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Nutritional and Metabolic Profiling of Pulmonary Tuberculosis

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Abstract

Nutritional and Metabolic Profiling of Pulmonary Tuberculosis

By Jennifer K. Frediani, M.S., R.D., ACSM-CES

The purpose of this dissertation was to explore 1) dietary intake in patients with pulmonary tuberculosis (TB) disease in Tbilisi, Georgia, through use of a novel dietary intake assessment instrument; 2) determine, via plasma metabolomics analysis, the metabolic relationships between subjects with active TB disease and control subjects without evidence of TB disease from a randomized, controlled clinical trial in Tbilisi, Georgia; and 3) to examine serial dietary and body composition indices over time in recently diagnosed TB patients, and as sub-aims, to examine differences between those with multidrug resistant TB (MDR-TB) and drug-susceptible TB and those receiving or not receiving high-dose vitamin D_3 (cholecalciferol) therapy, designed to enhance *Mtb* clearance and cure. All data were derived from a, double-blind, randomized, placebo controlled trial of high-dose vitamin D_3 therapy in patients with recently diagnosed pulmonary TB in Tbilisi, Georgia.

The dietary intake assessment instrument was validated in this population of Georgia TB disease patients by comparison with 24-recalls. Adjunctive high-dose vitamin D therapy did not affect changes in macronutrient intake or body composition over the course of treatment; however, total kilocalorie, protein and fat intake increased over the 16-week period in the TB patients overall. Body composition also improved over time with concurrent increases in body weight, BMI, fat mass and fat-free mass. In addition, a subsample of MDR-TB subjects was compared to drug-susceptible TB subjects. Although not statistically significant, there was a trend that suggested higher intakes of macronutrients but concomitantly lower body weight and fat-free mass over time in those with MDR-TB compared to their drug-susceptible TB counterparts.

We then incorporated dietary intake data with high-resolution LC-MS plasma metabolomics in a sub-sample of 17 patients with TB disease and their matched household contacts without apparent TB disease. In this study, mean 3-day daily total caloric and carbohydrate intake was significantly higher per kg body weight in TB disease subjects than their household contacts, and total fat and protein intake tended to be higher. Overall, our metabolomics study results did not appear to be a function of individual diets of subjects with TB or the healthy controls. We were able to differentiate metabolic profiles between those with TB disease and apparently healthy controls using high-resolution plasma metabolomics in human plasma. We were able to identify multiple metabolites relevant to *Mtb* disease and its unique metabolism, as well as D-series resolvins that may reflect a pathophysiologic response to TB disease in humans.

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Chapter 1 - INTRODUCTION

History and Epidemiology of Tuberculosis

Mycobacterium tuberculosis (*Mtb*) is an acid-fast, non-motile, aerobic bacillus that flourishes in high oxygen environments such as the lung as well as several different extrapulmonary sites including bone, lymph nodes, intestines, heart, kidneys, adrenal glands and the brain.[1] The organism, *Mtb,* was discovered by Robert Koch in 1882,[2] but the disease defined as symptomatic patients (e.g. productive cough, fever, malaise, weight loss) due to documented active *Mtb* infection in the lung (pulmonary TB) dates back to 460 BC.[3] Hippocrates described patients with "consumption" or "phthisis," defined by "wasting away due to coughing up blood and chest pain".[3] Historians believe Indo-Europeans transported the pathogen during their migration to Europe[4] and from there the urban explosion catapulted TB-related mortality during the early 19th century. Poor sanitation and poverty were to blame for this epidemic and once sanitation improved during the industrial revolution the incidence and prevalence of TB declined. Similarly, urban epicenters such as Boston and New York saw a decline in TB mortality towards the end of the 1800s. Prior to the development of modern treatments, sanatoria were used to segregate the infected and air and sunlight were thought to improve symptoms.[5] Widespread use of the Bacillus Calmette-Guérin (BCG) vaccine and antibiotics in the 1950s significantly contributed to decreased prevalence of systemic TB in the developed world. However, low income countries remain endemic today.[6]

In 2012, the incidence of TB reached 8.6 million people worldwide and resulted in 1.3 million deaths (**Figure 1.1**).[7] The majority of these cases are found in Africa, Asia and the Western Pacific and HIV co-infection contributes to a quarter of all TB-related deaths.[7] The emergence of HIV and drug-resistant TB strains, along with its lipid-rich impermeable cell wall, are strong factors enabling the virulence of the *Mtb* organism. The incidence of TB peaked in 2004 due to the HIV epidemic, the breakup of the Soviet

Union (and it's anti-TB clinic infrastructure) and the development of multi-drug and extensive-drug resistant TB (MDR-TB and XDR-TB, respectively).[8] MDR-TB is defined as an active infection with *Mtb* resistant to at least isoniazid and rifampicin, the most effective first-line anti-TB drugs. XDR-TB is categorized as an MDR strain that is also resistant to any second-line drugs, fluoroquinolones, and one of the injectable drugs capreomycin, kanamycin, or amikacin. A large percentage of MDR-TB cases develop in patients who have previously been treated for TB, especially in high-burden countries in Eastern Europe and Central Asia where this percentage can reach 50%.[7]

We conducted a double-blind, randomized, placebo-controlled clinical trial of high-dose cholecalciferol in patients with documented pulmonary TB in the country of Georgia, designated by the World Health Organization (WHO) as a current high-burden MDR-TB country.[7] A former Soviet Republic, Georgia had a population of 4.4 million in 2012 and a 158 per 100,000 TB prevalence rate, which puts them in the moderate range for TB burden. Despite very low HIV prevalence (0.3% in 2012[9]), 9% of all new cases and 31% of retreatment cases are diagnosed with MDR-TB.[10] The overall incidence of TB in Georgia has been on a steady decline since the early 1990s due to improved public health anti-TB programs of the National Center of Tuberculosis and Lung Disease (NCTBLD) and the country has a current incidence rate of 116 per 100,000.[10]

Nutrition Status and TB

Body weight, BMI and the relationship between malnutrition and infection have been widely studied within the scope of TB disease.[11-20] Evidence suggests that malnutrition, particularly as assessed by low body mass index (BMI), is associated with secondary immune dysfunction that increases the host's susceptibility to infections.[17,19,20] Body weight changes have been explored as a low-cost biomarker for both disease severity and treatment outcomes.[11,21] Further, low BMI (<18.5 kg/m^2) patients are at increased risk for TB-related mortality and treatment failure.[11,12,21]

Underweight or wasting is often associated with both relapse and death in TB disease.[20,22,23] TB patients diagnosed with moderate to severe malnutrition, defined as BMI ≤17 kg/m^2, had higher rates of TB-related death when compared to TB patients diagnosed with mild to moderate malnutrition.[23] Indicators of moderate to severe malnutrition at diagnosis of TB are associated with an increased risk of relapse.[11] Further, underweight patients who increased their weight by 5% during the first two months of chemotherapy had significantly less relapse than those who gained less than 5%.[11,21]

Although TB is classified as an infectious disease, many aspects of the disease have nutritional relevance. TB is associated with wasting[12], low BMI[18], food insecurity and poverty.[24,25] A recent Cochrane review concluded that there was not sufficient evidence warranting whole food supplementation for adult patients with TB.[26] This was based on five trials, two of which studied extrapulmonary TB and none of which exceeded the recommended calorie intake. Most of these trials were deemed to be poorly designed or lacked power to draw concrete conclusions. It is known that malnutrition is synonymous with low immune function and increased TB risk.[11,17-20]

Several micronutrients have been reported to play a vital role in immune function; although it remains unclear whether supplementation of single or combined

micronutrients facilitates immune function in patients with TB disease.[26] Despite this discrepancy, dietary intake has been little studied in TB patients. This could be due, in part, to lack of validated nutrient assessment tools in developing countries. In chapter 3 we discuss the need to further assess dietary intake using culturally-sensitive dietary intake assessment instruments.

Some research has suggested that patients with TB exhibit lower levels of certain micronutrients as compared to healthy controls. For instance, a study conducted in Ecuador by Koyanagi et al found that 46 smear positive patients with pulmonary TB had significantly lower serum concentrations of zinc (Zn), retinol and selenium as compared to 10 healthy Ecuadorian volunteers.[27] Additional research suggests that intake of specific vitamins and trace elements may favorably influence clinical outcomes in TB. A study conducted in Tanzania by Range et al suggested that supplementation with a multivitamin containing vitamins (A, B, C, D, E), and selenium, copper and Zn during treatment of pulmonary TB may reduce mortality (survival at 8 months post initiation of anti-TB therapy) in those co-infected with HIV.[28] Vitamin A plays a regulatory role in the conversion of naïve T cells to INF gamma secreting Type 1 helper T cells and also differentiation to Type 2 helper T cells.[29] Vitamin A metabolites, as well as vitamin D, may specifically inhibit mycobacterium.[30] Many dietary intervention studies have included vitamin A, either alone or as part of a multivitamin supplement, yet there is no conclusive evidence that vitamin A improves TB-related outcomes.[26]

Zinc, a trace element predominantly obtained from foods such as beef, pork, and legumes, plays an essential role in preventing upper respiratory infection and regulates gene expression.[31] Therefore, it is an important component in the signaling pathways of the immune system. Zinc deficiency has been shown to affect the levels of inflammatory cytokines tumor necrosis factor (TNF alpha) and interferon gamma (INF gamma),[27] substances which play a major role in the pathogenesis of granulomatous

diseases such as TB. Zinc deficiency is common in TB patients, and was found to correlate with more extensive cavitary disease.[32] Individuals suffering from TB with concomitant zinc deficiency were found to have higher levels of inflammatory markers such as C-reactive protein, signaling a more active inflammatory environment. This results in the redistribution of zinc to the liver and increased urinary zinc losses.[27] Studies have also suggested that zinc deficiency exacerbates the immunosuppressive effects of generalized malnutrition.[32] Several randomized clinical trials evaluated the role of zinc supplementation in improving clinical outcomes in TB with mixed results.[28,33,34]

Copper status is also important for immunity. A study conducted by Kassau et al in 150 TB patients, 74 who were co-infected with HIV, found that the copper and copper/zinc ratio was significantly higher ($P<0.05$) in serum of TB patients as compared to healthy controls.[35] Serum copper concentration and copper/zinc ratio declined significantly after anti-TB chemotherapy irrespective of HIV infection positivity ($P<0.05$). Another study conducted by Cernat et al found patients with active pulmonary TB had increased blood copper and ceruloplasmin levels when compared to a control group ($P<0.01$).[36] Wolschendorfa et al suggested *Mtb* is susceptible to copper and that mutant, copper-resistant *Mtb* are crucial for *Mtb* virulence.[37]

Selenium, an anti-oxidant found in high levels in beef, fish, poultry, and eggs, can reduce systemic oxidative stress. It has been shown to affect the immune response to viral pathogens in animal models. Furthermore, selenium-deficient mice demonstrate altered cytokine profiles and significantly decreased T cell proliferation in response to infection with intracellular pathogens as compared to controls.[38] Deficiencies in selenium and other antioxidants, such as vitamin C may also increase the likelihood of an individual progressing from a latent TB infection to active TB disease. A study conducted by van Lettow and colleagues suggested low selenium concentrations, high

HIV load, and high IL-6 concentrations are associated with anemia in adults with pulmonary TB in sub-Saharan Africa.[39] A recent double-blind, placebo-controlled clinical trial of vitamin E and selenium supplementation in patients with pulmonary TB demonstrated significant attenuation of oxidative stress indices in the supplemented group versus placebo-treated patients.[40] Although it is a well-known antioxidant, vitamin E has not definitively been shown to either inhibit or protect against mycobacterium and one study suggested it may even increase risk in certain populations.[30,40,41] Data on the relationship between vitamin E and TB remains controversial. Furthermore, little information is available about the relationship between total anti-oxidant status and specific TB treatment outcomes such as sputum conversion and relapse rates. Based on the above, albeit, limited evidence, however, it appears clear that nutritional status is a key factor involved in the pathogenesis of TB and recovery of individuals with active TB disease.

Pathogenesis and Immune Response

Pulmonary TB is an extremely contagious infection that is spread via droplet nuclei dispersed by an infected host coughing, sneezing, speaking or singing,[42] and *Mtb* can survive several hours outside of the host. Common symptoms include coughing with hemoptysis, chest pain, fever, chills, night sweats, fatigue, anorexia and weight loss.[43] The initial stages of infection have not been explicitly studied and our current knowledge is largely based on inference of observational clinical data. When an individual is exposed to *Mtb,* the innate immune system is activated and the pathogen undergoes phagocytosis by macrophages located in the lung alveoli. This process initiates an inflammatory response that recruits nearby mononuclear cells which invade the epithelium and contribute to the formation of the lung granulomas typically seen in TB. It is within these granulomas the *Mtb* can persist in latency for the remainder of the host's life. Risk for reactivation of the disease is increased in response to a suppressed immune system due to HIV, diabetes or other indications for immunosuppressive therapy.[44,45] The exact mechanisms underlying why some individuals have latent TB but not symptomatic active TB disease remain unknown, although immunosuppression, as occurs in HIV-co-infection, clearly plays a role.[46] In addition, generalized malnutrition is associated with an increased risk of developing active TB disease in subjects with latent TB.[46]

Neutrophils play a critical role in the innate immune response.[47] Antimicrobial peptides (AMP), such as cathelicidin (LL37) a major human endogenous AMP derived from the cathelicidin AMP gene family,[48-51] are produced by neutrophils (and macrophages and other immune cells) and can also trigger macrophage activation.[49-51] This provides a crucial link between the innate and acquired immune systems. As outlined below, adequate vitamin D nutriture (as determined by circulating 25 hydroxyvitamin D [25(OH)D] concentrations) has been shown to induce LL37, which in

turn activates macrophages to kill *Mtb* [49-52] thus providing a link between vitamin D nutriture and immunity.

The Role of Vitamin D Deficiency in TB Pathophysiology

Deficiency of vitamin D is now considered to be a worldwide epidemic in both high-income and low-income countries.[48,53,54] The majority of the body's vitamin D is made from skin-derived precursors (pre-vitamin D) after exposure to ultraviolet B light (UVB) from the sun. Vitamin D, in turn, is hydroxylated in liver to form 25-hydroxyvitamin D (25(OH)D), the primary circulating form of vitamin D. It is hydroxylated once more in the kidney and peripheral tissues (including macrophages) to form 1,25-dihydroxyvitamin D (or calcitriol), the active metabolite of vitamin D.[48] Only a few food sources are rich in vitamin D, and vitamin D-fortified foods are generally unavailable in low-income countries. Other factors that affect vitamin D status include dietary practices that result in poor dietary intake of vitamin D and factors that inhibit sun exposure including clothing, climate and dark skin pigmentation. The highest prevalence of vitamin D deficiency occurs in South Asia and the Middle East.[54] These risk factors and regions are in line with TB endemic regions and risk factors. It is thought that low serum 25(OH)D could be a risk factor for TB infection.[55,56]

Current evidence strongly suggests a poorly-understood, but potentially important, link between vitamin D status and TB.[48-50,57-63] Animal models of *Mycobacterium bovis* infection demonstrate increased lung colonization and lesion size in vitamin D deficient mice.[60] In a previous era, both sunlight and vitamin D were used as primary and adjunctive therapy in pulmonary TB, but these practices fell out of favor as effective anti-mycobacterials became widely available in the latter half of the twentieth century.[61] Unfortunately, the currently published literature on effectiveness of vitamin D therapy in patients with TB is difficult to interpret given their general poor quality or uncertain methodology, small sample sizes, subject and protocol heterogeneity, widely variable regimens of vitamin D treatment and mixed results.[61] Observational studies suggest that vitamin D deficiency increases susceptibility to TB and worsens TB

severity,[62-64] while patients presenting with active TB have significantly lower serum concentrations of 25(OH)D than healthy controls.[65,66] Rathored and colleagues concluded that patients with MDR-TB may have a higher prevalence of vitamin D receptor (VDR) polymorphisms and hypovitaminosis D compared to drug-sensitive TB patients. However, this study did not control for diet or sun exposure.[57]

Recently, there has been a resurgence of interest in vitamin D nutrition as an adjunctive therapy in TB (and other infectious diseases) given its potent effect to stimulate production of LL37. Studies in human monocytes, macrophages, and skin keratinocytes demonstrate that LL37 production is critical for bacterial clearance.[51,67-70] LL37 is chemotactic for neutrophils, monocytes and CD-4 helper T cells and exhibits direct broad-spectrum anti-microbial activity including against gram-positive and gram-negative microorganisms, fungi, and *Mtb*.[51,67-70] Recent reports indicate that vitamin D has a pronounced immunomodulatory role in the pathogenesis of TB within the human host via induction of local LL37.[46,48-50,69,70] Increased levels of 1,25-dihydroxyvitamin D are present in macrophages at sites of *Mtb* infection.[48,50] Upregulated production of 1,25-dihydroxyvitamin D in such cells occurs as a function of activation of cell surface toll-like receptor 2 (TLR2) by microbial (e.g. mycobacterial) TLR2-TLR1 ligands.[50,69,70] This, in turn, upregulates expression of both the nuclear VDR and the enzyme 1-α-hydroxylase (CYP27B1), which converts 25(OH)D to the active form, 1,25-dihydroxyvitamin D.[46,48,50] Adequate vitamin D status is required because the extracellular pool of 25(OH)D is the rate-limiting substrate that is shuttled into the cell for the synthesis of 1,25-dihydroxyvitamin D.[48,50] 1,25-dihydroxyvitamin D binds to the VDR and enters the nucleus to enhance transcription of vitamin D-dependent genes including the AMP cathelicidin.[46,48,50] The potent activity of vitamin D against *Mtb* in both human monocytes and macrophages *in vitro* has recently been demonstrated. Monocytes cultured in sera from African Americans deficient in vitamin D failed to demonstrate

upregulation of cathelicidin mRNA upon stimulation by *Mtb*-derived TLR2 ligands when compared to monocytes cultured in sera from vitamin D-replete individuals.[49] Addition of exogenous 25(OH)D to deficient sera restored levels of cathelicidin mRNA and the anti-mycobacterial activity of these cultured cells.[49,71] Klug-Micu and colleagues also tested this concept in vitamin D sufficient sera and this pathway was shown to be triggered by CD40 activation, which may link it to anti-mycobacterial response.[71] These findings support the hypothesis that therapy with orally-administered vitamin D, at doses designed to increase blood concentration of 25(OH)D to levels ≥ 30 ng/mL which is considered by many experts to be optimal lower limit for adequate vitamin D status,[48] may upregulate the cathelicidin/LL-37 synthetic pathway in humans, which, in turn, may potentially facilitate clearance of *Mtb* in persons with latent TB infection or active TB. Such data informed the design of the randomized clinical trial (RCT) that forms the basis for this dissertation research.

