value = 0.386). As explained, this model was only used in an explorative sense and the scores plot is displayed in Figure 3a to show the distribution of sample groups. Figure 3a indicates that the co-infected patients on ART, and not the untreated co-infected patients, lie farthest away from the healthy controls in terms of the serum metabolic profile and that the diseased groups were expectedly more heterogeneous than the healthy controls. All metabolites indicated as significant by the ANOVA also had a VIP > 1 considering the first five components of the PLS-DA. Although the metabolites listed in Table 3 are not predictive, they can be used to explain the variance between the groups in this cohort, and therefore the metabolic alterations induced during the disease states under investigation. Quantitative MSEA was used to facilitate the biological interpretation of the metabolites in Table 3 and indicated that the metabolic pathways of essential amino acids and glycerolipids were most significantly enriched (Figure 3b). 3,4-Dihydroxybutyric acid, phenylalanine, lysine, and tryptophan were selected, based on statistical significance and biological relevance, for correlation with other metabolites (Figure 4).

Table 3: The 47 serum metabolite markers that best describe the variation between the groups compared in this study.

Compound	Line graph of means 	ANOVA (BH-adjusted p-value)	Post-hoc (comparison where metabolite is significantly different. Figure 1)
Amino acids and related metabolites (of human and/or microbial origin)			
Phenylalanine (unique derivatised mass = 218)		0.002	①②③④
DL-Phenylalanine (unique derivatised mass = 120)		< 0.0001	①②③
2,6-di-tert-butyl-p-Cresol		0.021	②
Benzoic acid		0.007	②③
L-Norvaline		0.042	①②③
Tryptophan		0.013	②③
3-Indoleacetic acid		0.009	②③⑥
3-Indolepropionic acid		0.018	②③
3-Indolelactic acid		0.007	②③

Methionine		0-002	(2)(3)
Threonine		0-002	(2)(3)
Lysine		0-001	(2)(3)
2-Aminobutyric acid		0-039	(2)(3)
Hydroxyproline		0-002	(2)(3)
Proline, 1-methyl-, methyl ester		0-036	(1)(2)(5)
3-Hydroxyisovaleric acid		0-001	(1)(2)(3)
3-Hydroxypropionic acid		0-033	(2)(3)
Cadaverine		0-007	(3)(4)(6)
Energy metabolism			
Glycerol-3-phosphate		0-002	(2)(3)(4)
D-Glucuronic acid		0-002	(3)(4)(6)
Pyruvate		0-029	(1)(3)
Carbohydrates of exogenous or microbial origin			
Rhamnose		0-018	(1)(2)(3)
D-Mannitol		0-036	(1)(2)(5)
1-Deoxypentitol		0-001	(2)(3)(4)(6)
Tetrahydrofuran		< 0-0001	(2)(3)(4)(6)
2,4-Di-tert-butyl-phenol		0-001	(6)
D-Arabinose (unique derivatised mass = 204)		0-011	(1)
D-Arabinose (unique derivatised mass = 217)		0-050	(1)(4)
Fatty acid and lipid-related metabolites			
2,4-Dihydroxybutyric acid		0-044	(1)(2)
3,4-Dihydroxybutyric acid		< 0-0001	(1)(2)(3)(4)
alpha-Linolenic acid		0-018	(2)(4)(5)
1,E-11,Z-13-Octadecatriene (alpha-linolenic acid, unique derivatised mass = 67)		0-018	(1)(2)
Dodecanal		0-021	(2)(4)
Linoleyl alcohol		0-040	(2)
Capric monoethanolamide		0-043	(2)(5)
Glycerol (unique derivatised mass = 103)		0-002	(1)(2)(3)
Glycerol (unique derivatised mass = 73)		0-009	(1)(2)(3)
Unannotated/exogenous metabolites			
Acrylic acid 2-dimethylamino-ethyl ester		0-004	(2)(3)
Carbonic acid, 2-dimethylaminoethyl ethyl ester		0-044	(2)(3)
Carbonic acid, 2-dimethylaminoethyl propyl ester		0-046	(2)(3)
Cyclobutanecarboxylic acid, 2-dimethylaminoethyl ester		0-018	(2)(3)
Tetrahydro-pyran-2,4,5-triol		0-038	(2)(3)
2-Amino-1-methyl-1H-imidazol-4-ol		0-021	(3)
Analyte 622		0-002	(3)(6)
Analyte 628		0-002	(2)(3)(4)
Analyte 706		0-050	(2)(3)
Analyte 819		0-044	(3)

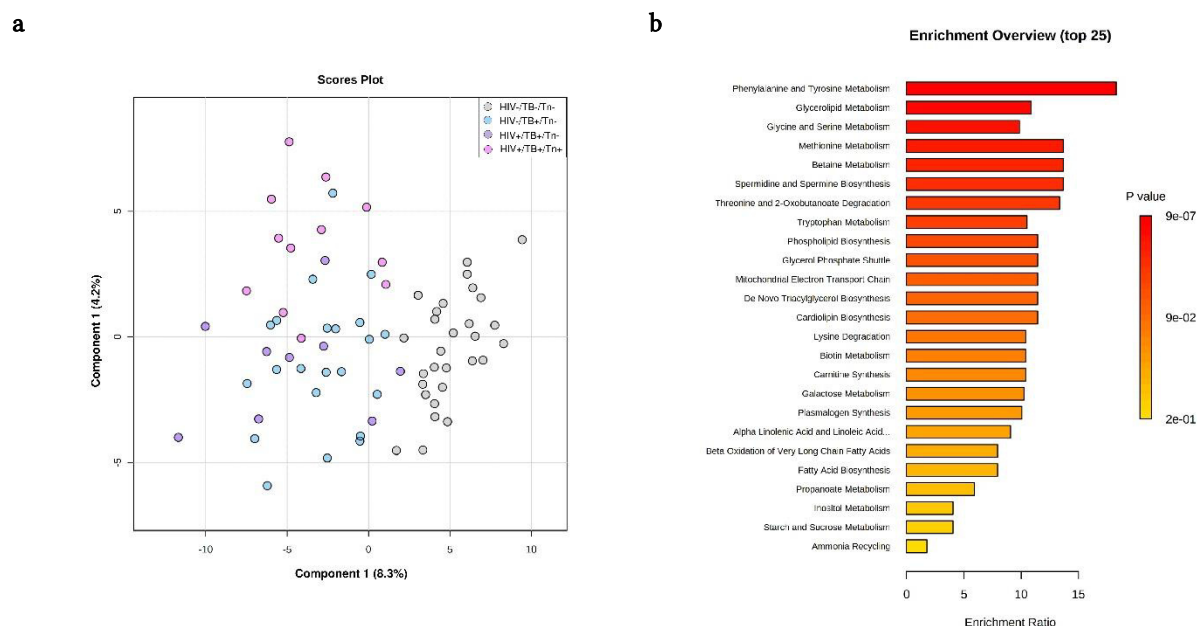


Figure 3: Multivariate and functional analyses relating to the metabolomics data. (a) PLS-DA scores plot indicating the metabolic profiles of the diseased groups are similar, but do not overlap completely. This implies that there are areas of uniqueness between the metabolic profiles of TB, untreated HIV/TB co-infected, and treated HIV/TB co-infected patients. (b) Overview of quantitative metabolite set enrichment analysis (for comparison 2, healthy controls versus untreated HIV/TB co-infected patients) identified as significant by ANOVA ($p < 0.05$).