There have been few randomized clinical trials involving vitamin D supplementation in active TB patients throughout the world. In the first study, Nursyam et al treated patients with pulmonary TB with high doses of vitamin D (10,000 IU daily) or placebo for six weeks in a double-blind trial.[72] All subjects were otherwise treated with conventional anti-TB drug therapy. All 34 vitamin D-treated patients (100%) converted sputum from acid-fast bacilli (AFB) positive to AFB negative by six weeks compared to only 77% of 25 placebo-treated patients.[72] Wejse and colleagues conducted a double-blind, randomized, clinical trial (RCT) that gave a total of 300,000 IU of cholecalciferol over three doses (baseline, 5 and 8 months) with concomitant anti-TB chemotherapy. Subjects included HIV-1 and HIV-2 infected patients as well as HIV-negative patients; the TB Score was chosen as the primary outcome and no differences were observed between the study groups (vitamin D vs placebo).[73] There were many limitations with this trial that have been addressed in subsequent trials. One is the low dose of vitamin

D: the safety of pharmacological doses had not been documented prior to the beginning of this trial and it therefore used conservative doses. The other major limitation is the primary outcome chosen. Investigating sputum culture conversion over time and at 8 weeks after initial therapy may have been a better choice due to its use in the literature to allow comparison of studies, although better predictors are certainly needed.[74]

Martineau et al have published extensively on the link between vitamin D and TB.[61,75-77] In their first study, they treated healthy household contacts of active TB patients with a single dose of 100,000 IU vitamin D2 versus placebo in a double-blind trial. Isolated monocytes/macrophages from the vitamin D-treated subjects contained significantly more 25(OH)D than controls and whole blood from the vitamin D-treated contacts was significantly more capable of suppressing proliferation of *Mtb in vitro* versus control subjects.[77] In the larger RCT, they gave four doses of 100,000 IU vitamin D3 (totaling 400,000 IU) at 7, 14, 28, and 42 days after start of antimicrobial therapy.[76] The investigators concluded that overall the vitamin D supplementation did not speed up sputum culture conversion; however it did accelerate conversion in those patients with the T*aq1* vitamin D receptor polymorphism of the *tt* genotype. The *tt* genotype patients were a small percentage of this study therefore further investigation is needed on potential genetic factors that may influence anti-TB effects of vitamin D supplementation.

The SUCCINCT study is the most recent randomized, double-blind, multi-center, placebo-controlled vitamin D trial published. The investigators gave two doses of 600,000 IU vitaminD_3 intramuscularly one month apart. Sputum culture conversion was a secondary outcome, weight gain being the primary outcome, and they found no difference in the overall groups (vitamin D vs. placebo). However, when stratified by level of deficiency (<30 ng/mL was deficient), the deficient group showed greater improvement of TB severity scores compared to those who were considered sufficient in vitamin D at baseline.[58]

Potential Utility of Metabolomics in TB

The small molecule metabolites that comprise the human metabolome is affected by pathology, genetic mutations, disease, environmental exposure, pharmaceuticals and diet.[78] Metabolomics can be defined as the study of small molecules involved in complex biological systems.[78] Metabolomics has recently been used in both the identification of infection and to predict the response to therapy in a number of infectious diseases, including *plasmodium falciparum*[79], onchocerciasis[80], malaria[81], *Escherichia coli*[82], and *Mycobacterium tuberculosis.*[83-87] This suggests the potential utility of metabolomics to further examine potential biomarkers for diagnosis and treatment response in TB.

Biomarkers for TB diagnostics, vaccines, and treatment progression are severely lacking, especially in low and middle-income countries. Currently, there are two approaches for TB diagnosis: identification of *Mtb* via bacterial culture, which can take up to eight weeks[88], and the host response to *Mtb* via skin test or interferon-gamma release assay. The medical community continues to rely on the pure protein derivative (PPD) or the tuberculin skin test (TST) as a screening marker for TB infection, but it cannot distinguish between active TB disease and latent TB infection (LTBI). In areas of high TB incidence, misdiagnosis can be as high as 40% due to lack of sensitivity and specificity of these tests.[89]

Three areas of TB biomarker research have been identified: those related to active TB disease diagnosis and treatment progression, those related to LTBI identification and reactivation risk, and biomarkers that can be utilized in vaccine research for uninfected individuals.[90] This call to action for additional biomarker research has spawned new strategies in biomarker development. Gene expression profiling and metabolic profiling have recently been used to differentiate between TB disease and healthy controls.[84,87,91-93] Recent, though limited, metabolomics analysis of

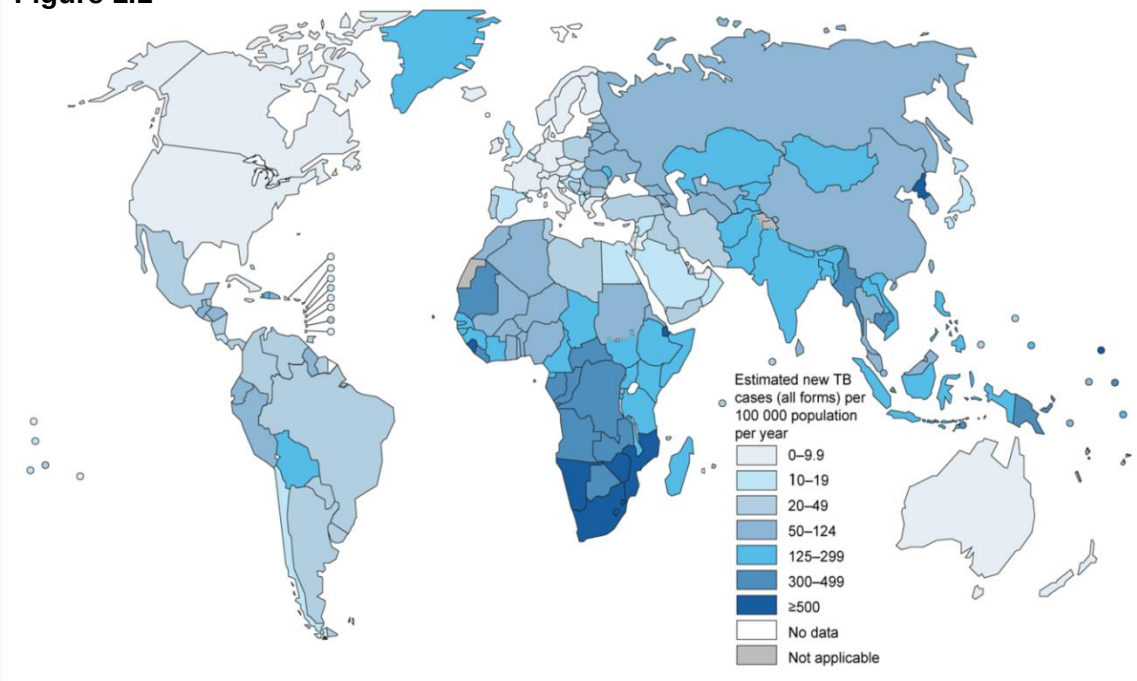
blood and sputum has enhanced TB research by providing new hypotheses and theories on *Mtb* metabolism[94] and identifying differing metabolic profiles for those with and without active TB.[18,20,21,84,87,93,95] Metabolomics analysis may provide insight into host-pathogen interactions. Sputum can provide information on *Mtb* metabolites from the organism, but it poses an extreme safety risk due to the contagious nature of *Mtb*. Plasma and sera samples can provide a safer approach to study *Mtb* metabolites as well as host changes in metabolism. In one recent human sputum study, 95 samples were collected from suspected TB cases and analyzed using two dimensional gas chromatography time of flight mass spectrometry (GCxGC-TOFMS).[85] Twenty-two common metabolites were detected and they included *Mtb* cell wall components, intermediates in the citric acid cycle/glyoxalate shunt pathway and neurotransmitters that may explain certain TB-related symptoms. Weiner et al. using low-resolution LC-MS approaches conducted a cross-sectional study using human serum samples from 44 subjects with active pulmonary TB, 46 subjects with latent TB infection, and 46 healthy controls from South Africa. They identified 20 significant metabolites distinguishing subjects with active TB and healthy controls out of a total of 428 identified metabolites. These included amino acids such as glutamine and tryptophan, which were lower in those with active TB. Although this set of metabolite biomarkers was confirmed *in vitro* and correlated with corresponding cytokines, the signature still lacks specificity because none of the metabolites appear to be exclusive to *Mtb*. Zhou et al. also examined serum from subjects with active TB and non-infected controls using ^{1}H NMR spectroscopy metabolomics methods. Seventeen significantly different metabolites were found between the two groups and the majority of these metabolites were related to the alternate citric acid cycle common in *Mtb*, including higher glutamate in TB subjects. Glutamate is used as an alternative energy source for the organism in the low-oxygen environment of the granuloma. Another recently published human study identified nine

significantly different metabolites between TB subjects and controls in sera and five that differed between start of treatment and after completion.[87] One metabolite, 5-oxoproline, was common between the two studies and may be a marker for disease severity as it is associated with cavitary disease. This study, although limited in sample size, signifies the most progress toward a metabolomics-defined biomarker in TB.

Purpose of Research

The purpose of this dissertation was to explore 1) dietary intake in patients with pulmonary TB disease in Tbilisi, Georgia, through use of a novel dietary intake assessment instrument; 2) to examine serial dietary and body composition indices over time in recently diagnosed TB patients, and as sub-aims, to examine differences between those with MDR-TB and drug-susceptible TB and those receiving or not receiving high-dose vitamin D_3 therapy, designed to enhance *Mtb* clearance and cure; and 3) to determine, via plasma metabolomics analysis, the metabolic relationships between subjects with active TB disease and control subjects without evidence of TB disease from a randomized, controlled clinical trial in Tbilisi, Georgia. All data were derived from a double-blind, randomized, placebo controlled trial of high-dose vitamin D_3 therapy in patients with recently diagnosed pulmonary TB in Tbilisi, Georgia. As described in the next several chapters, we developed a novel dietary assessment tool for this population in order to collect accurate dietary intake and collected body composition data, which has rarely been conducted in studies of patients with TB disease. We also used high-resolution LC-MS metabolomics methods to determine metabolic profile differences in a sub-sample of TB subjects and healthy household controls without evidence of TB disease.

Figure 1.1



WHO Tuberculosis Incidence 2012

Chapter 2: METHODS

Objectives and Hypotheses

The primary objectives of this dissertation are: 1) To develop and validate a novel dietary intake assessment instrument specific for Georgian culture in patients with pulmonary tuberculosis; 2) a) To evaluate macronutrient and body composition changes in patients with pulmonary TB disease in Tbilisi, Georgia over a 16 week period after diagnosis in comparison to a community household reference group; and b) to determine the influence on macronutrient intake and body composition of administration of high-dose vitamin D_3 and anti-TB drug susceptibility in patients with pulmonary TB disease studied during a randomized, controlled trial of high-dose vitamin D_3; and 3) To obtain pilot data on the utility of high-resolution metabolomics profiling of plasma to detect the presence of active TB disease compared to a community household contact reference group without apparent TB disease.

The specific hypotheses are:

1. The mean daily dietary intake of specific nutrients from the novel dietary assessment tool will positively correlate with the nutrient intakes using conventional dietary recall methods.
2. Body composition will demonstrate wasting (decrease fat mass and fat-free mass) despite the level of macronutrient intake in pulmonary disease patients in comparison to a community reference group of subjects without pulmonary TB disease. Subjects with multidrug resistant TB (MDR-TB) will demonstrate increased wasting compared to drug-sensitive TB disease subjects over time and vitamin D treatment will not influence these relationships.
3. High-resolution LC-MS-based plasma metabolomics methods will identify specific metabolites that distinguish patients without or with pulmonary TB.

Summary of Methods

Overall RCT Study Design

This was a randomized, double-blind, controlled intent-to-treat 16 week trial in Georgian adult patients with documented, newly diagnosed pulmonary tuberculosis (TB) carried out to test the clinical and nutritional efficacy of an adjunctive regimen of oral high-dose vitamin D_3 supplementation designed to optimize serum 25-hydroxyvitamin D levels to > 30 ng/mL. Study sites were two outpatient clinics in Tbilisi, Georgia**,** the National Center for Tuberculosis and Lung Diseases (NCTBLD) and the Ftizio-Pulmonologic Center. The study was approved by the institutional ethics committee of NCTBLD Tbilisi, Georgia and Emory University Institutional Review Board in Atlanta, GA, USA (IRB00014641). The study was supported by grants from the National Institutes of Health D43 TW007124, D43 TW007124-06S, K24 DK096574, and UL1 TR000454, and a grant from the Emory Global Health Institute.

We included study subjects ≥18 years of age who were documented as a new case of smear-positive pulmonary TB and were within ≤ 1 week of initiation anti-TB therapy. The subjects agreed to receive anti-TB therapy and attend all study visits in Tbilisi and sign the informed consent. We excluded those subjects who had received greater than 30 days of anti-TB therapy in their lifetime, were currently incarcerated and women who were currently pregnant or lactating. The subject's previous medical history could not include organ transplant, cancer during the previous five years, seizure disorder, cirrhosis, hypercalcemia, hyperparathyroidism, sarcoidosis, or nephrolithiasis, use of oral corticosteroids during the past 30 days, current use of cytotoxic or immunosuppressive drugs, current significant renal dysfunction (serum creatinine concentration >250 mmol/L) or requirement for dialysis therapy.

The patient, primary physicians, investigators and study coordinators were blinded to the treatment allocation. Randomization was conducted with a 1:1 treatment

allocation ratio by using a pseudo-random-number generator with permutated blocks. This minimized allocation bias and can prevent differences between treatment groups caused by certain patient characteristics.[96] Vitamin D_3 or identical placebo capsules were given concomitant with standard anti-TB drugs (RIPE- rifampin, isoniazid, pyrazinamide, and ethambutol) under direct observed therapy-short course (DOTS) as per the World Health Organization (WHO). The capsules were obtained from Biotech Pharmacal, Inc. and contained microcrystalline cellulose and 50,000 IU of cholecalciferol set in gelatin or cellulose alone. The vitamin D containing capsules were third-party tested for potency by Analytical Research Labs and were verified to contain 106.4% of the 50,000 IU indicated on the label. Vitamin D_3 was chosen over vitamin D_2 because D_3 (cholecalciferol) may be more bioavailable than D_2 (ergocalciferol).[97-99] Vitamin D_3 has a higher affinity for vitamin D binding protein than vitamin D_2, therefore vitamin D_3 has a longer half-life in circulation.[100] Study subjects were randomized to 50,000 IU of oral vitamin D_3 three times weekly for the first eight weeks followed by the same vitamin D_3 dose given every other week for a subsequent eight weeks versus placebo. This allowed for a substantial increase in 25(OH)D levels in the blood, reaching a mean peak level of ≈100 ng/dL by 8 weeks (not shown; data will be submitted in main RCT publication).

Data Collection and Measurements

Patient baseline and follow up visits were conducted after enrollment at weeks 0, 2, 4, 6, 8, 12 and 16 as shown on the study diagram (**Figure 2.1**). Data was collected using a standard data collection form during patient interviews and patient clinical records including history and standard TB forms. Patient data case report form (CRF) included information on patient's demographics, clinical data, microbiological and biochemical test results and safety data.

Patients were eligible to withdraw informed consent during whole study period. Study subjects were declared as lost to follow-up after missing visit and defaulted after not being able to be reached and interviewed within period of two weeks after a missed visit. All data were collected by two study coordinators transcribed into English and entered into a web-based CRF, developed by the Emory-based investigators and biostatistians.

Safety parameters were obtained by collecting a set of standardized questions, primarily symptoms related to possible hypercalcemia, from study subjects at the study visits in which blood sampling was performed for the presence of hypercalcemia. In addition, subjects were asked about symptoms of hypercalcemia on each study visit (i.e. nausea, vomiting, abdominal pain, confusion or renal colic). The Tbilisi-based investigators could discontinue any subject from study therapy at their discretion, if, in their professional opinion, the subject's health, safety, and/or well-being is threatened by continued use of study therapy. Blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing collection tubes and isolated plasma immediately stored frozen in a -80°C freezer prior to later batch shipment in dry ice from Tbilisi to Emory University, Atlanta, GA. Samples were never previously thawed, remained frozen during transit to Atlanta, and were stored at -80°C at Emory University. Blood (5 mL) was obtained for

measurement of serum calcium levels (standard laboratory methods) and 25(OH)D concentrations on weeks 2, 4, 8, 12 and 16.

Results of calcium analyses were available the same day and hypercalcemia was defined as >10.5 mg/dL (>2.6 mmol/L) and such patients were immediately discontinued from study drug but continued to be followed. Serum calcium levels >10.5 mg/dL (>2.6 mmol/L) at any of the follow up visits also resulted in study drug being discontinued, but the subject otherwise followed on the intent-to-treat protocol. Serum for 25(OH)D analyses was saved in 0.5 ml cryovials at -80C and shipped on dry ice to Emory University, Atlanta, GA, USA for further analyses.

Sputum AFB cultures were performed at weeks 0, 2, 4, 6, 8, 12 and 16. Two sputum specimens were obtained from each patient on baseline and each follow-up visits. Direct smears with Ziehl-Neelsen staining were examined by light microscopy at local microscopy centers of both Georgian sites. All samples were sent to the National Research Laboratory (NRL) for culture analyses on Löwenstein-Jensen (LJ) solid media in a BSL 2+ area, using standard methodologies. Positive cultures were confirmed to be *Mtb* complex using phenotypic tests. Drug susceptibility testing for first-line drugs was done using absolute concentration method on solid media with standard methodology (decontaminated in a BSL3 area with N-acetyl-L-cysteine-sodium hydroxide, centrifuged, and the sediment was then suspended in 1.5 ml of phosphate buffer, then inoculated on to LJ solid medium).[101] Since early 2011, Georgia has introduced molecular diagnostic test HAIN MTBDR*plus* in direct samples as the routine diagnostic test and it was used for earlier confirmation of MDR-TB among the later cohort of study subjects.[102]

Chapter 3 Specific Methods

Nutrient Intake Assessment

The nutrition assessment instrument was developed to capture the mean daily micronutrient and macronutrient intake over the previous three day period via face-to-face interviews by trained investigators. The dietary intake interviews were performed at baseline and again at the eight and sixteen-week time points, respectively. The instrument was designed prior to initiation of the RCT to assess nutrient intake in a low socioeconomic status, non-English speaking adult population. During the instrument developmental phase, we initially explored typical foods and meal patterns of adult Georgians by face-to-face and email discussions between the Georgian- and United States (U.S.)-based investigators involved in the RCT. The instrument was designed to follow principles routinely utilized by nutritionists and dietitians in standardized food record intake forms. In addition, food items (including beverages and snacks) consumed commonly in Georgian culture and typical recipes for these were included in the questionnaire as prompts. For example, **Table 3.1** in Chapter 3 outlines details of the questions for typically consumed tea and soup, respectively. A free text comment section at the end of the questionnaire as added to allow for additional details regarding recipes. The Georgian-based physician investigators were extensively trained prior to the initiation of the RCT by the registered dietitian investigator on the interview process via video training uploads (YouTube), demonstrations with mock face-to-face interviews, a comprehensive training DVD, and regular live training sessions via Skype. Standardized food models and common household measurement instruments were provided to the investigators in Tbilisi and used in the patient interviews to help to determine accurate serving sizes. The Georgian language face-to-face interviews with TB patients were completed within 30-40 min at outpatient research visits often in the company of the primary caregiver; food and beverage intake during the previous three

days was recalled and recorded in the case report form (CRF). The food intake data were then transcribed in English by the multilingual investigators into a web-based case report form (CRF) for review. Review of intake data for individual subjects took place within 1-3 days after data entry in Tbilisi since all subjects returned to the two outpatient TB clinics on a daily basis for directly observed anti-tuberculosis drug therapy. Data were analyzed using state-of-the-art dietary analysis software [Nutrition Data System for Research (NDSR), University of Minnesota, Minneapolis, MN]. Final calculations were completed using NDSR version 2011. The NDSR time-related database updates analytic data while maintaining nutrient profiles true to the version used for data collection. NDSR analyzes for specific quantities of over 160 different micronutrient and macronutrients and dietary compounds with well-described accuracy and completeness.[103] Mean daily intake was determined for each subject from the three day food recall questionnaire. Specific methods were used to enter the Georgian food items into the NDSR software program, which was developed for foods commonly consumed in the U.S. First, the format of the structured three day food recalls did not distinguish between different versions of the same food type. For example, beef was always entered as a trimmed sirloin if it was eaten on its own and as stew beef if consumed in a soup regardless of the cut of meat that was actually consumed. These assumptions were based on most common food servings given by the Georgian investigators during the development phase of the instrument. Some assumptions were also needed for food items that were specific to Georgian culture in order to find a similar item in the U.S.-based NDSR nutrient database. For example, “matsoni”, a concentrated yogurt food item, was entered as plain whole milk yogurt into the NDSR software. All milk intake was entered as a fresh whole milk to eliminate the extraneous micronutrients supplied by milk fortification in the U.S. captured by the NDSR database, but not present in the unfortified Georgian commercial milk supply.

Validation methodology

The validation methods were based on previous methods of similar studies as outlined below. A series of 24 hour recalls is considered the most cost effective and feasible gold standard for validating novel nutrition assessment tools.[104] A convenience sample of 31 enrolled pulmonary TB subjects who completed both the three-day food recall questionnaire at baseline and were able to also complete three consecutive 24-hour recalls during the following week were studied. This proportion (31/199 or 16% of total study subjects) is in line with previous diet intake tool validation studies in which 10-20% of the total patient population was studied.[105-108] This was a pilot study and therefore a prehoc sample size calculation was not performed. A series of three 24-hr recalls was conducted in the 31 subjects during the week following their baseline visit, at which the current assessment tool was previously completed. We chose to focus only on subjects following their baseline visit to reduce the potential "learning effect" of serial face-to-face interviews to provide recent food intake data. This format was utilized to capture three days of food intake data via both 24-hour recall and by the RCT assessment tool and to evaluate the impact of recalling foods eaten two and three days prior to the interview in the RCT tool.

Statistical analysis

The differences between the food questionnaire and food recall of each outcome were summarized by the mean difference (questionnaire-recall), the standard deviation (SD) of the differences, and the 95% agreement limits.[109] The differences between the two measurements and their mean for each outcome were summarized by use of scatterplots (Bland-Altman plots). A 1-sample paired t-test was used to compare the mean differences between the food questionnaire and food record recall measurements. The intra-class correlation coefficient (ICC) was also used as a measure of agreement and was estimated by variance components based on statistical modeling as described by Bartko.[110] The ICC is large (i.e., near 1) when there is little within participant variation. We used a varied approach at validation to encompass both individual variation and variation between the two tools. Reproducibility could not be assessed due to the nature of pulmonary TB. Patients tend to increase eating habits as they feel better therefore altering the food questionnaires as the disease and treatment progressed. All statistical analyzes were carried out using SAS software, Version 9.3. (Cary, NC, USA).

Chapter 4 Specific Methods

Nutrition and Body Composition Assessment

Dietary intake was documented from study participants at baseline, week 8 and week 16 of the clinical trial. The nutrient intake tool discussed in Chapter 3 was used for dietary intake assessment.[74] Subjects that exhibited a markedly elevated baseline mean daily caloric intake (defined *prehoc* as mean daily caloric intake of > 6000 kcal/day) were excluded from analysis. This calorie limit is higher than what is typically used in the U.S. TB patients are instructed to increase their food consumption as part of treatment causing the caloric mean to increase. We used Tukey's method for outliers to reach the 6000 kcal/day cut off.[111] The Georgian calendar consists of many celebrations and Georgians participate in extended meals (*supras)* where dietary consumption can last for hours; thus we established the upper limit of caloric intake to help to ensure representative dietary intake days.

Body weight and height were measured via electronic height and weight scale *(Tanita Inc;* Arlington Heights, Illinois, USA) at baseline and on weeks 4, 8, 12, and 16. Body mass index (BMI) was calculated using the standard formula. Bioelectrical impedance analysis (Bioelectrical Impedance Analyzer, Model Quantam X: RJL Systems, Clinton Township, MI, U.S.A) (BIA) was used to determine body composition; % body fat mass and % body fat free mass. BIA was the chosen method of body composition because of its ease and portability, making it an ideal, robust measure for a field study. Our Georgian staff was trained via video and various practice sessions on the new instrument. BIA allowed us to collect information on fat mass and fat free mass to enhance our conventional anthropometric methods. Kilograms of fat mass and fat free mass were calculated using the body weight recorded at the respective time point.

Statistical Analysis

Descriptive statistics (Student's t-tests for continuous variables and chi-square tests and Fisher's exact tests for categorical variables) were used where appropriate. PROC GLM was used to build models to determine differences in macronutrient and body composition variables between TB disease subjects and household contacts. Two models were developed; model I adjusted for age and gender, while model II adjusted for age, gender, employment status and smoking. We also tested for interactions with gender. Simple repeated linear models were used to look at changes in each variable over time. PROC MIXED was used for these and we assumed compound symmetry. Repeated measures analysis of variance was used to evaluate time and treatment group effects as well as interaction within the body composition and macronutrient variables. TB subjects were dichotomized on both gender and drug susceptibility to assess changes between those with and without MDR-TB and to assess gender differences. All statistics were completed using SAS software, Version 9.3. (Cary, NC, USA) and a *P*-value of <0.05 was determined significant. We chose PROC MIXED over PROC GLM to account for missing data because a value that is missing has no effect on the other values from the same subject.

Linear Relationship between Calorie Intake and % Calories from Protein and Body Composition Variables at Baseline

Prior to implementation of simple linear regression to investigate the relationship between outcome (calories (kcal/kg/day) or % calories from protein) and body composition variables (body weight (kg), BMI (kg/m^2) and fat-free mass (kg)) all assumptions were assessed. Scatterplots and residual analyses were used to help assess nonlinearity of the relationship and the appropriateness of the assumptions of normality for each outcome and constant variance of the outcome for each value of the body composition variables. Linear regression, implemented using SAS PROC REG,

was used to summarize the linear relationship between the outcome and body composition variable by estimating the regression coefficients [the intercept ± standard error; the linear slope ± the standard error and the 95% confidence interval for the slope]. A t-test was used to determine whether the estimated slope differed from zero. The coefficient of determination (R^2) was used to assess how much of the variation in outcome was accounted for by the body composition variable. The mean squared error (MSE) is the residual variance estimate (i.e., variance estimate among all patients having the same value for the predictor, e.g. BMI). The RMSE (root MSE) is the standard deviation around the regression line (the distance, on average of a data point from the fitted regression line). Analysis of covariance (i.e., linear regression of calories on body composition variable by MDR subgroup) was used to estimate and compare adjusted mean calories by MDR status. The adjusted mean outcome for a subgroup was defined as the predicted value obtained by evaluating the regression model for a subgroup at the mean body composition variable of the two MDR subgroups.

Chapter 5 Specific Metabolomics Analysis

Metabolomics Analysis

High-Resolution Metabolomics Profiling

Frozen plasma samples for 17 subjects with pulmonary TB disease and 17 smear-negative household contacts without TB disease were thawed and 65 µL plasma was treated with 130 µl acetonitrile (2:1, v/v) and contained an internal isotopic standard mixture (3.5 µL/sample), as previously described.[112] The internal standard mix for quality control consisted of 14 stable isotopic chemicals covering a broad range of chemical properties represented in small molecules, including [$^{13}C_6$]-D-glucose, [1,2-$^{13}C_2$]-palmitic acid, amino acids ([3,3-$^{13}C_2$]-cystine, [$^{13}C_5$]-L-glutamate, [2-^{15}N]-L-lysine dihydrochloride, [^{15}N,$^{13}C_5$]-L-methionine, [^{15}N]-L-tyrosine), [3,4-$^{13}C_2$]-cholesterol [$^{13}C_7$]-benzoic acid, [^{15}N]-indole, [trimethyl-$^{13}C_3$]-caffeine, [$^{15}N_2$]-uracil, [^{15}N]-choline chloride, and 2'-deoxyguanosine-$^{15}N_2$,$^{13}C_{10}$-5'-monophosphate.[112] Pooled human reference samples (NIST) were used to compare analysis variation. Samples were mixed and placed in ice for 30 min prior to centrifugation for 10 min (16,100 x *g* at 4ºC) to remove protein. The supernatants (10 µL), for each LC-MS run were then loaded onto a Shimadzu® autosampler maintained at 4°C and analyzed in triplicate using a LTQ-Velos Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA). Analysis was performed with a C18 chromatography column (Higgins Analytical, Targa, Mountain View, CA, USA, 2.1 x 10 cm) and C18 chromatography.[112] Elution was obtained with a formic acid/acetonitrile gradient at a flow rate of 0.35 ml/min for the initial 6 min and 0.5 ml/min for the remaining 4 min. The first 2-min period consisted of 5% solution A [2% (v/v) formic acid in water], 60% water, 35% acetonitrile, followed by a 4-min linear gradient to 5% solution A, 0% water, 95% acetonitrile. The final 4-min period was maintained at 5% solution A, 95% acetonitrile. The mass spectrometer was set to collect data from mass/charge ratio (*m/z*) 85 to 2000 daltons over the 10-minute

chromatography period. Electrospray ionization was used in the positive ion mode for detection, as outlined previously.[112,113]

Plasma samples were chosen over serum or other biological fluids because of the relative safety of the sample from *Mtb* contracture, accessibility, and contain metabolites from both short and long-term exposures.[114,115] Although this was an exploratory analysis, we anticipated finding hydrophobic metabolites such as vitamin D metabolites and other complex lipids which may be better detected from plasma. Blood collection in EDTA tubes was chosen as this buffer has been used most frequently in the high-resolution metabolomics analysis performed in the laboratory of Co-Advisor Dr. Jones to date.

Tandem Mass Spectrometry

Ion dissociation analysis by tandem LC-MS-MS was used in separate studies to verify identity of L-glutamate, which is also included in our findings.[113] Identity of specific D-series resolvins [resolvin D1 (RvD1), 7*S*, 8*R*,17*S*-trihydroxy-4*Z*, 9*E*, 11*E*, 13*Z*, 15*E*, 19*Z*-docosahexaenoic acid), resolvin D2 (RvD2), 7*S*, 16*R*, 17*S*-trihydroxy-4*Z*, 8*E*, 10*Z*, 12*E*, 14*E*,19*Z*- docosahexaenoic acid)] was confirmed using lipid mediator metabololipidomics analytical methods, as described by Serhan and colleagues.[116,117] Briefly, five deuterium-labeled internal standards (0.5 ng) were added to plasma aliquots [d_5-RvD2, d_8-5-hydroxyeicosatetraenoic acid (d_8-5-HETE), d_4-leukotriene B_4 (d_4-LTB_4), d_5-lipoxin A_4 (d_5-LXA_4) and d_4-prostaglandin E_2 (d_4-PGE_2) to facilitate quantification of mediator recovery. Samples were extracted using SPE columns, eluted with methyl formate, and organic solvent evaporated using a nitrogen stream. Samples were suspended in methanol for analysis by high-resolution liquid chromatography coupled with tandem mass spectroscopy (LC-MS/MS), using a QTrap 5500 machine (ABSciex, Framingham, MA).[116,117] To monitor and quantitate levels of the specialized pro-resolving lipid mediators (SPMs) derived from arachidonic acid, docosahexanoic acid

(DHA) and eicosapentanoic acid (EPA) in plasma samples,[118,119] multiple reaction monitoring (MRM) for signature ion fragments was performed with identification accomplished using LC retention time (RT), band shape and maximum absorbance wavelength (λmax) of UV and ≥ 6 diagnostic ions of tandem MS-MS spectrum. Quantification was determined based on peak MSM transition area and linear calibration curves, as described.[116,117]

Data Collection and Processing for High-resolution Metabolomics

Data from the LTQ-Velos Orbitrap was continuously collected over the 10-min chromatographic separation period and stored as .Raw files. The .Raw files were converted to .cdf format using Xcalibur file converter software (Thermo Fisher, San Diego, CA) and used for data extraction. Peak extraction and integration was performed using apLCMS with xMSanalyzer.[95,120] apLCMS (http://www.sph.emory.edu/apLCMS) is an adaptive processing software package designed for high resolution LC-MS data that performs data filtering, peak detection, and alignment and generates a feature table, where a feature is defined as the measured *m/z*, retention time, and integrated ion intensity. xMSanalyzer enhances the feature detection process by performing systematic data re-extraction and combining results from different parameter settings (http://userwww.service.emory.edu/~kuppal2/xMSanalyzer).

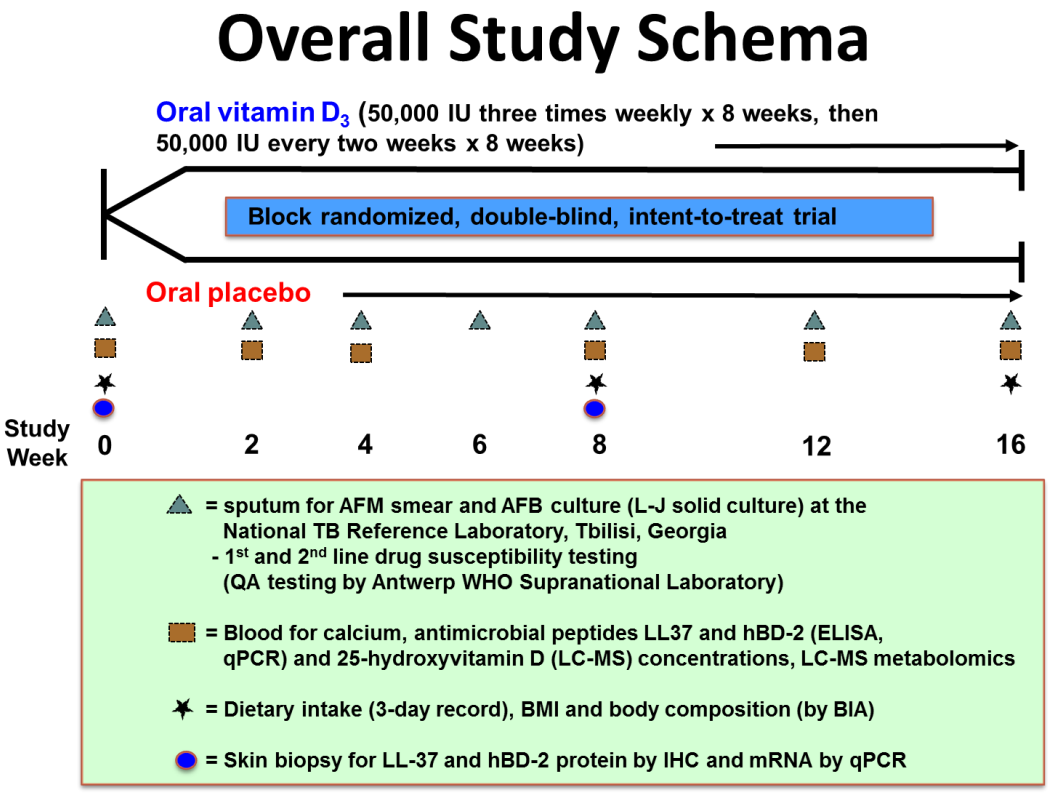
Statistical Analysis

Descriptive statistics for demographic and clinical data were performed (mean [SD]; n [%]). Two-tailed t-tests and two-tailed Fisher exact tests were used to compare patients with TB disease and their household contacts using SAS version 9.3 (Cary, NC, USA) for continuous and categorical data, respectively. LIMMA was used to identify differentially expressed metabolites between TB and HC subjects.[121] To account for multiple comparisons, *P* values were adjusted using the Hochberg and Benjamini false discovery rate (FDR; q=0.05) to distinguish statistically significant metabolites that differed between the two groups.[122] Two-way hierarchal clustering analysis (HCA) was performed using the differentially expressed metabolites to visualize patterns and detect clusters of co-regulated metabolites by disease state (TB disease compared to HC).[78,123] An untargeted metabolome-wide association study (MWAS) based on the Pearson correlation analysis of the differentially expressed metabolites with all detected metabolites in plasma of subjects with TB and household contacts was performed to understand the global association pattern of the discriminatory metabolites.[78] The statistical significance of correlations was determined using the Student's t-test method and visualized using a Manhattan plot where the x-axis corresponds to the metabolites (85-2000 *m/z*) and the y-axis corresponds to the negative $\log_{10}$ of the *P* value.[124,125] Targeted MWAS were also performed with anti-TB drugs differentially expressed in TB disease versus household contacts. FDR, HCA and Pearson correlations analyses were performed using R.[95,120]

Metabolite Annotation and Pathway Analysis

Putative metabolite identification of the discriminatory ions between TB and household contacts were determined using open-access Metlin ([126]; http://metlin.scripps.edu/) and the xMSannotator R package (manuscript submitted; http://userwww.service.emory.edu/~kuppal2/xMSannotator/). xMSannotator uses biological, chemical and pathway information with a suite of major small molecule databases including KEGG ([127]; http://www.genome.jp/kegg/), Human Metabolome Database (HMDB)[128], MetaCyc (http://www.metacyc.org) and ChemSpider (http://www.chemspider.com/).[129] Pathway analysis was performed using KEGG.[127]

Figure 2.1


Overall Study Schema
Oral vitamin D3 (50,000 IU three times weekly x 8 weeks, then 50,000 IU every two weeks x 8 weeks)
Block randomized, double-blind, intent-to-treat trial
Oral placebo
Study Week
0
2
4
6
8
12
16
= sputum for AFM smear and AFB culture (L-J solid culture) at the National TB Reference Laboratory, Tbilisi, Georgia
- 1st and 2nd line drug susceptibility testing
(QA testing by Antwerp WHO Supranational Laboratory)
= Blood for calcium, antimicrobial peptides LL37 and hBD-2 (ELISA, qPCR) and 25-hydroxyvitamin D (LC-MS) concentrations, LC-MS metabolomics
= Dietary intake (3-day record), BMI and body composition (by BIA)
= Skin biopsy for LL-37 and hBD-2 protein by IHC and mRNA by qPCR

Chapter 3 - A CULTURE-SPECIFIC NUTRIENT INTAKE ASSESSMENT INSTRUMENT IN PATIENTS WITH PULMONARY TUBERCULOSIS

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Non-Standard Abbreviation

TB - Tuberculosis

NDS-R – Nutrient Database System for Research

ICC – Intraclass Correlation Coefficient

MDR – Multi-drug resistant tuberculosis

FFQ – Food frequency questionnaire

NCTBLD - Georgian National Center for Tuberculosis and Lung Diseases

RCT - Randomized clinical trial

CRF – Case report form

ACTSI - Atlanta Clinical and Translational Science Institute

CCC - Concordance correlation coefficient

Abstract

Objective: To develop and evaluate a culture-specific nutrient intake assessment tool for use in adults with pulmonary tuberculosis (TB) in Tbilisi, Georgia. **Methods:** We developed an instrument to measure food intake over three consecutive days using a questionnaire format. The tool was then compared to 24 hour food recalls. Food intake data from 31 subjects with TB were analyzed using the Nutrient Database System for Research (NDS-R) dietary analysis program. Paired t-tests, Pearson correlations and intraclass correlation coefficients (ICC) were used to assess the agreement between the two methods of dietary intake for calculated nutrient intakes. **Results:** The Pearson correlation coefficient for mean daily caloric intake between the two methods was 0.37 (P = 0.04) with a mean difference of 171 kcals/day (p = 0.34). The ICC was 0.38 (95% CI: 0.03 to 0.64) suggesting the within-patient variability may be larger than between-patient variability. Results for mean daily intake of total fat, total carbohydrate, total protein, retinol, vitamins D and E, thiamine, calcium, sodium, iron, selenium, copper, and zinc between the two assessment methods were also similar. **Conclusions:** This novel nutrient intake assessment tool provided quantitative nutrient intake data from TB patients. These pilot data can inform larger studies in similar populations.