4 Discussion

Six of the nine HIV/TB co-infected patients had CD4 T cell counts below 200 cells/mm³, indicating a severe immunodeficiency and (by the immunological definition) the onset of acquired immunodeficiency syndrome (AIDS) (41). One patient was close to the immunological definition of AIDS, with a CD4 T count of 248 cells/mm³ and the remaining two patients had CD4 T cell counts above 500 cells/mm³. A CD4 count above 500 cells/mm³ is typical of healthy individuals (42) indicating that the disease state of these are less advanced compared to the others in this group. In the HIV/TB co-infected group on ART, all but two patients (CD4 T cell counts > 300 and > 500 cells/mm³ respectively) met the immunological definition of AIDS. Unfortunately, the cohort cannot be described based on VL since this information was not available for all patients. Given the small sample size, this study is explorative and does not attempt to identify predictive markers. As no comprehensive HIV/TB co-infection metabolomics profile has been published (at the time of

submission and to the best of our knowledge), this study will contribute to better understanding the metabolic responses of the host during HIV/TB co-infection and direct future studies on the topic.

The serum cytokine profiles of untreated HIV/TB co-infected patients (Figure 1, comparison 2) were mainly characterised by significantly increased pro-inflammatory cytokines of Th1 (IFN- γ) and Th2/immunoregulatory (IL-6) origin. IFN- γ is necessary, but not sufficient, for protection against *Mtb* infection (43, 44) and is essential to enhancing cytokine and other cellular immune responses during HIV infection (45). Increased levels of IL-6 is generally considered an indicator of HIV disease progression, as this cytokine positively correlates with VL (46), predicts mortality in HIV patients (47, 48), is higher in HIV-positive patients who develop cardiovascular disease, and in immunological non-responders (49, 50). These cytokines are significantly altered in the unstimulated plasma cytokine profiles of HIV/TB co-infected patients (16-19). Using IFN- γ and IL-6 in combination best discriminate HIV-patients with and without TB (19). Here, the serum metabolic profiles of untreated HIV/TB co-infected patients (comparison 2) were mainly characterised by changes in amino acids, metabolites originating from the gut microbiota, metabolites related to energy production, carbohydrates, and lipids (and related metabolites). This is in line with previous blood-based metabolomics studies done on untreated HIV (33, 34, 51, 52) and TB patients (26, 53-55). Since there is overlap between the blood metabolic profiles of HIV infection and TB patients (56), one can expect that HIV/TB co-infected patients would show similar or related metabolites to those reported in previous studies done on HIV infection or TB alone, although the trends (increased or decreased) may differ.

Here, IFN- γ was increased in the untreated HIV/TB co-infected group relative to healthy controls, albeit very slightly compared to the other diseased groups. This pro-inflammatory cytokine induces the enzymes guanosine triphosphate cyclohydrolase (GCH) and indoleamine-2,3-dioxygenase (IDO1) (57, 58). GCH produces tetrahydrobiopterin (BH₄) and neopterin, which are essential cofactors to enzymes responsible for the production of monoamine neurotransmitters and nitric oxide (58). Increased phenylalanine concentrations were observed in the untreated TB and untreated HIV/TB co-infected cohorts relative to healthy controls (Table 3 and Table S6). Elevated phenylalanine has been

reported in biofluids collected from untreated TB (53, 54, 59-61) and untreated HIV (51, 62, 63) patients. This has been ascribed to a decreased phenylalanine hydroxylase (PAH) activity (62, 63), due to a BH₄ deficiency (63) or compromised insulin secretion (61). Since the IFN-influenced BH₄ is sensitive to oxidation, such as during high levels of oxidative stress (OS) experienced during infection (64, 65), it may be highly labile in co-infected patients (66, 67). Macrophages and dendritic cells produce neopterin at the expense of BH₄. Neopterin is increased during both HIV infection (52) and TB (68) and this is even more pronounced during HIV/TB co-infection (69). Neopterin has also been shown to predict the development of active TB in HIV-positive patients six months before a dramatic increase in VL and reduction in CD4 T cell count. Although neopterin was not detected in this study, the continued immune activation during HIV/TB co-infection (7) likely contributes to BH₄ depletion via neopterin overproduction, decreasing PAH activity. Thus, the increased phenylalanine levels may be ascribed to OS and immune activation.

Microbiota metabolism is an essential component of human metabolism (70). Phenylalanine and tryptophan metabolism by gut microbiota yields cresol and indole intermediates (71-73). While previously shown to be elevated during HIV infection (62, 74, 75), p-cresol derivatives (2,4-di-tert-butyl-p-cresol and 2,4-di-tert-butyl-phenol) and benzoic acid (76) were reduced in the untreated HIV/TB co-infected group relative to healthy controls (Table 3). The impaired gut mucosal integrity, subsequent microbial translocation, and microbiome dysbiosis (which is also a feature of TB) induced by HIV infection (77-79) are generally reflected in the pathomorphological changes seen in the small intestine of HIV/TB co-infected patients. These changes include a reduction in the absorptive surface area, a reduced absorption rate due to sclerotic damage, a decreased functional activity of enterocytes, and increased oxidative damage to proteins, relative to healthy controls (80). Considering this, the decreased concentrations in the p-cresol derivatives [and of norvaline, another microbial product (81)] result from an altered microbiota and increased OS (Todoriko, Pidverbetska (80), given the antioxidant properties of these metabolites. The increased levels of IL-6 in this cohort, which is predictive of anorexia in co-infected patients (82), likely results in decreased food intake and therefore decreased

levels of phenolic antioxidants, which are typically used as food preservatives (83). The levels of IL-6 correlate with immune activation, the extent of dysbiosis (making extensive dysbiosis very likely in this cohort) and the activity of tryptophan catabolism (through kynurenine) during HIV infection (78).