Keywords: nutrition, diet, assessment, tuberculosis, micronutrient, macronutrient

Introduction

Tuberculosis (TB) is an enormous global health problem. In 2011, the World Health Organization (WHO) estimated that there were 8.7 million new cases of TB and 1.4 million deaths attributable to TB disease, with the overwhelming majority of cases occurring in low- and middle-income countries.[10] The country of Georgia, a former Soviet republic, has been designated by the World Health Organization (WHO) as a one of 27 high-burden countries for multidrug-resistant (MDR)-TB. The annual incidence rate of TB in Georgia exceeds 100 cases per 100,000.[102]

Malnutrition is a risk factor for the development of TB disease. The link between nutritional status and TB has long been appreciated, but remains an emerging area of study that has focused on investigations of related biomarkers and nutrient supplementation trials. A recent Cochrane review on the quality of evidence of trials on nutrient supplementation in TB concluded there is insufficient evidence to determine whether an increase in energy intake improves patient outcomes; further, rigorous research on the clinical impact of various strategies for micronutrient supplementation in patients with TB was found to be limited.[26] Surprisingly little data are available in the literature on habitual macronutrient and micronutrient intake in patients with TB. One study from Singapore focused on energy intake in TB patients using the 24-hour recall method.[81] In a pilot study in patients with pulmonary TB in Tbilisi, Georgia (using the nutrient intake assessment tool described in detail in this report), we estimated that vitamin D intake from diet was markedly lower than the Recommended Dietary Allowance (RDA) for this micronutrient, concomitant with a high prevalence of vitamin D insufficiency (low plasma 25 hydroxyvitamin D concentrations) in this patient population.[32]

Accurate dietary intake data is historically difficult to obtain and continues to be particularly problematic in subjects studied in the developing world due to lack of training

and resources, logistical issues and lack of validated nutrient content of certain food items. Several studies have been conducted involving the validity of self-administered food frequency questionnaires (FFQ) in various populations of patients without TB, but involve the validation of only a few macro- or micronutrients.[105-108,130-134] These studies used a variety of validating instruments, including nutrition-related biomarkers,[131] three day food records,[105] serial 24 hour recalls,[107] and use of food journal data in comparison to specific FFQs.[108,132-134] The study of Schroder et al, in a Spanish population, was the only investigation that validated both a FFQ and a structured 72-hour recall using three-day food records.[9]

The purpose of this study focused on the development of a novel instrument to serially estimate micronutrient and macronutrient intake data from a generally low income, non-English speaking, Georgian population via a structured interview process administered by trained personnel. We also sought to assess the validity of this structured 72-hour recall tool in a specific population—namely patients with pulmonary TB in Tbilisi, Georgia. The tool was developed as a component of a current double-blind, randomized, controlled study assessing the efficacy of high-dose vitamin D treatment to enhance *Mycobacterium tuberculosis* clearance in patients with pulmonary TB in Tbilisi, Georgia (clinicaltrials.gov identifier NCT00918086).[32]

Methods

Study Subjects

Subjects were recruited from the Georgian National Center for Tuberculosis and Lung Diseases (NCTBLD) and the Tbilisi Ftizio-Pulmonologic Center (an outpatient TB clinic) in Tbilisi, Georgia. The inclusion criteria included age $\geq$ 18 years, documented new case of smear-positive pulmonary TB, ≤ 1 week of anti-TB therapy, agreement to receive anti-TB therapy in Tbilisi, completion of the 72-hour recall instrument at baseline (week 1) and the serial 24-hour recalls during week 2, and a signed informed consent. Exclusion criteria included > 30 days of TB therapy, current pregnancy or lactation status, history of organ transplant, cancer during the previous 5 years, seizure disorder, cirrhosis, hypercalcemia, hyperparathyroidism, sarcoidosis, or nephrolithiasis, use of oral corticosteroids during the past 30 days, current use of cytotoxic or immunosuppressive drugs, current significant renal dysfunction (serum creatinine concentration >250 mmol/L), requirement for dialysis therapy, current incarceration, markedly elevated week 1 mean daily caloric intake (defined prehoc as mean daily caloric intake of > 6000 kcal/day) and inability to complete all study visits in Tbilisi. The Institutional Review Boards from Emory University in Atlanta, USA and the NCTBLD Ethics Committee in Tbilisi approved the study protocol. All subjects provided written informed consent in their native language for participation in the study.

Nutritional Assessment

The nutrition assessment instrument was developed to capture the mean daily micronutrient and macronutrient intake over the previous three-day period via face-to-face interviews by trained investigators. The dietary intake interviews were performed at baseline and again at the eight and sixteen-week time points of the randomized clinical trial (RCT). The instrument was designed prior to initiation of the RCT to assess nutrient intake in a low socioeconomic status, non-English speaking adult population. During the

instrument developmental phase, we initially explored typical foods and meal patterns of adult Georgians by face-to-face and email discussions between the Georgian- and United States (U.S.)-based investigators involved in the RCT. The instrument was designed to follow principles routinely utilized by nutritionists and dietitians in standardized food record intake forms. In addition, food items (including beverages and snacks) consumed commonly in Georgian culture and typical recipes for these were included in the questionnaire as prompts. For example, **Table 3.1** outlines details of the questions for typically consumed tea and soup, respectively**.** A free text comment section at the end of the questionnaire as added to allow for additional details regarding recipes.

The Georgian-based physician investigators were extensively trained prior to the initiation of the RCT by the registered dietitian investigator on the interview process via video training uploads (YouTube), demonstrations with mock face-to-face interviews, a comprehensive training DVD, and regular live training sessions via Skype. TRZ also conducted face-to-face training sessions with the Georgian investigators on the specific methodologies at the NCTBLD in Tbilisi during a study initiation visit prior to beginning the RCT. Standardized food models and common household measurement instruments were provided to the investigators in Tbilisi and used in the patient interviews to help to determine accurate serving sizes.

The Georgian language face-to-face interviews with TB patients were completed within 30-40 minutes at outpatient research visits; food and beverage intake during the previous three days was recalled and recorded in the case report form (CRF). The food intake data were then transcribed in English by the multilingual investigators into a web-based CRF for review in the Bionutrition Unit of the Atlanta Clinical and Translational Science Institute (ACTSI) by the U.S.-based research dietitian investigator. Review of intake data for individual subjects took place within 1-3 days after data entry in Tbilisi

since all subjects returned to the two outpatient TB clinics on a daily basis for directly observed anti-tuberculosis drug therapy and vitamin D or placebo administration per standard clinical care guidelines and the RCT protocol. Communications between the dietitian in Atlanta and the interviewer investigators in Tbilisi to clarify any questions regarding the specific food intake item entries were discussed via email or Skype telephone conferences in real time. As needed, the Tbilisi-based investigators then discussed the food items to be clarified with the specific study subjects in person during their daily visits. The clarified information was reported directly to the ACTSI via email or Skype calls during the Monday-Friday workweek.

Data were analyzed at the ACTSI using state-of-the-art dietary analysis software [Nutrition Data System for Research (NDSR), University of Minnesota, Minneapolis, MN]. Final calculations were completed using NDSR version 2011. The NDSR time-related database updates analytic data while maintaining nutrient profiles true to the version used for data collection. NDSR analyzes for specific quantities of over 160 different micronutrient and macronutrients and dietary compounds with well-described accuracy and completeness.[103] Mean daily intake was determined for each subject from the three-day food recall questionnaire. Specific methods were used to enter the Georgian food items into the NDSR software program, which was developed for foods commonly consumed in the U.S. First, the format of the structured three-day food recalls did not distinguish between different versions of the same food type. For example, beef was always entered as a trimmed sirloin if it was eaten on its own and as stew beef if consumed in a soup regardless of the cut of meat that was actually consumed. These assumptions were based on most common food servings given by the Georgian investigators during the development phase of the instrument. Some assumptions were also needed for food items that were specific to Georgian culture in order to find a similar item in the U.S.-based NDSR nutrient database. For example,

"matsoni", a concentrated yogurt food item, was entered as plain whole milk yogurt into the NDSR software. All milk intake was entered as a fresh whole milk to eliminate the extraneous micronutrients supplied by milk fortification in the U.S. captured by the NDSR database, but not present in the unfortified Georgian commercial milk supply.

Validation Methodology

The goal of the current study was to assess the validation of the nutrient intake assessment tool in a subset of the total 199 subjects entered into the full RCT. During the course of the ongoing RCT and prior to data collection for this report, two U.S.-based investigators conducted additional face-to-face training sessions with the Georgian interviewer-investigators in Tbilisi to standardize the conduct of typical 24 hour recalls. Expertise was validated by the registered dietitian investigator, who also gave guidance as needed throughout the validation data collection process. A convenience sample of 31 enrolled study subjects who completed both the three-day food recall questionnaire at baseline and were able to also complete three consecutive face-to-face 24-hr recalls during the following week. This proportion (31/199 or 16% of total study subjects) is in line with previous diet intake tool validation studies in which 10-20% of the total patient population was studied.[105-108] This study was a pilot study and therefore a *prehoc* sample size calculation was not performed.

A series of three standardized and conventional 24-hour recalls was conducted in the 31 pulmonary TB subjects during the week following their baseline visit, at which the current assessment tool was previously completed. We chose to focus only on subjects following their baseline visit to reduce the potential "learning effect" of serial face-to-face interviews to provide recent food intake data. This format was utilized to capture three days of food intake data via both 24-hour recall and by the RCT assessment tool and to evaluate the impact of recalling foods eaten two and three days prior to the interview in the RCT tool.

Statistical Analysis

The differences between the food questionnaire and food record recall of each outcome were summarized by the mean difference (questionnaire – recall), the standard deviation (SD) of the differences, and the 95% agreement limits.[109] The differences between the two measurements and their mean for each outcome were summarized by use of scatterplots (Bland-Altman plots). A 1-sample paired t-test was used to compare the mean differences between the food questionnaire and food record recall measurements. The intra-class correlation coefficient (ICC) was also used as a measure of agreement and was estimated by variance components based on statistical modeling as described by Bartko.[110] The ICC is large (i.e., near 1) when there is little within-participant variation. All statistical analyses were carried out using SAS software, Version 9.3. (Cary, NC, USA).

Results

A total of 31 subjects were included in this study; demographic information for these individuals is shown in **Table 3.2**. Nineteen subjects (61%) were male and about half were unemployed and/or of low socioeconomic status (income < 3000 Georgian lari/yr, or $1800 USD/yr). The mean and standard deviation of calculated daily intake of specific macronutrients and micronutrients (vitamins, minerals and trace elements) from both the 72-hour nutrient intake assessment tool and the paired three 24-hour recalls, with mean differences between the methods, the upper and lower 95% agreement limits and *P* values between the two methods are shown in **Table 3.3**. The *P* value reflects the 1-sample paired t-test between the means of the intakes for each nutrient estimated by the new nutrient intake instrument and the conventional 24-hour recalls. There were no significant differences between assessment methods for estimated mean daily intakes of total calories, total fat, total carbohydrate, total protein, retinol, vitamins D and E, thiamine, calcium, sodium, iron, selenium, copper, and zinc. In contrast, mean daily intake for vitamin C and potassium were overestimated by the nutrient intake instrument by approximately 43 and 22%, respectively, compared to the mean of the three 24-hour recalls (**Table 3.3**).

The ICC and Pearson R values for each nutrient contrasting the two intake assessment methods and the 95% confidence limits for these are illustrated in **Figure 3.1**. The Pearson correlation coefficient (R value) for mean daily caloric intake between the two dietary intake methods was 0.37 (P = 0.04). The mean difference for calories (nutrient intake tool minus 24 hour recall data) was 171 kcal/day and not significantly different from zero (p = 0.34). The ICC value was 0.38 (95% CI: 0.03 to 0.64) suggesting the within-patient variability may be larger than the between-patient variability (**Figure 3.1**). The ICC and Pearson R values for all nutrients ranged from 0.13 to 0.46 and thus showed good agreement between the two nutrient intake assessment methods.

Discussion

There are very limited data on evaluation and comparison of multi-day food recalls in resource limited countries, such as Georgia, especially in adult populations with specific disease states. The majority of such studies published to date, including the few from industrialized countries, are related to FFQs and not specifically to multi-day food recalls, as we have done in Tbilisi. Studies validating FFQs, including reports by Ogawa et al[132] from rural Japan and Pandey et al[133] from northern India, administered the FFQ on two separate occasions along with a three to five day food journal to compare nutrient intake to that calculated from the FFQs. These studies differed in the length of time between administering the validating questionnaires (e.g. from one month to one year later). In addition, there was no determined sample size in these reports which range from 23 participants in the northern Indian study[13] to 138 participants in the Danish study of Biltoft-Jensen et al.[14]

To our knowledge, our study provides the first such data from a patient population with TB and is also the first study from a former Soviet republic. Taken together, our data in this study provide confidence that the three-day food recall questionnaire developed to assess serial macronutrient and micronutrient nutrient intake from adults living in Tbilisi, Georgia has utility and relative accuracy for this purpose. A limitation of our study, despite the comprehensive data obtained from two methods, was the relatively small sample size. Larger studies in this and other Georgian adult populations will be necessary to accurately estimate interrater and intrarater reliability.

Collecting reasonably accurate food intake data from specific populations is a difficult problem due to language, logistical and cultural barriers, including the translation of meaningful information between native investigators working in a low- resource country to investigators in the data coordinating center in a developed country.[130,133] In our case, Georgia does not have a professional discipline of dietetics or clinical nutrition

in health professions education to help guide development of the tool we incorporated. Accurate food composition tables derived in Georgia are non-existent and compiling accurate tables is expensive, adding to the challenges of nutrient intake analysis. In this Georgia-U.S. collaboration, we utilized bilateral face-to-face instruction of the Georgian team of investigators with experienced U.S.-based clinical nutrition professionals and electronic tools in both the development of the data collection instrument and for methodological training prior to study initiation.

The steps we outline in this report could potentially be used to develop nutrient intake assessment tools in other low- or medium-resource countries without a developed capability for such studies. Low literacy populations may benefit from an interviewer process, as we incorporated here, whereas more educated populations may be able to self-report on a designated form that can be easily translated. A structured multiple-day recall format can be useful in remote populations where no previous method for nutrient intake exists. Utilizing classification techniques and dividing sample data into quartiles is one method to interpret such data. In our study, the interview method was developed to take advantage of the serial availability of subjects to the Georgian physician-investigators (who worked at the NCTBLD and were responsible for the TB care of the subjects) for daily directly observed anti-TB therapy per WHO protocols. We designed the nutrient intake assessment instrument questionnaire and study methods for a low-socioeconomic status and poorly educated patient population. The method we used took advantage of an extensive, complete and well-established U.S.-based nutrient analysis software resource (NDSR) given the lack of such a database from Georgia.

Our method (a hybrid between a conventional three-day dietary history and a face-to-face 24-hour recall method) may be superior to developing a new traditional FFQ in countries, such as Georgia, without a nutrient composition database of usual food items. This method allows the investigator to obtain information on several consecutive

days of food intake in a concise, structured manner and can be done serially throughout a study. Data can be easily analyzed and interpreted, using a resource such as NDSR for relatively quantitative nutrient intake information. Although developing new FFQs for a particular population may be an important tool, these may be difficult to design and analyze initially due to their complex nature.[108,132,133] For example, FFQ development requires previous data collection, often in the form of 24-hour recalls, in addition to cultural insight from in-country partners. FFQs are also considered semi-quantitative and nutrient values may not be directly related to outcomes.[108,132,133] The instrument described in this report is analyzed exactly the same as a food record and can be utilized similarly.

A strength of our specific nutrient intake instrument is that it provided a picture of the habitual diet of a patient population within a previously little-studied culture in terms of dietary intake. The major limitation to this study, in addition to our small sample size, is that our method is novel and therefore there is little reference for comparison of accuracy, particularly given the lack of habitual dietary intake data available in healthy adult Georgians. On the other hand, more conventional methods were felt to not be feasible in the population with TB that we studied. In summary, the novel nutrient intake assessment tool described here appeared to provide accurate quantitative nutrient intake data from TB patients in Georgia. These pilot data can be used to inform larger studies of nutrient intake in Georgians and also to further assess agreement between dietary intake assessment methods in this population. Further, the approach we used could potentially be a model for development of other culture-specific nutrient intake assessment tools in other countries.

Acknowledgments

Statement of Authorship

JKF carried out study training, participated in development of the tool, conducted nutrient data analyses, and drafted the manuscript. NT participated in development of the tool and contributed to overall study organization in Tbilisi. ES and MK carried out the study in Tbilisi. GH participated in the design of the study, including the case report form, and overall study coordination. KAE and NS performed the statistical analysis. UR helped to conceive the study design. HMB and VT helped to conceive the study design and participated in overall design and coordination. TRZ helped to conceive the study, participated in overall design, coordination and development of the tool, and contributed to drafting the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest Statement

Jennifer K. Frediani, Nestani Tukvadze, Ekaterina Sanikidze, Maia Kipiani, Gautam Hebbar, Kirk A. Easley, Neeta Shenvi, Usha Ramakrishnan, Vin Tangpricha, Henry M. Blumberg and Thomas R. Ziegler have no conflicts of interest.

Table 3.1. Example questions in Georgian food intake instrument

Q2. How many glasses of tea did you have? __ __ __ __number of glasses __ __ __ __ ml volume of each glass

If 0 glasses, skip to Question 3

Below are questions about what you added to the tea. Each question should be the amount added per glass (one glass)

Did you add sugar? Yes No

If yes, how much?

If the sample spoon is 15 ml, how many spoonfuls did you have? __ __number of spoonfuls __converted to ml

Did you add fruit syrup? Yes No

If yes, how much?

If the sample spoonfull is 15 ml, how many spoonfulls did you have? __ __number of spoonfulls __converted to ml

Q12. Soup " Borshi "

Did you have this in the last three days? Yes No **If no, Skip to the next dish**

How much did you have at one time ? __ __ __ __ __ ml

How many times have you had this recipe for dinner in the last three days? __ __ times

Below are typical ingredients in borshi. Please comment if there are any major differences in ingredients from what you eat. Yes No

1 kg Beef, 1 kg cabbage, 100g carrots, 200g red beetroot, 1 kg potatoes, 0.5 kg tomatoes, 300 g onion,30 g garlic,100 g of greens, sour cream 50-100 mg per serving black pepper, salt to taste

Table 3.2. Demographic characteristics

Characteristic	Total Sample (n=31)
Age mean(SD)	33 (11%)
% Male n(%)	19 (61%)
Ethnicity n(%)	
Georgian	29 (94%)
Education n(%)	
Secondary	11 (35%)
Some college or university	20 (65%)
Yearly Income n(%)	
(1000 lari = 478 euro or 604 USD)	
<1000 lari	10 (32%)
1000-3000 lari	9 (29%)
3001-10,000 lari	9 (29%)
10001-20000 lari	3 (10%)
Employment Status n(%)	
Employed	15 (48%)
Unemployed	16 (52%)
Marital Status n(%)	
Single/never married	15 (48%)

Table 3.3: Nutrient intake and agreement between nutrient intake assessment methods

Nutrients	24 hour recall Mean (SD)	Nutrient Intake Instrument Mean (SD)	Mean Difference Mean (SD)	LL Agreement, UL Agreement	P-value
Calories (kcal)	2983 (830)	3153 (940)	171 (990)	(-2288, 2489)	.34
Total Fat (g)	124 (51)	127 (57)	3 (56)	(-109, 115)	.77
Total Carbohydrate (g)	386 (93)	417 (102)	31 (115)	(-199, 262)	.14
Total Protein (g)	91 (37)	97 (35)	6(38)	(-71, 83)	.41
Retinol (mcg)	725 (812)	679 (836)	-47 (1086)	(-2220, 2126)	.81
Vitamin C (mg)	96 (63)	137 (76)	41 (92)	(-142, 225)	.02
Vitamin D (mcg)	3.2 (2.4)	5.1 (7.0)	1.9 (6.7)	(-11.5, 15.3)	.12
Vitamin E (mg)	9.8 (3.5)	11.4 (4.9)	1.5 (5.5)	(-9.5, 12.5)	.14
Thiamine (mg)	2.4 (0.7)	2.6 (0.6)	0.2 (0.7)	(-1.2, 1.6)	.23
Calcium (mg)	1221 (410)	1260 (446)	39 (487)	(-935, 1012)	.66
Sodium (mg)	3728 (1141)	4029 (1135)	301 (1246)	(-2192, 2794)	.19
Potassium (mg)	2925 (866)	3566 (1122)	641 (1208)	(-1776, 3057)	<.01
Iron (mg)	19.2 (5.1)	21.3 (5.9)	2.1 (6.7)	(-11.3, 15.5)	.09
Selenium (mcg)	142 (60)	143 (49)	1.3 (65)	(-129, 131)	.92

Copper (mg)	2.2 (1.3)	2.3 (1.2)	0.2 (1.6)	(-3, 3.4)	.54
Zinc (mg)	11.7 (4.1)	12.2 (4.0)	0.5 (4.8)	(-9.1, 10.1)	.57

LL= lower limit; UL= upper limit of 95% confidence intervals

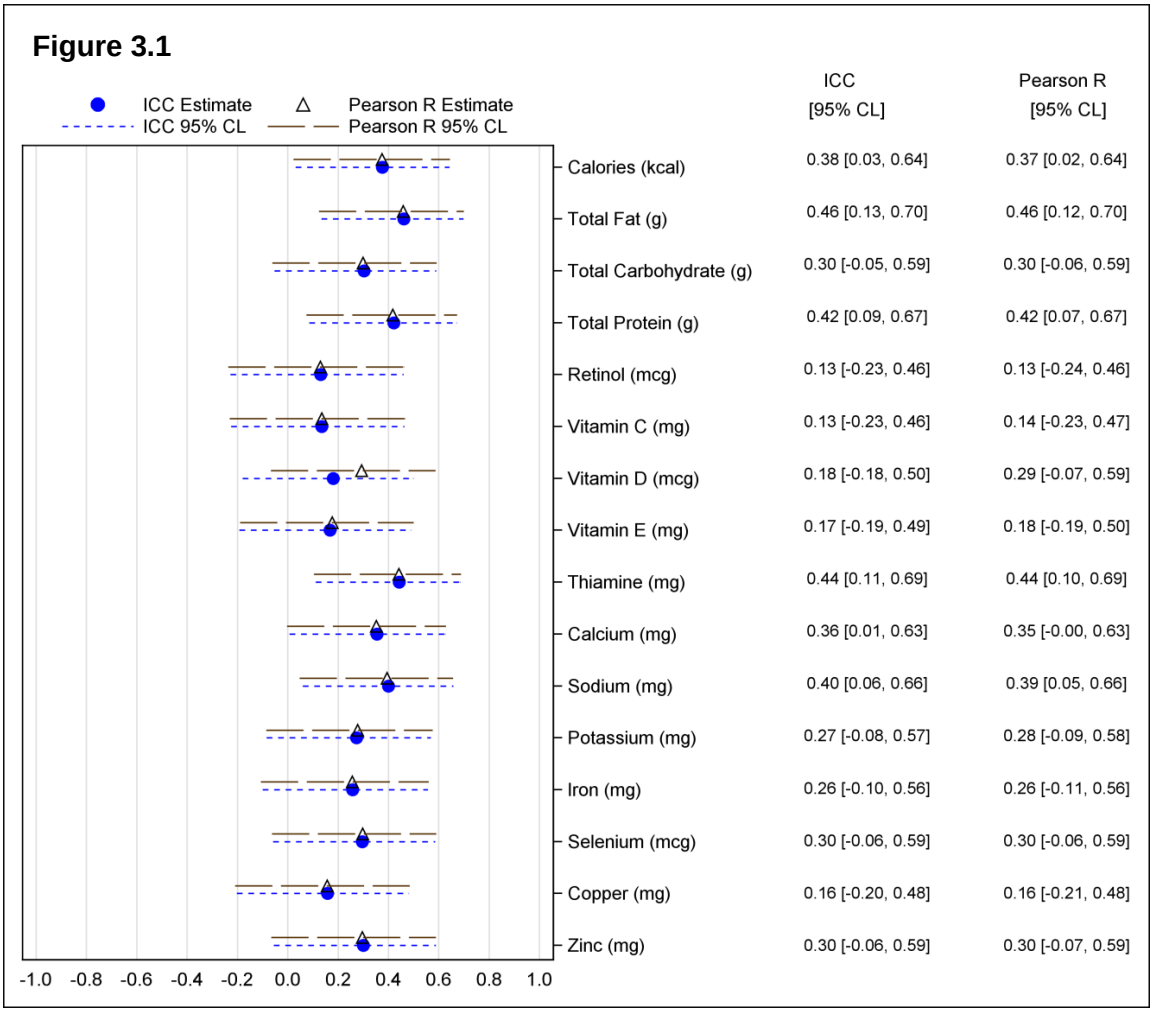

Figure 3.1. Intraclass correlation coefficient (ICC) and Pearson R estimate with 95% confidence levels for mean of three 24-hr dietary recalls compared to 72-hr questionnaire method

Chapter 4 - MACRONUTRIENT INTAKE AND BODY COMPOSITION OVER TIME IN ADULTS WITH PULMONARY TUBERCULOSIS DISEASE

Planned submission

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Running Title: Macronutrient Intake in Tuberculosis

Abbreviations:

TB – Tuberculosis

MDR-TB – Multidrug resistant tuberculosis

IU – International Units

BMI – Body mass index

BIA – Bioelectrical impedance analysis

Kcal – Kilocalories

CHO - Carbohydrates

Vit D – Cholecalciferol

Mtb – Mycobacterium tuberculosis

CRF – Case report form

AFB - Acid-fast bacilli

Abstract

Background: Malnutrition is common in patients with active tuberculosis (TB) disease, yet little information is available on habitual dietary intake or body composition changes over time. **Objective:** To evaluate macronutrient intake and body composition indexes in TB disease subjects over time. **Methods:** Subjects with pulmonary TB disease (n=191; 23 with MDR-TB) enrolled in a randomized clinical trial of high-dose cholecalciferol versus placebo were studied. Asymptomatic, sputum and culture negative household contacts (n=36) were studied for baseline comparison. In subjects with TB disease, food intake was obtained and macronutrient intake calculated at baseline, 8 and 16 weeks. Serial body composition was assessed by body mass index (BMI; kg/m^2) and bioelectrical impedance analysis (BIA). Descriptive statistics, repeated measures ANOVA for changes over time and linear regression for dietary intake and body composition comparisons were used. **Results:** At baseline, mean daily kilocalories (kcal), protein, fat and carbohydrate (CHO) intakes were significantly higher, and body weight, BMI, fat-free mass and fat mass were significantly lower, respectively between active TB subjects and controls. These remained significant after adjusting for age, gender, employment status and smoking. In all TB subjects, baseline mean daily intake of kcal, fat and protein were considered adequate according to US Dietary Reference Intakes and increased over time (time effect $p<0.0001$ for protein, treatment effect, NS). BMI increased over time (time effect $p<0.0001$, treatment effect, NS). There were no significant differences in % fat mass or % fat-free mass between drug-sensitive-TB and MDR-TB groups at baseline or over time. However, MDR-TB patients exhibited lower body weight and fat-free mass over time, despite similar daily intake of kcal, protein, and fat. Calorie intake and % calories from protein were compared to body weight, BMI and fat-free mass at baseline, weeks 8 and 16, but no nutritionally significant changes were

found. **Conclusions:** Macronutrient intake was higher in TB patients. Overall, as macronutrient intake of the TB subjects increased over time and there was a parallel increase in BMI, while body composition proportions were maintained. However, MDR-TB subjects demonstrated concomitantly decreased body weight and fat-free mass over time despite increased macronutrient intake. Thus, individuals with MDR-TB demonstrate a blunted anabolism in response to macronutrient intake, reflecting the catabolic nature of TB disease.

Introduction

Tuberculosis (TB) has long been associated with a decrease in body mass. The relationship between malnutrition and infection, especially in TB, is well established.[11,17-20] Evidence suggests that malnutrition, particularly low body mass index (BMI), can lead to secondary immune dysfunction that increases the host's susceptibility to infections.[17,19,20] Weight changes during treatment have been explored as a low-cost biomarker for both disease severity and treatment outcomes.[11,21] Further, it has been shown that those with low BMI (<18.5 kg/m^2) are at increased risk for TB-related mortality and treatment failure.[11,12,21] Despite the abundance of literature investigating the relationship between drug-sensitive TB and body composition, there are few studies exploring the relationship between nutrient intake and body composition in individuals with multi-drug resistant TB (MDR-TB) or in comparison to patients with drug-sensitive TB.

Macronutrients, especially protein and energy intake are critical factors involved in susceptibility to infection, but remain poorly studied in in HIV, TB, and malaria, the leading causes of infectious disease-related mortality worldwide.[17] Determination of accurate data regarding habitual dietary and nutrient intake has not been well characterized in patients with TB. The majority of studies in TB have measured body weight and BMI as a marker of nutrition status. While a few found nutritional supplementation may improve treatment outcomes in patients with TB,[16,81] a recent Cochrane review concluded there was not sufficient evidence to recommend general nutritional supplementation in patients with active TB.[26] Given the limited and inconclusive data, we sought to determine how macronutrient intake and body composition change during TB treatment and how time to appropriate treatment may play a role in pulmonary TB-associated wasting.

Methods

Study Subjects

Subjects were from a double blind, randomized, controlled, prospective trial of high-dose vitamin D_3 (cholecalciferol) treatment of patients with pulmonary TB disease (clinicaltrials.gov identifier NCT00918086). They were recruited between November 2009 and January 2011 from the Georgia National Center for Tuberculosis and Lung Diseases (NCTBLD) and an affiliated outpatient TB clinic in Tbilisi, Georgia.

Inclusion criteria were: 1) age ≥ 18 years; 2) newly diagnosed TB as determined by a positive AFB sputum smear and later confirmed by positive culture; 3) patient received ≤ 7 days of treatment with anti-TB drug therapy prior to entry; 4) subject has signed the informed consent. Exclusion criteria were: 1) patient has had previous diagnosis of TB disease, current extrapulmonary TB, requirement for TB surgery; 2) patient is currently pregnant or lactating or has a history of hypercalcemia, nephrolithiasis, hyperparathyroidism, sarcoidosis, organ transplant, hepatic cirrhosis, seizures, or cancer in the past 5 years; 3) patient has a serum creatinine concentration >250 mmol/L or requires renal replacement therapy; 4); patient required corticosteroid use in the past 30 days; 5) current use of cytotoxic or immunosuppressive drugs; and 7) current incarceration. TB subjects receiving vitamin D_3 (Vit D) were given 50,000 IU of oral vitamin D_3 weekly for 8 consecutive weeks, followed by 50,000 IU of vitamin D_3 every two weeks for 8 consecutive weeks, for a total dose of 1.4M IU vitamin D_3 during the 16-week period of study. The control TB disease group received an identical placebo capsule at the same time points as the Vit D group.

A group of 36 asymptomatic household contacts of the TB patients served as a non-TB disease control group. These individuals were AFB sputum smear and culture negative. They accompanied the TB patient to the clinic and were thus a convenience sample.

Sputum Culture and Drug Susceptibility Testing

Two sputum specimens were obtained from each TB suspect. Direct sputum smears were examined by light microscopy. All sputum samples were sent to the NRL for culture, using standard methodologies.[102] Drug susceptibility testing (DST) for first-line anti-TB drugs (isoniazid, rifampicin, ethambutol) and second-line drugs was done using absolute concentration method on solid media, as previously described.[135]

Nutritional and Body Composition Assessment

Dietary intake was documented from study participants at baseline, week 8 and week 16 of the clinical trial. A validated food/nutrient intake instrument which captures composition of specific foods and meal patterns common in Georgian culture was developed specifically for this TB patient population.[74] Trained study coordinators conducted one-on-one interviews using appropriate food model to determine all food intake of subjects in the three days prior to the specified study visits. After interviews were completed, data was entered into a web-based case report form (CRF) and sent to the US-based registered dietitian for review. Quantitated food intake data were subsequently analyzed using the Nutrition Data System for Research (NDSR) nutrient intake software (University of Minnesota, Minneapolis, MN). Final calculations were completed using NDSR version 2011. Mean daily intake of kilocalories, total protein, fat and carbohydrate were determined.

The body mass index [BMI; body weight (kg)/height (m^2)] was calculated in all subjects using data obtained from a calibrated research stadiometer and digital body weight scale system *(Tanita Inc;* Arlington Heights, Illinois, USA) at baseline and on weeks 4, 8, 12, and 16. Bioelectrical impedance analysis (Bioelectrical Impedance Analyzer, Model Quantam X: RJL Systems, Clinton Township, MI, USA) (BIA) was used to determine body composition; percent body fat mass and percent body fat-free mass.

Fat mass and fat-free mass were calculated using the kilogram weight of the subject at the respective time point.

Ethics Statement

This study was approved by the Institutional Review Board of Emory University (Atlanta, GA, USA) and the Georgian NCTBLD Ethics Committee (Tbilisi, Georgia). All subjects provided written informed consent for participation in the study.

Statistical Analysis

Subjects that exhibited a markedly elevated baseline mean daily caloric intake (defined *prehoc* as mean daily caloric intake of > 6000 kcal/day) were excluded from analysis. Descriptive statistics (Student's t-tests for continuous variables and chi-square tests and Fisher's exact tests for categorical variables) were used where appropriate. PROC GLM was used to build models to determine differences in macronutrient and body composition variables between TB disease subjects and household contacts. Two models were developed; model I adjusted for age and gender, while model II adjusted for age, gender, employment status and smoking. We also tested for interactions with gender. Repeated measures analysis of variance was used to evaluate time and treatment group effects within the body composition and macronutrient variables. TB subjects were dichotomized by both gender and drug susceptibility to assess changes between those with and without MDR-TB. Time to appropriate treatment in MDR-TB subjects was defined as correct treatment within 56 days from the study baseline visit. All statistics were completed using SAS software, Version 9.3. (Cary, NC, USA) and a *P*-value of <0.05 was determined significant. Linear regression was used to summarize the linear relationship between the outcome and body composition variable by estimating the regression coefficients. A t-test was used to determine whether the estimated slope differed from zero. The coefficient of determination (R^2) was used to assess how much of the variation in outcome was accounted for by the body

composition variable. Analysis of covariance was used to estimate and compare adjusted mean calories by MDR status.

Results

Comparison of TB Disease Subjects with Household Contacts

The demographic characteristics of 191 subjects with newly diagnosed pulmonary TB disease and 36 household contacts are shown in **Table 4.1**. The TB disease subjects' mean age was 34 years, 64% were male, 47% were currently unemployed and individual annual income for over 75% was less than 3,000 USD annually. A significant proportion of TB disease subjects were current tobacco smokers. The household contact cohort was older than the TB disease subjects (by 5 years on average), smoked less, and had modestly higher employment status but similar individual annual income (**Table 4.1**). These two groups were also stratified by gender (**Table 4.2**). Comparison by gender showed that men were older and more likely to smoke in both groups.

Subjects with TB disease consumed significantly greater amounts of total calories, protein, fat and CHO than the household contact group during the three days prior to the baseline study visit (**Table 4.3**). This was reflected in the overall significantly higher macronutrient intake of the TB disease cohort relative to the household contact group (**Table 4.3**). Total energy intake (kcal/kg/day) was 17.6% higher in the TB disease subjects than the control group, while protein, total fat and CHO intake were 28.5%, 31.6%, and 32.1% higher than household contacts, respectively. Due to differences in demographics between TB patients and household contacts (**Table 4.1**), multiple models were developed. The final model adjusted for age, gender, employment status, and smoking and all macronutrient variables remained significant (calories- P=0.008; protein- P=0.04; fat-P=0.02; and CHO- P=0.001). Tests for interaction for gender were not statistically significant. However, gender was kept in the second model due to gender differences often seen in dietary intake.

Despite higher macronutrient dietary intake, subjects with TB disease demonstrated significantly lower body weight (18.6% lower), BMI (24.4% lower) and

particularly fat mass (61.2% lower in grams; 41.5% as percentage of total mass); fat-free mass (1.4% higher in grams; 14.9% as a percentage of total mass) was similar between the groups (**Table 4.3**). These comparisons remained significant after adjusting for differences in age, gender, smoking and employment status.

There were no differences in either macronutrient intake or the body composition indexes among TB disease patients who received high-dose vitamin D_3 compared to those receiving placebo (**Table 4.4**). Therefore, all TB subject data were combined for multivariate analyses. Protein intake significantly increased over the course of the 16-week study in this TB disease cohort (p=0.030), whereas CHO intake remained stable (**Figure 4.1**). Intake of dietary calories and fat tended to increase over the 16-week study, but this was not statistically significant (**Figure 4.1**). As shown in **Figure 4.2,** body weight, BMI, and fat-free mass significantly increased in the TB disease subjects over time (all p<0.0001), while fat mass remained relatively constant.