An altered tryptophan metabolism via the kynurenine pathway as a result of increased IDO1 activity is a well-established consequence of various inflammatory conditions, including HIV (33, 34, 51, 84-86), TB (59, 61, 68, 87, 88) and HIV/TB co-infection (26, 27). The indole products of tryptophan catabolism in the gut microbiota can be produced endogenously in an IL-4 inducible manner in T cells and dendritic cells (89). The importance of tryptophan catabolism by the gut microbiota during co-infection is reflected in the decreased levels of tryptophan, 3-indolepropionic acid (IPA), 3-indoleacetic acid (IAA), and 3-indolelactic acid (ILA) in the untreated HIV/TB co-infected group when compared to the healthy controls. Tryptophan was decreased in all diseased groups relative to healthy controls, and this was most pronounced in the untreated HIV/TB group, although not statistically significant (Table 3). There was also a significant positive correlation between IAA, ILA and tryptophan (Figure 4). IPA, IAA, and ILA all have roles as antioxidants, ameliorate inflammation (90-93), and regulate intestinal immunity by activating the aryl-hydrocarbon receptor transcription factor (89, 94). Increased IPA has been associated with enhanced intestinal barrier integrity (95) and has mycobacterial-specific antibiotic activity (92), thus its decrease could relate to the loss of gut barrier integrity induced by HIV (96), intestinal damage observed in co-infected patients (80), and possibly TB progression in HIV-patients (92). Typically, the catabolic products of tryptophan would increase as tryptophan decreases, however, the advanced disease state (as per CD4 T cell counts in Table 1), high levels of OS (64, 65), microbiome dysbiosis (78, 79), and decreased endogenous production of these metabolites due to the decreased levels of IL-4 (Table 2, not statistically significant) in the co-infected patients may account for this observation. These data and previous reports confirm that HIV/TB co-infected patients have a significantly increased tryptophan catabolism (26, 27) compared to patients with either disease state alone, and suggests that the gut microbiota-derived tryptophan

catabolites are important biomarkers for the co-infected state. This could be used in conjunction with the kynurenine/tryptophan ratio, which has been suggested as a biomarker for co-infection (27).

HIV infection causes Th17 cell depletion, resulting in an imbalanced Th17:T regulatory cell (Treg) ratio which has been correlated to the induction of IDO1 and increased levels of microbial products in HIV-patients (97). The IDO1 activation initiates a feedback loop that contributes to systemic inflammation and immunosuppression (induced by the higher numbers of Tregs compared to Th17 cells). Seeing that these changes result in irreversible sclerotic damage in the small intestine (80), targeting IDO1 (26) and maintaining gut health should be considered in the development of host-directed therapies.

Apart from its previously mentioned association with anorexia, IL-6 is also an indicator of severe inflammation (82), and negatively affects muscle turnover (resulting in net muscle breakdown) (35, 98). The mechanisms underlying the higher risk of malnutrition (99) and more severe cachexia (100, 101) experienced by HIV/TB co-infected patients remain poorly understood. Prolonged periods of high energy requirements, such as during chronic infection and inflammation, with concomitant infection-associated anorexia, malaise and malabsorption, results in proteolysis for energy production. In TB patients with high inflammation (those with IL-6 and other pro-inflammatory cytokines) the amino acid levels are comparatively lower (59). The use of stored amino acids for energy is a last resort resource and limited since essential proteins and muscle mass needs to be preserved. During the initial stages of starvation, the free amino acids would increase but quickly deplete due to poor feeding and malabsorption. Decreased levels of the essential amino acids methionine, threonine (and their catabolic product, 2-aminobutyric acid), and lysine suggest such a state in the untreated HIV/TB co-infected cohort (Table 3). These results, the decreased 3-hydroxypropionic acid and hydroxyproline, increased levels of a proline derivative (proline, 1-methyl methyl ester) and 3-hydroxyisovaleric acid (a leucine catabolite) (Table 3), all support an impaired protein balance in HIV/TB co-infected individuals (23, 24). Decreased energy availability is also evident by the decreased levels of pyruvate (although not significant) and glycerol-3-phosphate

detected in this patient group comparatively. Glycerol-3-phosphate is used for the rapid regeneration of reducing equivalents for oxidative phosphorylation (102) in the brain and skeletal muscle. The glycerol-3-phosphate shuttle also links fatty acid metabolism, which is typically dysregulated during HIV infection (22) and TB (103), to glycolysis and oxidative phosphorylation (104), allowing fatty acids to be preferentially used for energy.

The catabolism of the neurotransmitter 4-aminobutyric acid (GABA) results in the production of 3,4-dihydroxybutyric acid (DHBA) and 2,4-DHBA (105). These hydroxy fatty acids were increased significantly in the untreated co-infected group relative to healthy controls, and their levels were highest in this group compared to the other diseased groups. 3,4-DHBA induces a feeling of satiety possibly due to its structural similarity to 3-hydroxybutyric acid (106), which is used as an alternative fuel substrate during fasting. These metabolites are also linked to poor overall health and nutritional status induced by an extremely low energy intake (107). This further indicates cachexia, which is characteristic of both HIV infection and TB (108). Elevated 3,4-DHBA has also been detected in the urine of unsuccessfully treated TB patients (109), further confirming its association with a progressive phenotype. However, the exact mechanism of increased 3,4-DHBA and 2,4-DHBA concentrations have yet to be clarified. Lysine, the second most significantly altered essential amino acid, is abundant in muscle tissue and is essential for carnitine synthesis, and therefore for fatty acid metabolism (110). As such, 3,4-DHBA and lysine were subjected to correlation analysis to determine if this could yield new insight into the mechanisms of cachexia. 3,4-DHBA correlated positively with 2,4-DHBA, phenylalanine, 3-hydroxyisovaleric acid, and various microbial metabolites (Figure 4), while negatively correlating with tryptophan, IAA, threonine, 2-ketoisocaproic acid (another intermediate of leucine catabolism), and tetrahydrofuran. Biologically, and in light of how IL-6 and IFN- γ relate to these metabolites, 3,4-DHBA would increase with more pronounced inflammation (as seen in comparisons 4 and 5) and microbial product translocation. Lysine correlated with other amino acids and the intermediates in their catabolic pathways (Figure 4), among which 2-hydroxybutyric acid, 2-ketobutyric acid, and 5-oxoproline associated with alterations to glutathione metabolism, OS, and

energy distress (22, 59, 111). Lysine also correlated with 3,4-DHBA (adjusted p-value = 0.004), although not at the same level of significance as for the other correlations discussed. How this would relate to body composition changes and cachexia is unknown, and further investigation into the origin and effects of 3,4-DHBA in the context of cachexia in HIV infection and TB is needed.

A general decrease in the lipid-related metabolites was observed in the untreated HIV/TB group relative to the healthy controls, reflecting a lack of nutrition. Alpha-linolenic acid is an essential polyunsaturated fatty acid (PUFA) and is an important structural component of cell membranes (112). The low CD4 counts found in HIV-positive patients (Table 1) also result in reduced alpha-linolenic acid, as the phospholipid membranes of CD4 cells are composed of alpha-linolenic acid (113). An alpha-linolenic acid deficiency is associated with viral inactivation failure during infection, resulting in virus proliferation and immunodeficiency (114). PUFAs and glycerophospholipids are also important for distinguishing co-infected patients who develop immune reconstitution inflammatory syndrome from those who do not (38). Decreased levels of dodecanal and linoleyl alcohol were observed in the untreated co-infected group (Table 3), implicating decanoic and octadecanoic acid metabolism (115) as alternative energy substrates (116). These fatty acids are typically produced as intermediates of glycerophospholipid (specifically plasmalogens) metabolism and indicate alterations in membrane turnover. Previously reported decreased plasmalogens during HIV infection (34, 117), is supported by the reduced capric monoethanolamide concentrations in our study, an intermediate of plasmalogen phospholipids (118). These lipids are essential to membrane structure and function, immunity, and serve as antioxidants to protect against lipid peroxidation (115).

The altered carbohydrate metabolites that distinguished the untreated co-infected patients from healthy controls were of microbial and/or exogenous origin, and included increased rhamnose (119), mannitol (120), and 1-deoxypentitol (121), as well as decreased tetrahydrofuran (122, 123) and 2-deoxy-D-pentofuranose. The increased mannitol may additionally indicate subclinical co-infections in the co-infected patients and/or a more virulent microbiota composition (124, 125). Gut inflammation also has an impact on mannitol permeability in HIV-patients (77), which could explain

the increased mannitol observed here. The altered levels of *Mtb*-related metabolites are likely due to a higher bacterial load in the untreated HIV/TB co-infected patients, the majority of which were severely immunosuppressed. Tetrahydrofuran is typically elevated in poor quality drinking water, such as in the rural community from where the samples were collected (126, 127). However, the decreased concentration detected in the various diseased groups may result from decreased water intake, either via direct consumption or food preparation/intake, associated with the aforementioned cachexia.

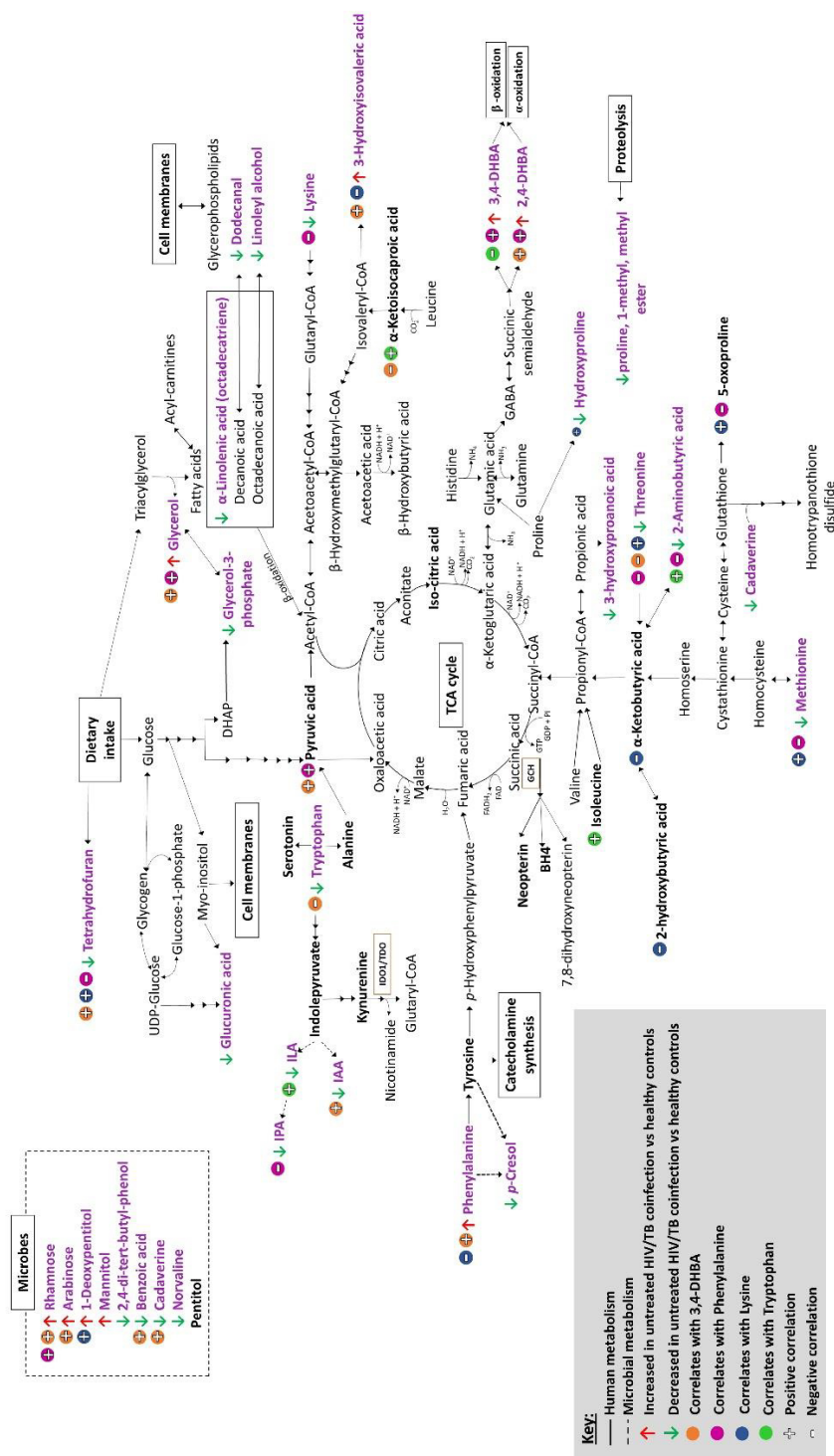


Figure 4: A summary of the metabolic effects of HIV/TB co-infection (comparison 2) and the correlations of 3,4-dihydroxybutyric acid, phenylalanine, lysine, and tryptophan with other metabolites. Abbreviations: 3,4-DHBA: 3,4-dihydroxybutyric acid; 2,4-DHBA: 2,4-dihydroxybutyric acid; IDO1: indoleamine-2,3-dioxygenase 1; TDO: tryptophan dioxygenase; IFN-γ: interferon-gamma; GCH: GTP cyclohydrolase; IPA: 3-indolepropionic acid; ILA: 3-indolelactic acid; IAA: 3-indoleacetic acid. When considering the immunometabolic effects of HIV