To explore the impact of MDR-TB on the study endpoints, subjects with TB disease were dichotomized as a function of drug susceptibility (drug susceptible-TB n=169; MDR-TB n=23) and dietary macronutrient intake and body composition indexes were compared over time. Dietary protein intake was significantly greater over time (p=0.0042), but there was no significant effect of drug susceptibility on this response (**Figure 4.3**). Total intake of calories and CHO tended to be greater in MDR-TB (not significant), while fat intake was similar over time. Overall, there was a clear pattern for the two TB subject cohorts to consume more dietary kcal, protein, fat and CHO over time, which was most evident in the MDR-TB subjects at week 16 for (kcal and CHO)(**Figure 4.3**). We also dichotomized the MDR-TB group by time to correct treatment regimen (<56 days n=11; >56 days n=12) and found no differences between groups at any time point or over time.

There was a significant effect for time for body weight, BMI and fat-free mass to increase in TB disease subjects, but this effect on both body weight and fat-free mass was significantly blunted in MDR-TB compared to drug-susceptible (**Figure 4.4**). Fat mass was not significantly altered over time or as a function of drug susceptibility. The fat-free mass data also showed a significant interaction effect of MDR-TB status and time ($p = 0.044$). Therefore, pairwise comparisons were presented over time between the two TB groups at each time point. In the drug-susceptible TB subjects, there was a significant difference from baseline (increased fat-free mass) at weeks 12 and 16 ($p=0.0009$ and $p<0.0001$, respectively) and between week 8 and 16 ($p=0.044$). In contrast, fat-free mass did not change over time in the MDR-TB patients. In addition, fat-free mass was significantly lower in the MDR-TB cohort than the drug-susceptible cohort at both week 4 and week 16 ($p=0.046$) (**Figure 4.4**).

Linear regression methods were used to compare outcomes (calorie intake and % calories from protein) with several body composition variables at baseline, week 8 and week 16. (**Table 4.5**) When comparing calorie intake with BMI at baseline among all TB patients, the mean change in calorie intake was 2.03 kcal/kg/day per 1 unit increase in BMI (1 unit=5 kg/m^2) and this negative slope is statistically different from zero ($P <0.001$). This relationship was similar for males versus females. This change declined at weeks 8 and 16, but remained statistically significant. Comparisons between calorie intake and both body weight and fat-free mass were similar. The comparison between calorie intake and fat-free mass showed a lower change at baseline (-0.56 kcal/kg/day per unit of fat-free mass (1 unit= 10 kg) and continued to decline by time point. We stratified by drug susceptibility for the comparisons between calorie intake and % calories from protein with fat-free mass and there was a significant difference (P=0.04) between the groups when calorie intake was compared to fat-free mass. At baseline, calorie intake decreased as fat-free mass increased for the drug-sensitive subjects

(Pearson R=-0.3; r-square=0.09; slope=-0.66 (0.16); *P*<0.001), while calorie intake increased as fat-free mass increased for those with MDR-TB (Pearson R=0.2; r-square=0.05; slope=0.61 (SE- 0.61); *P*= 0.33) and these two slopes were significantly different (*P*=0.04). (**Figure 4.5**) When % calories from protein were compared to fat-free mass we did not see differences by MDR status.

Discussion

In this study, we provide data describing dietary intake and body composition changes over the initial 16-week period in patients receiving anti-TB therapy, with or without high-dose vitamin D_3. As macronutrient intake of the TB subjects increased over time, there was a parallel increase in BMI, while body composition proportions were maintained after repeated measures ANOVA. However, MDR-TB subjects demonstrated concomitantly decreased body weight and fat-free mass over time despite increased macronutrient intake. Thus, individuals with MDR-TB demonstrate a blunted anabolism in response to macronutrient intake, reflecting the catabolic nature of TB disease.

Body weight and BMI have been widely studied within the scope of TB disease.[11-16] However, our study adds to the limited data on serial body composition changes during the early phase after pulmonary TB diagnosis. The baseline body composition data show that, despite the increased caloric and macronutrient intake and after adjusting for gender, age, employment status and smoking, adults with recently diagnosed TB disease have significantly lower body weight and BMI, and markedly lower body fat and fat-free mass compared to asymptomatic adults from the same community. This suggests that the catabolic effects of newly diagnosed TB disease is primarily reflected by loss of body fat, and suggests that enhanced lipolysis occurs early in the disease course. The serial macronutrient intake and body composition data (**Figures 4.1 and 4.2**) show that body weight, BMI and fat-free mass all rose over time as patients are treated with anti-TB drugs, concomitant with an increase in dietary protein and tendencies for calorie and fat intake to rise. Fat mass rose to a lesser extent (NS), perhaps reflecting ongoing lipolysis during the 16-week period.

Fat-free mass is largely composed of lean tissue and primarily skeletal muscle, and may have been maintained in these individuals by the concomitant increase in

dietary calories and protein sources. Studies that measure lean body mass composition directly (e.g. dual energy x-ray absorptiometry),[136] skeletal muscle function,[137] metabolomics profiles[78] and protein and fat kinetic studies using stable isotopes[138] over time would be of interest to better define body composition changes, functional consequences and macronutrient metabolism in TB disease.

Our study is the first on nutritional status of patients with TB disease in Georgia, which is also a low-burden country with respect to concomitant HIV and TB infection.[10,135,139] Thus, our data may not be generalizable to TB patient populations with a high burden of HIV co-infection. Only 24% of our sample was underweight (BMI < 18.5 kg/m^2), which is less than has been found in TB cohorts from other lower-middle income countries.[14,15,140] In the country of Georgia, food is plentiful and relatively inexpensive therefore wasting due to food insecurity is relatively uncommon compared to other parts of the world, and this is confirmed by the overall increase in food/nutrient intake over time in our cohort.

A recent Cochrane review of 23 trials, five of which evaluated macronutrient intake in a cross-sectional manner, determined there was inconclusive evidence for the efficacy or effectiveness of supplementation with whole foods to improve outcomes in patients with TB disease.[26] In a recent cross-sectional study, evaluating nutrient intake in women with concomitant TB/HIV versus controls in Uganda, Mupere and colleagues performed a single 24-hour dietary recall and measured BMI, lean body mass and fat mass. They concluded lower acute energy and protein intake was associated with worsened body wasting (determined by BMI) and TB disease severity (determined by TB Score), and suggested that wasting was not a determinant of nutrient intake but a result of the inflammatory process and its associated anorexia.[20] Our study shows that in the full cohort, body weight, BMI and fat-free mass increased over time with largely unchanged fat mass (from a low baseline percentage of body fat). This occurred in association with

a significant increase in dietary protein and more modest increases in calories and fat over time. Anecdotal information obtained by the investigators via discussions with TB disease subjects and their caregivers revealed that with initial symptoms of illness consistent with active infection and/or following formal diagnosis, subjects with pulmonary TB are encouraged by physicians and household members to consume more food in general. Whether fat-free mass/lean body mass or fat mass can be increased further in this population with supplementation with either certain foods or defined nutrient products (e.g. to provide additional high-biological value protein sources and/or calories from fat and CHO sources) would be of interest.

Georgia is a lower-middle income country that has been designated by the WHO as a high-burden country for MDR-TB.[10,135,141] Previous studies have not analyzed dietary intake or body composition in patients with MDR-TB over time. To our knowledge, the current study also provides the first data comparing macronutrient intake and body composition over time in patients with MDR-TB disease in comparison to patients with drug-sensitive TB disease. Of interest, Gler et al indicated that weight gain was associated with better response to MDR-TB treatment.[21] Despite a strong trend toward increased mean daily intake of calories, particularly as CHO sources over time in the MDR-TB subjects versus drug-sensitive subjects, these individuals gained significantly less body weight and fat-free mass. These data suggest that MDR-TB patients were more catabolic than the drug- sensitive TB patients, but the reason(s) underlying the evident blunted response to macronutrient intake are unclear. After analysis of covariance (**Table 4.6 and Figure 4.5**), there was a very small change in calorie intake (0.5 kcal/kg/day) per unit of fat-free mass (10 kg), but such minor changes are not nutritionally significant. We were limited by sample size when dichotomizing by drug susceptibility and this may have contributed to the lack of meaningful differences between these two TB disease cohorts. Further longitudinal regression analysis would

help explain the relationship between dietary intake and body composition over time. Larger studies focused on MDR-TB as compared to drug- sensitive TB disease patients are needed to confirm our data and further define the body composition responses. Studies which serially measure energy expenditure (e.g. via indirect calorimetry), macronutrient metabolic pathways (e.g. via metabolomics profiling of plasma),[78] blood concentrations of pro-inflammatory markers and other indices of a catabolic response,[142] and lean body mass (e.g. by dual energy x-ray absorptiometry) in MDR-TB compared to drug-sensitive TB disease patients would be of interest. It would also be helpful to study this relationship over a longer period of time.

Our study has several limitations. Despite the overall large sample size, our study contained a small cohort of patients with MDR-TB. Our cohort also demonstrated a relatively low rate of frank generalized malnutrition (BMI < 18.5 kg/m^2) and had a low rate of HIV-co-infection. Thus, our results cannot be generalized to TB disease populations with higher rates of malnutrition, food insecurity and HIV co-infection. There was a 14% loss to follow-up in our clinical trial; however, this is similar to other studies conducted in this part of the world. A systematic review published by the World Health Organization found an average 13% lost to follow-up rate for TB studies conducted in Asia.[143] There were no significant differences in demographic characteristics between MDR-TB and drug-sensitive TB at any time points. Thus, differences seen at week 16 in **Figures 4.3 and 4.4** are likely due to the differences in *Mtb* drug resistance among these individuals. Although our dietary intake data was obtained from a validated tool,[74] self-reported intake is inherently prone to bias. Another limitation of our study is that we did not assess long-term relapse or cure rates and had only two deaths in the short 16-week study period. Longer-term follow-up of our cohort is in progress to address whether the body composition measures and dietary intake we determined during the

first eight weeks of anti-TB drug therapy are associated with rates of disease relapse and mortality.

In conclusion, patients with recently diagnosed pulmonary TB disease in the country of Georgia consume greater amounts of calories, protein, CHO and fat than asymptomatic controls without evidence of TB disease. Despite this food consumption behavior, TB disease patients exhibit lower body weight and BMI, which appears to be largely explained by loss of body fat. Serial data over 16 weeks shows a general modest increase in macronutrient intake in TB disease patients which, in association with anti-TB drug therapy, is linked with significantly increased body weight, BMI and fat-free mass and maintenance of body fat and body composition proportions in the overall cohort. However, our data show for the first time that this anabolic response is blunted in individuals with MDR-TB, despite presumably adequate macronutrient intake per kilogram body weight. This strongly suggests that anabolism is less efficient in MDR-TB, likely due to ongoing catabolic responses with poorly controlled TB disease.

Table 4.1. Subject Demographics

Characteristic	TB Disease Subjects (n=191)	Household Contacts (n=36)	P-value
Age [mean(SD)]	36 (12)	41 (14)	<0.01
Male Gender	123 (64%)	12 (33%)	<0.01
Ethnicity			
Georgian	176 (92%)	33 (92%)	0.37
Other	16 (8%)	3 (8%)	
Education			
Secondary or less	83 (43%)	11 (30%)	0.13
College	28 (15%)	10 (28%)	
University	81 (42%)	15 (42%)	
Employment Status			
Employed	78 (41%)	17 (47%)	<0.01
Unemployed	93 (47%)	11 (31%)	
Other	21 (12%)	8 (22%)	
Annual Income			
<1000 lari (~600USD)	55 (28%)	9 (25%)	0.97
1000-5000 lari	92 (48%)	18 (50%)	
5001-10,000 lari	40 (21%)	8 (22%)	
>10,000 lari	5 (3%)	1 (3%)	
Marital Status			
Single	83 (43%)	4 (11%)	<0.01

Married	90 (47%)	31 (86%)	
Divorced/Widow	19 (10%)	1 (3%)	
Current Smoker	137 (71%)	14 (39%)	<0.01

Table 4.2. Demographics between TB Subjects and Household Contacts Stratified by Gender

	TB subjects			Household contacts		
Characteristic	**Male (n=124)**	**Female (n=68)**	***P*-value**	**Male (n=12)**	**Female (n=24)**	***P*-value**
Age [mean(SD)]	38 (13)	33 (8)	<0.01	35 (10)	45 (15)	<0.01
Ethnicity						
Georgian	114 (92%)	62 (91%)	0.29	11 (92%)	22 (92%)	0.41
Other	10 (8%)	6 (9%)		1 (8%)	2 (8%)	
Education						
Secondary or less	60 (48%)	23 (34%)	0.16	5 (42%)	6 (25%)	0.34
College	16 (13%)	10 (15%)		4 (33%)	6 (25%)	
University	46 (37%)	35 (51%)		3 (25%)	12 (50%)	
Employment Status						
Employed	48 (39%)	30 (44%)	<0.01	8 (67%)	9 (38%)	0.12
Unemployed	69 (56%)	24 (35%)		4 (33%)	7 (29%)	
Other	7 (5%)	14 (21%)		0 (0%)	8 (33%)	

Annual Income						
<1000 lari (~600USD)	35 (28%)	20 (29%)	0.36	2 (17%)	7 (29%)	0.93
1000-5000 lari	65 (52%)	27 (40%)		7 (58%)	11 (46%)	
5001-10,000 lari	21 (17%)	19 (28%)		3 (25%)	5 (21%)	
>10,000 lari	3 (3%)	2 (3%)		0 (0%)	1 (4%)	
Marital Status						
Single	55 (44%)	28 (41%)	0.64	2 (17%)	2 (8%)	0.73
Married	59 (48%)	31 (46%)		10 (83%)	21 (88%)	
Divorced/Widow	10 (8%)	9 (13%)		0 (0%)	1 (4%)	
Smoker	109 (88%)	28 (41%)	<0.01	8 (67%)	6 (25%)	0.03

Table 4.3. Dietary Macronutrient Intake and Body Composition: Comparisons between Subjects with TB Disease and Household Contacts

Characteristic	TB Disease Subjects	Household Contacts	P-value	Model I* Adjusted P-value	Model II** Adjusted P-value
Total Calories (kcal/kg/day)	56.2 (23.2)	41.7 (15.0)	<0.01	<0.01	<0.01
Protein (g/kg/day)	1.6 (0.73)	1.2 (0.48)	<0.01	0.02	0.04
Total Fat (g/kg/day)	2.2 (1.2)	1.6 (0.66)	<0.01	0.02	0.02
Carbohydrate (g/kg/day)	7.6 (3.2)	5.5 (2.0)	<0.01	<0.01	<0.01
Body Composition					
Body Weight (kg)	62.4 (12.2)	75.2 (17.5)	<0.01	<0.01	<0.01
BMI (kg/m^2)	20.9 (3.6)	26.7 (6.0)	<0.01	<0.01	<0.01
Fat-Free Mass (kg / % of total kg body weight)	48.8 (10.2) / 79 (8)	49.5 (12.5) / 68 (9)	0.73 / <0.01	<0.01/ <0.01	0.02/ <0.01
Fat Mass (kg / % of total kg body weight)	13.6 (8.6) / 21 (8)	25.6 (11.1) / 32 (9)	<0.01 /<0.01	<0.01/ <0.01	<0.01/ <0.01

*Model I adjusted for age and gender

**Model II adjusted for age, gender, employment status and smoking

Table 4.4. Dietary Macronutrient Intake and Body Composition Comparisons between Vitamin D and Placebo Treatment Groups of TB Disease Subjects

Characteristic	Vitamin D	Placebo	P-value
Total Calories (kcal/kg/day)*			
Baseline	58 (25)	54 (22)	0.25
Week 8	61 (23)	56 (21)	0.16
Week 16	61 (22)	57 (20)	0.24
Protein (g/kg/day)*			
Baseline	1.7 (0.75)	1.6 (0.71)	0.27
Week 8	1.8 (0.79)	1.7 (0.69)	0.28
Week 16	1.8 (0.64)	1.8 (0.64)	0.57
Total Fat (g/kg/day)*			
Baseline	2.3 (1.3)	2.2 (1.1)	0.44
Week 8	2.4 (1.1)	2.3 (1.0)	0.35
Week 16	2.5 (1.1)	2.3 (1.0)	0.24
Carbohydrate (g/kg/day)*			
Baseline	7.9 (3.3)	7.4 (6.8)	0.23
Week 8	8.2 (3.2)	7.4 (2.7)	0.12
Week 16	7.9 (2.8)	7.4 (2.6)	0.28
Body Composition			
Body Weight (kg)**			
Baseline	62.5 (11.1)	62.4 (13.3)	0.99
Week 8	64.5 (12.0)	65.2 (13.1)	0.74

Week 16	65.3 (12.2)	66.1 (13.8)	0.72
BMI (kg/m^2)**			
Baseline	21.0 (3.6)	20.8 (3.6)	0.65
Week 8	21.8 (3.9)	21.9 (3.7)	0.90
Week 16	22.1 (4.0)	22.1 (3.8)	0.93
Fat-Free Mass (kg / % of total kg body weight)**			
Baseline	49.4 (9.5) / 79 (8)	48.3 (11.0) / 79 (9)	0.45 / 0.90
Week 8	51.0 (10.2) / 79 (8)	49.1 (12.5) / 78 (8)	0.30 / 0.60
Week 16	51.3 (10.1) / 79 (8)	51.5 (10.7) / 78 (8)	0.89 / 0.75
Fat Mass (kg / % of total kg body weight)**			
Baseline	13.1 (6.1) / 21 (8)	14.2 (10.5) / 21 (8)	0.38 / 0.91
Week 8	13.6 (6.3) / 21 (8)	16.1 (11.8) / 22 (8)	0.10 / 0.59
Week 16	14.1 (7.0) / 21 (8)	14.6 (8.1) / 22 (8)	0.67 / 0.75

*Sample sizes for the determination of specific dietary intake characteristics at each time point varied: Baseline; Vit D=97; Placebo=94; Week 8; Vit D=79; Placebo=81; Week 16; Vit D=71; Placebo=75. Mean (SD).

**Sample sizes for the determination of specific body composition characteristics at each time point varied: Baseline; Vit D=97; Placebo=94; Week 8; Vit D=81; Placebo=83; Week 16; Vit D=79; Placebo=83. Mean (SD).