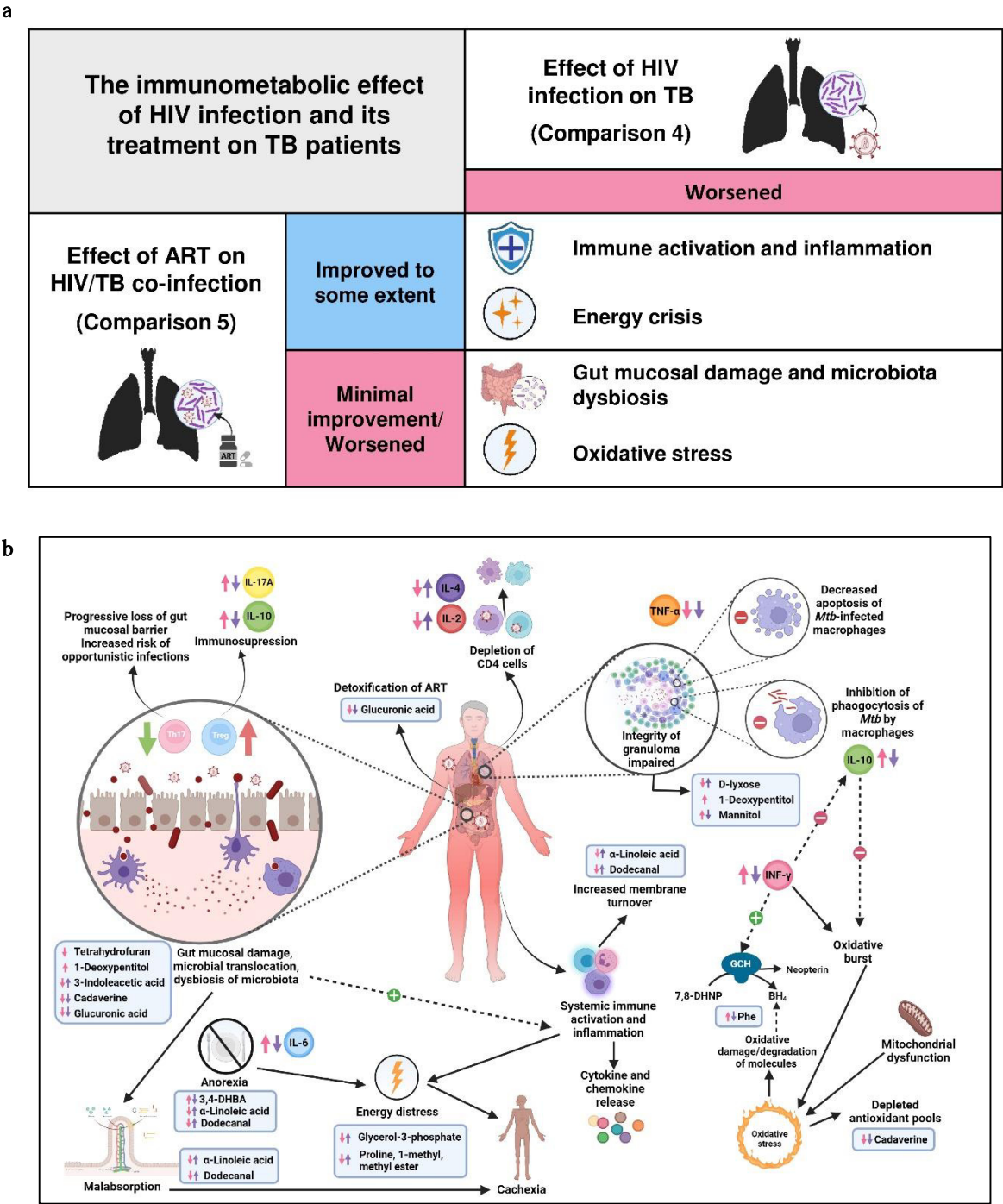
infection and its treatment on the host, there were no statistically significant differences between the cytokine profiles of the diseased groups (Figure 1, comparisons 4–6), however, trends could be identified (Table 2). HIV infection is primarily associated with altered IL-6 and IL-10, and IL-17 to some extent (128, 129), whereas TB induces changes to IFN- γ , TNF- α , IL-6, and IL-17 (18, 44, 130). The relatively low IL-2 and IL-4 in the untreated co-infected group, and the increasing trend of these with ART, result from reduced CD4 T cells and ART-induced immune reconstitution, respectively, as these cytokines are predominantly produced by activated CD4 T cells (131) and are involved in cell proliferation (132, 133). However, these cytokines did not correlate with the CD4 T cell count (Figure 2d). The source of IL-10 is dynamic as it can be produced by various cell types including Th1, Th2, Th17, Tregs, B cells and macrophages (134). The levels of IL-10 and IL-6, which inhibit TNF- α secretion (135, 136), were higher in the untreated co-infected group relative to all other groups, which contributed to the reduced TNF- α in the co-infected patients relative to the TB patients. This could also contribute to TB progression and increased immune activation in co-infected patients, as overexpression of IL-10 has been linked to an inability to control *Mtb* (137), and TNF- α is essential to maintaining granuloma integrity (138). Although IL-17A levels were only slightly higher in the untreated HIV/TB co-infected group relative to the TB group, its levels in the HIV/TB co-infected group on ART was comparable to that of healthy individuals and correlated positively with IL-6, IFN- γ and negatively with the CD4 T cell count. IL-17 is important in the success of ART in HIV/TB co-infected patients, showing the highest number of correlation network connections to other cytokines in the untreated co-infected patients, which were reduced upon suppressive ART (20). This strengthens the association between IL-17 and VL, which correlate positively in HIV-patients (129). The decreased Th17:Treg ratios in HIV-patients described earlier create the expectation that IL-17 levels would decrease in the HIV/TB co-infected patients compared to the TB patients. However, some subsets of Tregs also produce IL-17 and may be partly responsible for its increase in the co-infected group relative to the TB group, especially since these cells were also found to decrease when ART commences (139), which is in line with the trend observed here. These trends suggest a failure of immunoregulation, as regulatory and pro-inflammatory cytokines are concomitantly elevated,

without showing significant improvement once ART is started. This supports previous reports of improved T cell levels when commencing ART, however their heterogeneity and functionality are not fully restored (140).

The metabolite changes to tetrahydrofuran, 1-deoxypentitol, glucuronic acid, glycerol-3-phosphate, cadaverine, IAA, alpha-linolenic acid and dodecanal were unique to the untreated HIV/TB co-infected patients when compared to those with TB. These metabolites represent the unique metabolic effects caused by HIV infection and/or the synergistic effects of HIV and *Mtb* during co-infection. 3,4-DHBA and phenylalanine, which were previously elevated in TB patients relative to healthy controls (comparison 1), were further increased (with statistical significance) in co-infected patients (comparison 4), implying that these are metabolic effects of HIV infection and TB, as supported by literature (51, 56, 59, 141, 142). As previously mentioned, increased phenylalanine and neopterin are associated with a progressive phenotype, and neopterin inversely correlates with a CD4 T cell count (69). This implies that phenylalanine and neopterin could be used in combination as prognostic markers in co-infected patients, or as indicators of TB in HIV-patients. This requires further investigation, given the above-mentioned limitations of this study.

Patients on ART experience inflammation and immune activation, although to a lesser degree than before treatment, as active viral replication is repressed (143). Although ART did not generally alter the metabolic profile of the HIV/TB co-infected patients (comparison 5), the levels of alpha-linolenic acid, dodecanal and proline, 1-methyl, methyl ester were significantly different and partially recovered (although not to the levels in healthy controls). Also, mannitol levels were comparatively lower than those in the TB group (Table 3; similar to the level in healthy controls).

The metabolites identified as significantly different in comparisons 4 and 5, but which did not occur in the untreated co-infection profile, discussed in the previous section, will now be placed into biological context. Figure 5 summarises the immunometabolic effect of HIV infection and its treatment on patients with TB.



419 Figure 5: The immunometabolic effect of HIV infection and its treatment on TB. (a) A summary of the
420 implications of the immunometabolic data obtained from comparisons 4 and 5. (b) A summary of the
421 immunometabolic effect of HIV infection and its treatment on TB in the context of previous literature. Pink
422 arrows represent the effect of HIV infection on TB (comparison 4) and purple arrows represent the effect of
423 ART on HIV/TB co-infection (comparison 5), as observed in this investigation. Abbreviations: Mtb:
424 Mycobacterium tuberculosis; ART: antiretroviral therapy; Th: T helper cell; Treg: T regulatory cell; IL:
425 interleukin; TNF: tumour necrosis factor; BH₄: tetrahydrobiopterin; 7,8-DHNP: 7,8-dihydroneoterin-phosphate;

426 *GCH: guanosine triphosphate hydroxylase; Phe: phenylalanine; 3,4-DHBA: 3,4-dihydroxybutyric acid.*
427 Cadaverine, a microbially-produced antioxidant polyamine, was increased in the TB patients relative
428 to healthy controls (although not significantly; Table 3). This metabolite has been detected in HIV
429 infection (62) and *Mtb*-related metabolomics studies (144, 145). Cadaverine was detected in reduced
430 amounts in the untreated co-infected patients relative to the TB patients (comparison 4) and even
431 further reduced in the co-infected patients on ART (although not significantly; comparison 5). Since
432 the gut microbiota produces cadaverine, the reduction observed here is most likely ascribed to the
433 altered gut health and microbiota composition in these groups (146). Cadaverine forms part of the
434 glutathione metabolic pathway, and as such, its reduction may also be associated with OS, which is
435 known to persist even during ART (147). Furthermore, decreased levels of glutathione with
436 concomitantly increased indicators of lipid peroxidation has been reported in co-infected patients
437 relative to patients with HIV infection only (with and without ART) (64), which further supports
438 exacerbated OS during HIV/TB co-infection.

439 Glucuronic acid, a metabolite of glucose involved in xenobiotic detoxification (148), was reduced in
440 the untreated and treated HIV/TB co-infected patients relative to the TB group (comparisons 4 and
441 6). Various members of the gut microbiota can cleave glucuronic acid from its conjugated xenobiotic
442 or steroid during detoxification. The activity of the beta-glucuronidase responsible for this cleavage
443 is variable among species (149, 150). Thus, if the composition or functionality of the microbiome was
444 altered in such a way as to decrease the number of species able to perform this cleavage (151), it will
445 alter the levels of unconjugated glucuronic acid. Increased and chronic xenobiotic detoxification due
446 to the leaky gut caused by HIV, as well as the continual detoxification of ART in treated patients (152)
447 contributes to the reduced levels of this metabolite.

448 Interestingly, D-arabinose, an essential component of the *Mtb* cell wall (153), was elevated in the TB
449 patients relative to healthy controls (comparison 1) but reduced in HIV/TB co-infected patients
450 (comparison 4). TB patients and HIV/TB co-infected patients on ART better phagocytose *Mtb*,
451 releasing their cell wall components into the bloodstream, compared to the severely
452 immunocompromised untreated HIV/TB. The absence of HIV (in TB patients) or reduced viral activity

(in patients on ART) contributes to this, as the Nef protein of HIV inhibits macrophage phagocytosis (154) and clearance of the phagocytosed bacteria via autophagy (12), which also explains the trends in the concentrations of D-arabinose observed here.

When the co-infected patients on ART were compared to the healthy controls (comparison 3), the qualitative metabolic profiles were comparatively similar to that of untreated co-infected patients, with some quantitative differences and additional alterations to 2-amino-1-methyl-imidazol-4-ol (decreased, biological function unknown) and pyruvate (increased). Pyruvate was elevated in all diseased groups relative to healthy controls but decreased in the untreated co-infected patients relative to those with TB only (comparison 4). This trend in a metabolite central to the regulation of energy metabolism further stresses the significant effect of HIV infection on energy metabolism. In the HIV/TB group on ART, the levels of phenylalanine increased relative to healthy controls but were similar to that of TB patients. This indicates that ART relieved some of the immune activation induced by uncontrolled active HIV replication, but that the effects of TB, residual effects of HIV (of those treated co-infected patients for whom VL was available, only one was virally suppressed), and of ART itself (as the majority of patients had been on ART for more than one year) are still evident.