Table 4.5. Changes in Calorie Intake for Body Weight and Body Composition Variables by Visit

Body Composition Variable	Slopes and Standard Errors by Visit		
	Baseline	**Week 8**	**Week 16**
Body Weight (kg)	-0.76 ±0.13*	-0.47 ±0.14*	-0.58 ±0.12*
BMI (kg/m^2)	-2.03 ±0.45*	-1.30 ±0.47*	-1.55 ±0.42*
Fat-Free Mass (kg)	-0.56 ±0.16*	-0.26 ±0.15	-0.44 ±0.16*

*P-value (significantly different from zero) ≤ 0.01

Figure 4.1

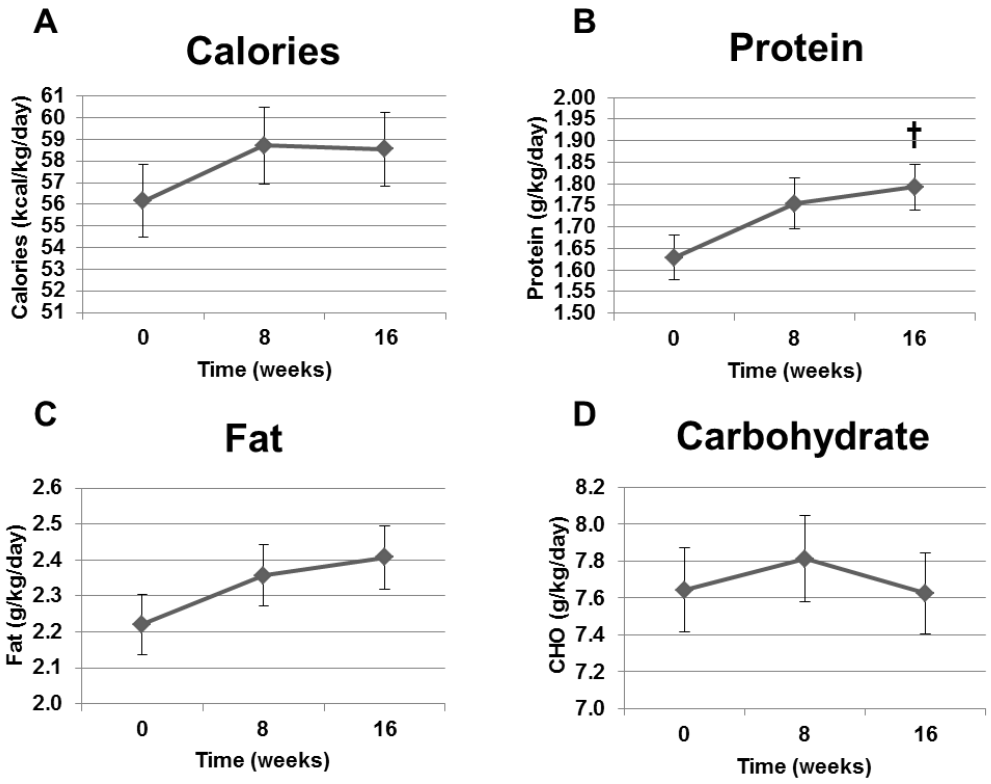

Figure 4.1. Mean daily calorie, protein, fat and carbohydrate intake in TB disease patients over time. Panel A: Calories (kcal/kg/day). Panel B: Total protein (g/kg/day). Panel C: total fat (g/kg/day). Panel D: Total carbohydrate (g/kg/day). Two-factor mixed-model repeated-measures analysis of variance was used for statistical analysis. Sample sizes were 191, 160, and 146 for time points 0, 8, and 16 weeks, respectively. Data as mean ± SEM. † Significant effect of time (p=0.030). CHO=carbohydrate.

Figure 4.2

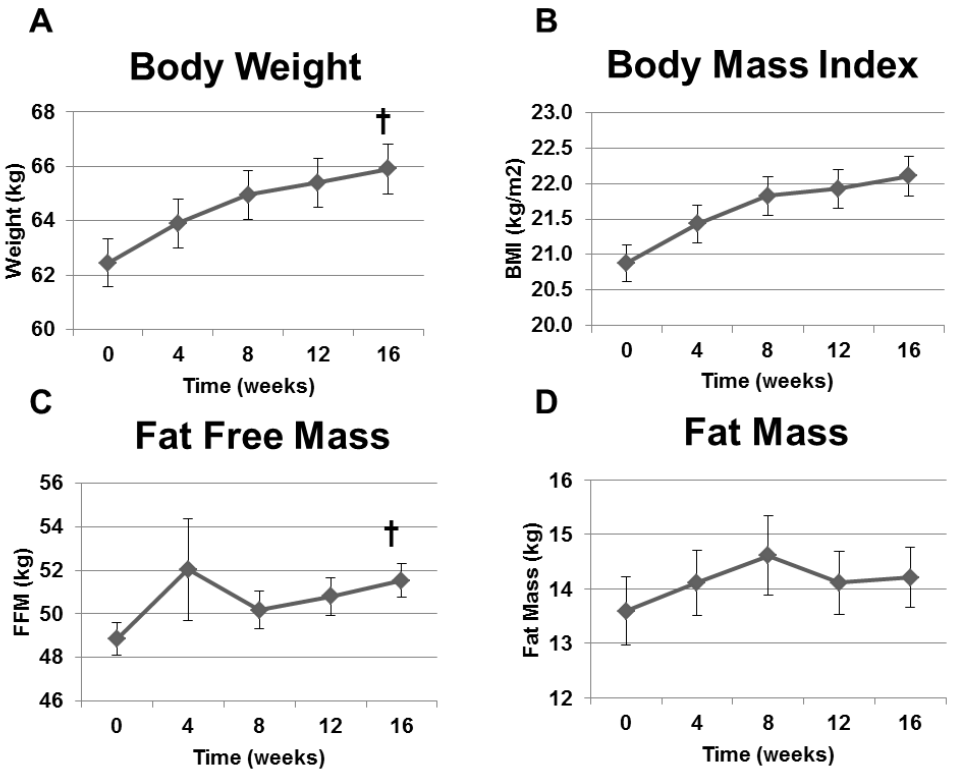

Figure 4.2. Body composition indexes in TB disease patients over time. Panel A: Body weight (kg, p<0.0001). Panel B: BMI (kg/m^2, *p*<0.0001). Panel C: Fat-free mass (kg, p<0.0001). Panel D: Fat mass (kg, NS). Two-factor mixed-model repeated-measures analysis of variance was used for statistical analysis. Sample sizes were 191, 170, 165, 145, and 162 for time points 0, 4, 8, 12, and 16 weeks, respectively. Data as mean ± SEM. † Significant effect of time. BMI=body mass index; kg=kilograms; NS= not significant.

Figure 4.3

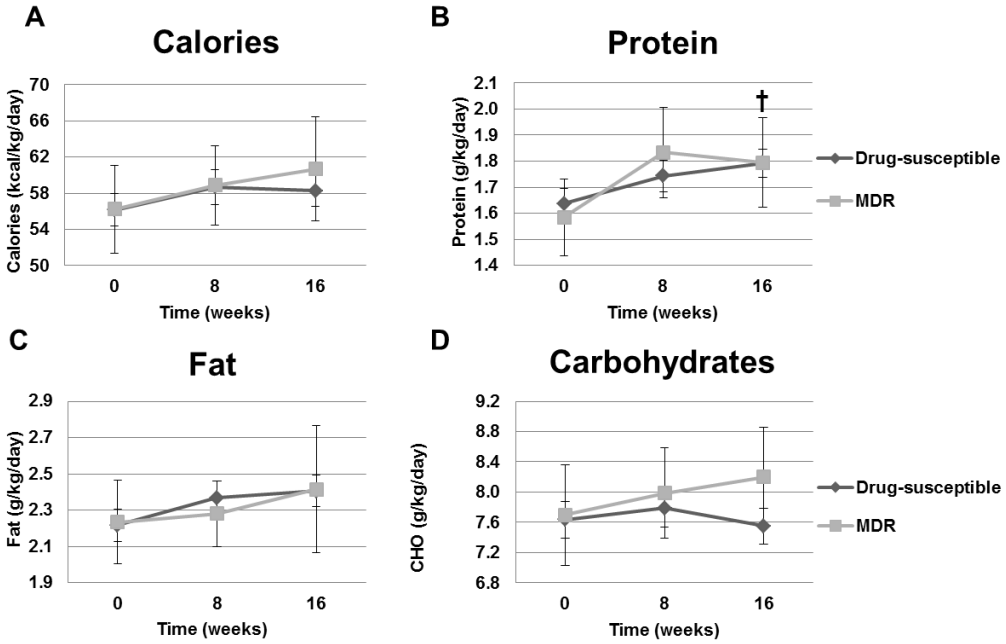

Figure 4.3. Mean daily calorie, protein, fat and carbohydrate intake in TB disease patients as a function of drug susceptibility over time. Panel A: Calories (kcal/kg/day). Panel B: Total protein (g/kg/day, p=0.0042 effect of time). Panel C: total fat (g/kg/day). Panel D: Total carbohydrate (g/kg/day). Two-factor mixed-model repeated-measures analysis of variance was used for statistical analysis. Sample sizes in the drug-susceptible group were 168, 143, and 132 for time points 0, 8, and 16 weeks, respectively; sample sizes for the MDR-TB group were 23, 17, and 14 for time points 0, 8, and 16 weeks, respectively. † Significant effect of time. MDR-TB= Multi-drug resistant-TB.

Figure 4.4

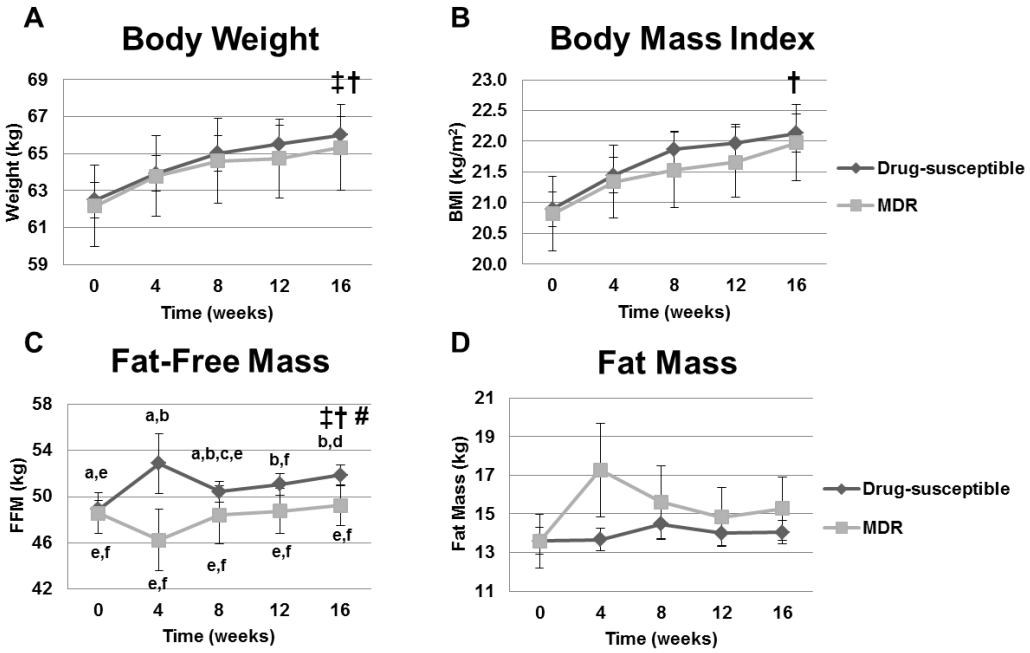

Figure 4.4. Body composition indexes in TB disease patients as a function of drug susceptibility over time. Panel A: Body weight (kg, $p<0.0001$ effect of time, $p= 0.048$ effect of drug susceptibility. Panel B: BMI (kg/m^2, $p<0.0001$ effect of time). Panel C: Fat-free mass (kg, $p =0.0502$ effect of time, $p =0.0070$ effect of drug susceptibility and $p= 0.0249$ for interaction). Given the interaction effect, pairwise comparisons were made over time between the two TB groups at each time point for fat-free mass. Values for fat-free mass at individual time points within and between the two drug-susceptibility groups that do not share the same letters are significantly different. Panel D: Fat mass (kg, NS). Two-factor mixed-model repeated-measures analysis of variance was used for statistical analysis. Sample sizes for drug susceptible group were 168, 153, 147, 131, and 145 for

time points 0, 4, 8, 12, and 16 weeks, respectively; sample sizes for the MDR-TB group were 23, 17, 18, 14, and 17 for time points 0, 4, 8, 12, and 16 weeks, respectively. † Significant effect of time, ‡ Significant effect of drug-susceptibility,# significant interaction between time and drug-susceptibility.

Figure 4.5.

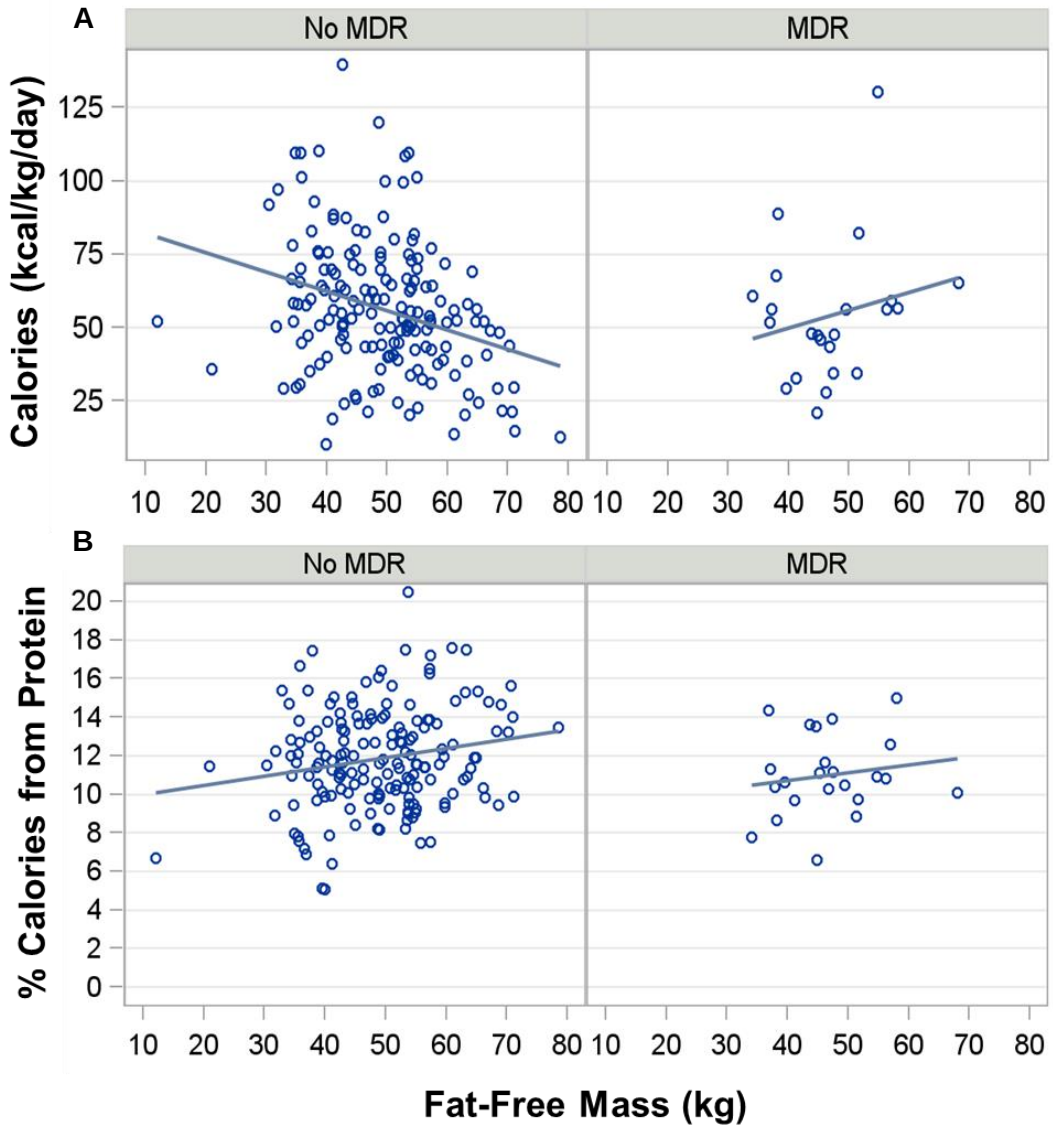

Figure 4.5 Comparison of the associations between a) calorie intake and b) % calories from protein by fat-free mass (kg) in TB patients by MDR status. Calorie intake (kcal/kg/day): MDR (n=23): Pearson R= 0.21, r-square= 0.05, slope= 0.61 (SE 0.61), *P*-value= 0.33. No MDR (n=168): Pearson R= -0.30, r-square= 0.09, slope= -0.66 (SE 0.16), *P*-value<0.0001. *P*-value for comparing 2 slopes=0.04. % Calories from protein: MDR (n=23): Pearson R= 0.19, r-square= 0.02, slope= 0.04 (SE 0.06), *P*-value= 0.48.

No MDR (n=168): Pearson R= 0.19, r-square= 0.04, slope= 0.05 (SE 0.02), *P*-value= 0.014. *P*-value for comparing 2 slopes=0.90.

CHAPTER 5: PLASMA METABOLOMICS OF HUMAN PULMONARY TUBERCULOSIS

Planned submission

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Key words: Metabolomics, *Mycobacterium tuberculosis*, Mycolate, Resolvins, Tuberculosis

Abstract

Background: Characterization of metabolites during tuberculosis (TB) disease may identify new pathophysiologic pathways involved in infection as well as biomarkers of TB onset, progression and resolution. Such data may inform development of new anti-tuberculosis drugs. **Methods:** Plasma samples from adults with newly diagnosed pulmonary TB disease (within 7 days of anti-TB drug initiation) and their asymptomatic, sputum culture-negative household contacts were analyzed using liquid chromatography high-resolution mass spectrometry (LC-MS) to identify metabolites. Statistical and bioinformatics methods were used to select accurate mass/charge (*m/z*) ions (metabolites) that were significantly different between the two groups at a false discovery rate (FDR) of $q<0.05$. Two-way hierarchical cluster analysis (HCA) was used to identify clusters of ions contributing to separation of cases and controls, and metabolomics databases were used to match these ions to known metabolites. Identity of specific D-series resolvins was confirmed using LC-MS/MS analysis. **Results:** We selected 17 adult patients with culture positive pulmonary TB disease and 17 of their household contacts. Over 23,000 metabolites were detected in untargeted metabolomic analysis and 61 were significantly different between the two groups ($q<0.05$). HCA revealed 8 metabolite clusters containing metabolites largely upregulated in patients with TB disease, including anti-TB drugs, glutamate, choline derivatives, likely *Mycobacterium tuberculosis*-derived cell wall glycolipids (trehalose-6-mycolate and phosphatidylinositol) and pro-resolving lipid mediators of inflammation (D-series resolvins RvD1 and RvD2), known to stimulate resolution, efferocytosis and microbial killing. The resolvins were confirmed to be RvD1, aspirin-triggered RvD1, and RvD2. **Conclusions:** This study shows that high-resolution metabolomic analysis can differentiate patients with active TB disease from their asymptomatic household contacts. Specific metabolites upregulated

in the plasma of patients with active TB disease have potential as biomarkers and may reveal pathways involved in TB disease pathogenesis and resolution.