When comparing the co-infected patients on ART to the TB patients (comparison 6), significantly reduced tetrahydrofuran, glucuronic acid, cadaverine, IAA and elevated 1-deoxypentitol and 2,4-di-tert-butyl-phenol were detected. This implies reduced IAA in the co-infected group on ART relative to the TB group, but it trended back to TB levels, i.e. was less significantly decreased than in the untreated co-infected group (see comparison 4 above). Although not statistically significant, IPA also trended back to a level comparable with that of TB patients, while ILA was comparable between the untreated and treated co-infected groups. Thus, the alterations to ILA, IPA, and ILA are more pronounced in combination with HIV infection than during TB alone, with only ILA not being altered when commencing ART.

To conclude, the cytokine profile of untreated HIV/TB co-infected individuals was mainly characterised by elevated IL-6 and IFN- γ , cytokines that are typically increased during HIV infection,

TB, and HIV/TB co-infection. There were no significant differences between the cytokine levels of the diseased groups, although the observed trends suggest a failure of immunoregulation. The roles of IL-10 and IL-17 during co-infection warrant further investigation in light of the trends observed here and those reported in previous literature. The metabolic profile of the co-infected individuals shows significant overlap with profiles previously described for HIV infection and TB, with major areas affected including amino acids, lipids, and their related intermediates and metabolites related to central energy metabolism and gut dysbiosis. These alterations suggest considerable energy distress, reflected in amino acid catabolism and partially explained by high levels of the satiety-inducing metabolites 3,4-DHBA and 2-DHBA. These metabolites also suggest a mechanism by which cachexia is mediated in HIV infection, TB, and HIV/TB co-infection. The large proportion of the altered microbial metabolites, suggests that gut microbiome dysbiosis, mucosal damage, and potential microbial translocation may be more severe during HIV/TB co-infection. This may be mediated by, and contribute to, increased immune activation, inflammation, and subsequent OS, due to concurrent HIV and *Mtb* infection. Some of these metabolites could be related to *Mtb* directly, reflecting the increased success of *Mtb* during co-infection. The energy distress also contributes to the lipid alterations observed, but as these are mainly related to phospholipids, it most likely is associated with the chronic immune activation in these patients, as immune cells are perpetually being produced and remodelled for activation. This study provides a preliminary overview of the immunometabolic profile of HIV/TB co-infected patients, untreated and treated for HIV, and as such, these findings need to be investigated in greater detail. Further investigation on this very important global health issue could benefit using (i) a larger cohort, (ii) with VL and CD4 T cell counts for all patients (including HIV-negative TB patients), (iii) with other clinical information (such as body composition measurements, chest X-rays, bacterial load, etc.), (iv) in a longitudinal context, and (v) with confirmatory experiments, such as enzyme assays.

5 Methods

5.1 Ethics

The investigation was done according to the Declaration of Helsinki and International Conference of Harmonisation guidelines. Ethical approval was obtained from the Ethics Committees of the University of Cape Town (reference no: 680/2013) and the North-West University for a larger study focused on HIV/TB co-infection metabolomics (NWU-00355-20-A1), as well as for this sub-study (NWU-00355-20-A1-01).

5.2 Study design, subjects, and sample preparation

Serum samples were collected by the South African Tuberculosis Vaccine Initiative (SATVI) and the Desmond Tutu HIV Centre at the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, according to standard operating procedures. Anonymised serum samples were transported on dry ice to the North-West University, Focus Area of Human Metabolomics: Laboratory of Infectious and Acquired Diseases, where it was immediately frozen at -80°C until the day of analysis. All patients and controls were South African adults (male and female, between the ages of 18 and 69), residing in the Masiphumelele and Ocean View Townships in Cape Town, in the Western Cape Province of South Africa. All participants gave written and informed consent, received a financial incentive at the time of diagnosis and a follow-up visit, and received counselling and immediate treatment in case of positive test results.

The samples were divided into groups based on HIV, TB, and HIV treatment status (all samples were untreated for TB). TB was diagnosed using GeneXpert, while all HIV/TB co-infected individuals had a serum-confirmed HIV status. Given the rural background of patients, and therefore limited access to healthcare, the exclusion criteria were not extensive. Patients were excluded from the study if they: (a) consumed any over-the-counter or chronic medication, except for one patient taking non-steroidal anti-inflammatory drugs (trimethoprim/sulfamethoxazole) and one taking antibiotics

(amoxicillin/clavulanic acid); (b) had another disease that is similar to those under investigation (e.g. asthma) — one patient was diagnosed with nephrotic syndrome and was not excluded; or (c) were pregnant or lactating. Given that the burden of both HIV infection and TB is highest in resource-limited areas (3, 155), these exclusion criteria increases the likelihood that the results of this study will be robust.

The demographical and clinical summary of the cohort is given in Table 1 (see Table S2 for detailed information for the HIV-positive cohorts). The serum cytokine and metabolic profiles of 29 healthy controls (HIV-/TB-/Tn-), 22 untreated HIV-negative, TB-positive individuals (HIV-/TB+/Tn-), 9 untreated HIV/TB co-infected patients (HIV+/TB+/Tn-) and 12 HIV/TB co-infected patients on ART (HIV+/TB+/Tn+) were measured. Obtaining untreated HIV, TB and co-infected samples is extremely difficult in the light of the “Test and Treat” policy for HIV infection and latent TB, as recommended by the World Health Organization (156) (157, 158), hence the small sample sizes. Regarding the ART regimens, all patients received a fixed-dose combination of two nucleoside reverse transcriptase inhibitors (NRTIs; Tenofovir and Emtricitabine) and one non-nucleoside reverse transcriptase inhibitor (Efavirenz), except for two patients who received two NRTIs (Abacavir with Lamivudine and Combivir, respectively) and two protease inhibitors (Lopinavir/Ritonavir).

5.2.1 Cytokine analyses and data processing

The Human Th1/Th2/Th17 cytometric bead array (CBA) kit (catalogue number: 560484, lot: 0064427 and 0202260, BD Biosciences, San Jose, California) was used to measure the levels of seven bead populations (namely IL-2, IL-4, IL-6, IL-10, TNF- α , IFN- γ and IL-17A) simultaneously. Sample preparation and CBA analysis were performed as per the manufacturer’s instructions and as previously described (128). The samples were then analysed in a randomised order on a BD Accuri C6 Plus flow cytometer using BD Accuri C6 Plus CSampler software (version 1.0.23.1). Before data acquisition on the day of analysis, instrument performance was assessed by performing an automated quality control (QC) procedure using Cytometer Setup and Tracking beads (catalogue number: 661414, lot: 9155868). Thresholds were set on 500 000 for forward (FSC) and side scatter (SSC), which

allow for the exclusion of debris and background signal. A dot plot of FSC versus SSC and a histogram of event count versus allophycocyanin (APC) (indicating the seven bead populations) allowed the bead population to be defined and gated. Using a medium flow rate of 35 μ l/min, 3 000 events were collected per sample within the defined gate. After every four samples, the instrument was backflushed to purge the flow cell and avoid any build-up of background signals. A dot plot of PE (detection antibody) and APC (bead populations) showed the seven bead populations as well as their shifts in concentration. Following sample acquisition, the data were exported as flow cytometry standard files, and processed using BD FCAP Array version 3.0 software. The bead library corresponding to the CBA kit was used to assign beads to peaks and identify the seven bead populations (corresponding to each cytokine measured) in each sample. Debris filtering was performed to remove any background noise. The standard series was defined and five parameter logistic regression standard curves were plotted for each bead population, using an R^2 cut-off of 98% for fitting accuracy, to quantify the cytokine levels of the unknown samples. The calculated concentrations were then exported and subjected to statistical analyses.

5.2.2 Metabolomics analyses and data processing

Extraction and analysis of samples were done as previously described (116). Briefly, serum samples were extracted using a methanol:chloroform:water (1:3:1) solution (in which 3-phenylbutyric acid was dissolved as internal standard) and proteins were precipitated using acetonitrile. Extracts were dried under a stream of nitrogen gas and derivatised using methoxyamine and N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylsilyl chloride. Derivatised samples were placed into the tray of the Gerstel Multi-Purpose Sampler (Gerstel GmbH and co.KG, Eberhard-Gerstel-Platz 1, D-4573 Mülheim an der Ruhr, Germany) coupled to a two-dimensional gas chromatographer time-of-flight mass spectrometer (GCxGC-TOFMS; Pegasus 4D; LECO Corporation, St. Joseph, MI, USA). The GCxGC-TOFMS uses an Agilent 7890A gas chromatograph (Agilent, Atlanta, GA) and a time-of-flight mass spectrometer from LECO Corporation (St. Joseph, MI, USA) and is coupled to a cryogenic cooler. One microliter of all samples and each QC (a sample created by pooling 10 μ l of each sample

in one tube, one aliquot of this was used per sample batch) were injected with a 1:5 split ratio, at an inlet temperature of 270°C (kept constant throughout the run). Initially, one injection of a mixture of fatty acyl methyl esters (FAMES) and three QC sample injections (to prime the system) was done. The following sequence was then repeated for all sample batches: an injection of hexane (to avoid carry-over), a QC injection, 5–6 randomised samples, hexane again, three QC injections, and finally a FAMES injection. A Restek Rxi-5Sil-MS primary capillary column (28.759m, 0.25 mm internal diameter and 0.25 µm film thickness) and a Restek Rxi-17 secondary capillary column (1.380m, 0.25 mm internal diameter and 0.25 µm film thickness) were used to achieve compound separation. The oven, modulator and mass spectrometer programming were done as previously described (116), except for hot and cold pulses of nitrogen gas every 3 s for 1.5 s.

The data generated was processed using ChromaTOF software version 4.32 (LECO Corporation). Peak detection and deconvolution were performed before aligning all samples. The National Institute of Standards and Technology (159) mass spectral libraries and that of the Potchefstroom Laboratory for Inborn Errors of Metabolism were searched to identify peaks. The dataset was normalised using mass spectral total useful signal (160) to reduce technical variation and make samples more comparable. The normalised dataset was then subjected to various clean-up steps, including a 50% zero filter (161), batch effect correction using quantile equating (162) and QC coefficient of variation (CV) filter (61).

5.3 Statistical analyses

The distributions of nominal variables were assessed using Fisher's exact tests in IBM SPSS Statistics 27 software. Missing value replacement, data pre-treatment, and statistical analysis were performed on MetaboAnalyst (version 5.0). As per default, for the cytokine and metabolomics datasets, missing values were replaced by one-fifth of the minimum value in the dataset for each variable and is based on the hypothesis that these missing values are likely not truly zero but the result of compound abundances falling below the detection limit of the instrument (144). The cytokine dataset was log-transformed, while the metabolomics dataset was log-transformed and autoscaled to achieve a

normalised distribution, as this is a prerequisite for the accurate usage of various statistical analyses. Univariate analyses included ANOVA with correction for multiple testing by the Benjamini-Hochberg (BH) approach [variables were considered statistically significant if the adjusted p-value was < 0.05] and Fisher's least significant difference for post-hoc analysis. The BH method was also used to correct for multiple testing for all other statistical tests done in this experiment. Pearson's r correlations were also performed for cytokines and metabolites. Cytokine correlations were considered significant when the BH-adjusted p-value was < 0.05 . The metabolic correlations were done selectively due to the size of the dataset. Metabolites indicated as significant by ANOVA with a particular biological significance were correlated to all other metabolites. Metabolic correlations were only considered significant if the adjusted p-value was < 0.005 , to further reduce the number of metabolite markers to a usable amount for data interpretation. Correlation analysis was done between the CD4 T cell counts (for all HIV-positive patients), all cytokines and all metabolite markers. Multivariate analyses included PCA (used to assess the inherent structure of the data) and PLS-DA. PLS-DA models were subjected to leave-one-out cross-validation and permutation testing (precision accuracy during training) but given the small sample size, was not used to identify predictive markers. Instead, PLS-DA VIP values, which directly correlate with the importance of a metabolite in differentiating the groups (163), were used in an explorative sense. That is, to assess the distribution of groups and determine whether the cytokines and metabolites identified as statistically significant in the univariate analyses retained significance in a multivariate statistical setting. Quantitative metabolite set enrichment analysis (MSEA), using the Small Molecule Pathway Database (SMPDB) was also performed on those metabolites identified as significant when ANOVA was done.

6 Contributors

CL performed cytokine experiments, processed all the cytokine and metabolomics data, performed statistical analysis and drafted the paper. LL, DTL, and AW conceptualised the study and critically reviewed the results and manuscript. All authors were not precluded from accessing data in the study and approved the final version to be submitted for publication.

7 Declaration of interests

LL received National Research Foundation (NRF)-Thuthuka funding for the larger study related to this manuscript. Funding was paid to the North-West University (grant no 129871). CL, DTL and AW have no conflicts of interest to declare.

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9 Data sharing statement

All data that form part of this paper and its supplementary material are free to obtain. Datasets are available on Dryad (doi:10.5061/dryad.zw3r22891).

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