Introduction

The global burden of tuberculosis (TB) is vast with an estimated 8.6 million new TB cases and 1.3 million deaths due to the disease in 2012.[144,145] An estimated one third of the world population has latent TB infection, and thus are at risk for reactivation TB disease. Challenges in global TB control include the emergence and spread of drug resistant TB, HIV/TB co-infection and lack of adequate tools including lack of an effective vaccine and lack of simple point of care diagnostics.[1] The rising global presence of multidrug resistant TB (MDR-TB) and extensively drug resistant-TB (XDR-TB) strains of *Mycobacterium tuberculosis (Mtb*) constitute a public health crisis, particularly in high-burden countries.[1,144,145]

The 2013 World Health Organization (WHO) Global Tuberculosis Report calls for rapid development and implementation of innovative strategies and tools for better prevention, diagnosis and treatment of TB.[144,145] Unfortunately, progress remains slow, despite the large number of new diagnostic tools and anti-TB treatment regimens, vaccines, and drugs in development or under investigation.[146-150] Further, there are no well-validated or specific biomarkers for TB that can predict or discriminate transition from latent TB to active TB disease or that are useful to monitor efficacy of anti-TB drug treatments.[151,152]

Metabolomics analysis uses nuclear magnetic resonance (NMR) spectroscopy-based or mass spectrometry (MS)-based technologies to identify hundreds to thousands of small-molecule metabolites in biofluids or tissues, coupled with biostatistics and bioinformatics to identify potential biomarkers and metabolic pathways of disease.[78,120,153] Individualized metabolic phenotyping using metabolomics analysis of plasma or serum samples has been used to differentiate individuals with various chronic diseases from control subjects, such as in Parkinson's disease,[124] diabetes mellitus,[154] and age-related macular degeneration.[125] Further, targeted or untargeted NMR- or MS-

based metabolomics methods have recently been used to distinguish the presence of specific infectious diseases, to predict therapeutic responses to anti-microbial agents, and to explore host-pathogen metabolic interactions, including in malaria,[155,156] chronic *Pseudomonas aeruginosa* pulmonary infection,[157] HIV,[158] and sepsis.[159]

Targeted metabolomics methods have been used to characterize specific metabolites and regulated or unique metabolic pathways endogenous to the *Mtb* organism itself in cell culture studies,[160-163] as well as *Mtb* metabolites possibly involved in drug resistance.[164] Metabolomics analysis has provided an important approach to identify metabolic profiles associated with *Mtb* disease in animal models of TB using NMR[86,165] and in human TB infection or active disease using NMR-based analysis of serum[93] or MS-based analysis of sputum[85] or serum.[84,87] These studies have variously demonstrated differential small metabolite profiles in blood associated with TB disease compared to uninfected controls[84,86,87,93,165] and have identified apparent *Mtb*-derived metabolites in sputum of patients with culture positive pulmonary disease.[85]

Recently, we have developed high-resolution, high-throughput MS methods, advanced data extraction algorithms, and annotation software that allows detection of over 20,000 accurate mass metabolites in human plasma.[78,95,112,113,120] Although a limitation is the logistical challenge of absolute verification of all putative metabolites (which include many unidentified metabolites),[78,112,153] this robust approach has the potential to identify metabolites heretofore not known to be present in blood or otherwise linked to disease as well as potential pathophysiologic pathways[166] that may be linked with TB disease and can then be targeted for further verification and utility.

We designed this study to explore these enhanced metabolomics capabilities in a small number of subjects with newly diagnosed and laboratory-confirmed pulmonary TB and their matched household contacts at the National Center for Tuberculosis and Lung Disease (NCTBLD), Tbilisi, Georgia. Georgia is a lower-middle income country that has

been designated by the WHO as a high-burden country for MDR-TB.[102,135,144,167] Our purpose was to conduct a proof-of-principle study to test the utility of high-resolution MS-based metabolomics of plasma to differentiate subjects with active TB disease from asymptomatic household contacts without apparent TB disease and to determine potential metabolic features that may reflect host-*Mtb* metabolic interactions.

Materials and Methods

Ethics Statement

This study was approved by the Institutional Review Board of Emory University (Atlanta, GA, USA) and the Georgian NCTBLD Ethics Committee (Tbilisi, Georgia). All subjects provided written informed consent for participation in the study.

Study Participants

Study participants for this ancillary metabolomics study were selected from a double blind, randomized, controlled, prospective trial of high-dose cholecalciferol treatment of patients with pulmonary TB disease (clinicaltrials.gov identifier NCT00918086).[32] Inclusion criteria for patients were age ≥ 18 years, newly diagnosed TB as determined by a positive acid-fast bacilli (AFB) sputum smear, and later confirmed by a positive sputum culture for *Mtb* (performed at the Georgian National TB Reference Laboratory [NRL]),[102] ≤ 7 days of treatment with anti-TB drug therapy. Exclusion criteria included >7 days of anti-TB therapy (life time), hypercalcemia, nephrolithiasis, hyperparathyroidism, sarcoidosis, history of organ transplant, liver cirrhosis, requirement of hemodialysis, cancer in past 5 years, seizures, current pregnancy or lactation, serum creatinine >250 mmol/L, corticosteroid use in the past 30 days, current use of cytotoxic or immunosuppressive drugs, and current incarceration. Inclusion criteria for household contacts (controls) included lack of symptoms suggestive of TB disease or any other acute illness and documented negative sputum smear and culture. We chose a convenience sample of 17 TB subjects that had data available for their matched household contact.

Sputum Culture, Drug Susceptibility Testing and First-Line Anti-TB Drug Therapy

Two sputum specimens were obtained from each patient subsequently confirmed to have active pulmonary TB disease. Direct sputum smears with Ziehl-Neelsen staining were examined by light microscopy. All sputum samples were sent to the National

Research Laboratory for culture on Löwenstein-Jensen (LJ)-based solid media, using standard methodologies as previously described.[102,135] Positive cultures were confirmed to be *Mtb* complex using phenotypic tests, as outlined elsewhere.[102] Drug susceptibility testing (DST) for first-line anti-TB drugs (isoniazid, rifampicin, ethambutol) was done using absolute concentration method and for second-line drugs with proportion method on solid media with standard methodology, as previously described.[135]

To compare individuals with TB disease to those without evident TB disease, we also obtained blood from 17 asymptomatic household contacts of all studied TB disease subjects (typically a close relative) and were documented to be sputum *Mtb* culture negative.

Plasma Sample Collection

Peripheral blood samples were obtained by venipuncture from all 17 subjects with TB disease and well as asymptomatic household contacts without TB disease. Blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes, centrifuged and isolated plasma immediately stored frozen at -80°C. Samples were subsequently shipped on dry ice from Tbilisi to Emory University, Atlanta, GA, USA. Samples were never previously thawed, remained frozen during transit, and were frozen at -80°C in Atlanta prior to metabolomics analysis.

Macronutrient Intake and Body Mass Index Assessment

Mean daily dietary intake of macronutrients (total calories, protein, fat and carbohydrate) was estimated in the three days prior to the blood collection using a validated culture-specific nutrient intake assessment instrument using the Nutrition Data System for Research software, version 2011, as previously described.[32,74] Body mass index [BMI; body weight (kg)/height (m^2)] was calculated at entry in all subjects using data obtained from a calibrated research stadiometer and digital body weight scale system (Tanita Inc; Arlington Heights, Illinois, USA).

Metabolomics Analysis

High-Resolution Metabolomics

Thawed plasma (65 µL) was treated with 130 µl acetonitrile (2:1, v/v) containing an internal isotopic standard mixture (3.5 µL/sample), as previously described.[112] Briefly, the internal standard mix for quality control consisted of 14 stable isotopic chemicals covering a broad range of chemical properties represented in small molecules.[112] Samples were mixed and placed in ice for 30 min prior to centrifugation for 10 min (16,100 x *g* at 4ºC) to remove protein. The supernatants (10 µL), for each high-resolution LC-MS analysis were then loaded onto an autosampler maintained at 4°C and analyzed in triplicate using a LTQ-Velos Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA) and C18 chromatography (Higgins Analytical, Targa, Mountain View, CA, USA, 2.1 x 10 cm). Elution was obtained with a formic acid/acetonitrile gradient [112] at a flow rate of 0.35 ml/min for the initial 6 min and 0.5 ml/min for the remaining 4 min. The first 2-min period consisted of 5% solution A [2% (v/v) formic acid in water], 60% water, 35% acetonitrile, followed by a 4-min linear gradient to 5% solution A, 0% water, 95% acetonitrile. The final 4-min period was maintained at 5% solution A, 95% acetonitrile. The mass spectrometer was set to collect data from mass/charge ratio (*m/z*) 85 to 2000 daltons over the 10-minute chromatography period. Electrospray ionization was used in positive ion mode for detection, as outlined.[112,113]

Tandem Mass Spectrometry

Ion dissociation analysis by tandem LC-MS/MS was used in separate studies to positively identify L-glutamate.[113] Identity of specific D-series resolvins [resolvin D1 (RvD1), 7*S*, 8*R*,17*S*-trihydroxy-4*Z*, 9*E*, 11*E*, 13*Z*, 15*E*, 19*Z*-docosahexaenoic acid), resolvin D2 (RvD2), 7*S*, 16*R*, 17*S*-trihydroxy-4*Z*, 8*E*, 10*Z*, 12*E*, 14*E*,19*Z*-docosahexaenoic acid) and the aspirin-triggered RvD1 (AT-RvD1), 7*S*, 8*R*,17*R*-trihydroxy-4*Z*, 9*E*, 11*E*, 13*Z*, 15*E*, 19*Z*-docosahexaenoic acid] was confirmed using lipid

mediator metabololipidomics analytical methods, as described by Serhan and colleagues.[116,117] Briefly, five deuterium-labeled internal standards (0.5 ng) were added to plasma aliquots [d_5-RvD2, d_8-5-hydroxyeicosatetraenoic acid (d_8-5-HETE), d_4-leukotriene B_4 (d_4-LTB_4), d_5-lipoxin A_4 (d_5-LXA_4) and d_4-prostaglandin E_2 (d_4-PGE_2) to facilitate quantification of mediator recovery. Samples were extracted using SPE columns, eluted with methyl formate, and organic solvent evaporated using a nitrogen stream. Samples were suspended in methanol for analysis by high-resolution liquid chromatography coupled with tandem mass spectroscopy (LC-MS/MS), using a QTrap ABI 5500 (ABSciex, Framingham, MA).[116,117] To monitor and quantify levels of the specialized pro-resolving lipid mediators (SPMs) derived from arachidonic acid, docosahexanoic acid (DHA) and eicosapentanoic acid (EPA) in plasma samples,[118,119] multiple reaction monitoring (MRM) for signature ion fragments was performed, with identification accomplished using LC retention time (RT), band shape and maximum absorbance wavelength (λmax) of UV, and ≥ 6 diagnostic ions of MS/MS spectrum. Quantification was determined based on peak MRM transition area and linear calibration curves.[116,117]

Data Collection and Processing for High-Resolution Metabolomics

Data from the LTQ-Velos Orbitrap MS were continuously collected over the 10-min chromatographic separation period and stored as .Raw files. The .Raw files were converted to .cdf format using Xcalibur file converter software (Thermo Fisher, San Diego, CA) and used for data extraction. Peak extraction and integration were performed using apLCMS with xMSanalyzer.[95,120] apLCMS (http://www.sph.emory.edu/apLCMS) is an adaptive processing software package designed for high resolution LC-MS data that performs data filtering, peak detection, and alignment and generates a feature table, where a feature is defined as the measured *m/z*, RT, and integrated ion intensity. We define a metabolite as any chemical in a biological system, where some metabolites may

be gene-directed or environmental chemicals.[78] xMSanalyzer enhances the feature detection process by performing systematic data re-extraction and combining results from different parameter settings (http://userwww.service.emory.edu/~kuppal2/xMSanalyzer).

Statistical Analysis

Descriptive statistics for demographic and clinical data were performed. Two-tailed t-tests and two-tailed Fisher exact tests were used to compare metabolomics results from patients with TB disease and their household contacts using SAS version 9.3 (Cary, NC, USA) for continuous and categorical data, respectively. LIMMA, a package within the R framework for differential expression analysis, was used to identify differentially expressed metabolites between TB cases and household contacts.[121] To account for multiple comparisons, *P* values were adjusted using the Benjamini and Hochberg false discovery rate (FDR; q=0.05) to distinguish statistically significant metabolites that differed between the two groups.[122] Two-way hierarchal clustering analysis (HCA) was performed using the differentially expressed metabolites to visualize patterns and detect clusters of co-regulated metabolites by disease state.[78,123] An untargeted metabolome-wide association study (MWAS) based on the Pearson correlation analysis of the differentially expressed metabolites with all detected metabolites in plasma of subjects with TB disease and HCA was performed to understand the global association pattern of the discriminatory metabolites.[78] The statistical significance of correlations was determined using the Student's t-test method and visualized using a Manhattan plot where the x-axis corresponds to the metabolites (85-2000 *m/z*) and the y-axis corresponds to the negative $\log_{10}$ of the p-value.[124,125] Targeted MWAS were also performed with anti-TB drugs differentially expressed in TB disease versus HC subjects. FDR, HCA and Pearson correlations analyses were performed using R.[95,120]

Metabolite Annotation and Pathway Analysis

Putative metabolite identification of the discriminatory ions between TB and household contacts were determined using open-access Metlin ([126]; http://metlin.scripps.edu/) and the xMSannotator R package (manuscript submitted; http://userwww.service.emory.edu/~kuppal2/xMSannotator/). xMSannotator uses biological, chemical and pathway information with a suite of major small molecule databases including KEGG ([127]; http://www.genome.jp/kegg/), Human Metabolome Database (HMDB),[128] MetaCyc (http://www.metacyc.org) and ChemSpider (http://www.chemspider.com/) to enhance reliability of ion annotation relative to that obtained by matching high-resolution *m/z* to metabolites in Metlin.[129] Pathway analysis was performed using KEGG.[127]

Results

Subject Characteristics

The mean age of TB patients (35±12 years) and gender characteristics of the 17 subjects with TB disease did not differ significantly from the 17 household contacts (**Table 5.1**). All subjects were Caucasian; there were no differences between the two groups in terms of annual income and maximum educational level attained (not shown). Patients with active TB disease were more likely to smoke and less likely to be currently employed compared to their household contacts (**Table 5.1**). No subject took vitamin or mineral supplements; however, the three-day food intake analysis confirmed patients with TB consumed significantly greater total calories per kg of body weight daily, primarily due to increased carbohydrate sources compared to household contacts. Mean daily protein and fat intake was also higher in the subjects with TB disease, but these intakes were not statistically different between groups (**Table 5.1**). The average BMI in the TB disease cohort was markedly lower than the BMI of the matched housemates, likely reflecting the catabolic nature of TB disease,[1] despite the overall higher caloric intake.[74]

High-Resolution Metabolomics Data

Extraction of mass spectral data derived from C18 chromatography with apLCMS and xMSanalyzer yielded 23,241 metabolites. Statistical analysis of the 34 subjects with FDR at q=0.05 showed that 61 metabolites differed between TB disease subjects and household contacts. **Figure 5.1, panel A** depicts a Manhattan plot charting the –log *P* value for each *m/z*. The 61 metabolites are depicted as green dots above the dashed blue line signifying FDR q=0.05. The dashed red and green lines represent FDR cut-offs of q =0.1 and q=0.2, respectively. **Supplemental Table 5.S1** lists these significantly different metabolites with *m/z,* respective RT, relative log intensities, median coefficient of variation, and negative log *P* value.

Figure 5.1, panel B shows box and whisker plots of log(2) intensities for six selected significant metabolites comparing the median and interquartile range for metabolite intensities in household contacts (left boxplot within each individual metabolite) versus TB disease subjects (right boxplot within each individual metabolite). The individual *m/z* and putative metabolite identifications from Metlin are shown. The upper panel shows selected metabolites that were increased in TB subjects: from left to right, the amino acid L-glutamate, a D-series resolvin,[116-119] and a mycobacterium-specific cell wall glycolipid trehalose-6-mycolate.[168,169] The lower panel shows intensities of the metabolite matching phosphatidylinositol (PI), a key phospholipid present the cell wall of mycobacteria[168,169] that was increased in TB subjects. This *m/z* matched to six putative PI molecules of different carbon chain lengths. The lower panel also shows two unidentified metabolites that were decreased in TB subjects relative to household contacts.

Hierarchical cluster analysis of plasma metabolites differentiating pulmonary TB disease from control subjects

Two-way hierarchical cluster analysis (HCA) of the 61 significant metabolites is shown in **Figure 5.2A**. On the x-axis, the 17 persons with active TB disease (top light green bar on right) cluster separately from the 17 household contacts (top red bar on left). On the y-axis, the 61 metabolites are separated into eight clusters. The pie chart shown in **Figure 5.2B** depicts the distribution of the 61 significant metabolites different between the two study groups within pan-metabolome categories.[78] Of interest, the largest category (47%) of this classification did not match to known metabolites in the Metlin or xMSannotator metabolite databases. Metabolites classified within intermediary metabolism and those derived from pharmaceutical agents each represented 17% of the distribution.

Table 5.2 lists the 59 metabolites with RT> 30 sec grouped according to HCA cluster, and signifies cluster number, m/z, RT and their putative metabolite Metlin match.[126] As shown in the HCA heat map (**Figure 5.2A**), accurate mass metabolite matches within cluster 1 to 6 were all significantly increased in subjects with TB disease. In contrast, clusters 7 and 8 contained primarily of unidentified metabolites, all of which were decreased in those with TB disease. Metabolite cluster 5 contained *m/z* matches to the anti-TB drugs rifampin and ethambutol (both the Na^+ and the H^+ adducts of these agents were detected). The *m/z* for both pyrazinamide (*m/z* 124.0498) and isoniazid (*m/z* 120.0548) were detected in the full panel of metabolites, but did not statistically differ between those with TB disease compared to household contacts. Given that all those with TB disease were treated with a combination of isoniazid, rifampin, pyrazinamide, and ethambutol started within one week prior to the plasma sampling, we speculated that the anti-TB drugs and/or their metabolites should correlate. Therefore, we performed a MWAS study to determine the association of the *m/z* for pyrazinamide with the full panel of detected metabolites. **Figure 5.3** shows that many metabolites were highly correlated with the pyrazinamide metabolite *m/z* (17 metabolites showed a highly significant -log *P* >7 correlation Pearson R value). Metabolites with the highest -log *P* for correlation with the pyrazinamide *m/z* matched to phosphatidylethanolamine (*m/z* 792.5638), acetyl-hydrazine; a known isoniazid metabolite[170,171] (*m/z* 97.0389), the anti-TB drug isoniazid (*m/z* 120.0548), an unidentified metabolite (*m/z* 562.2247) and the anti-TB drug ethambutol (*m/z* 205.1898). The *m/z* to another known isoniazid metabolite, isoniazid pyruvate (*m/z* 208.0682),[170] was also significantly correlated with the pyrazinamide metabolite (not shown in figure).

Verification studies for D-series resolvins and glutamate

One of the 61 significant plasma metabolites that differentiated TB disease from controls was *m/z* 399.2116, which matched to several possible D-series resolvins using

the Metlin metabolite database. We refined the annotation by using xMSannotator, which matched the resolvin *m/z* to two specific resolvins, RvD1 and RvD2. As shown in **Figure 5.4**, verification studies confirmed the positive identification of RvD1, RvD2, and aspirin-triggered resolvin D1 (AT-RvD1) in the plasma from subjects with TB disease. Glutamate (*m/z* 148.0594) was verified by MS/MS and fragmentation of co-eluting [$^{13}C_5$]-L-glutamate added in the internal standard mix for LC-MS.[112] **Figure 5.5** depicts an abbreviated diagram of this pathway from KEGG with five *m/z* matches we found in plasma of TB disease subjects noted in red.

Concomitant detection of putative* Mtb *cell wall-derived metabolites and the D-series resolvin metabolite in plasma of subjects with drug-sensitive or drug-resistant TB disease

Co-detection of the *m/z* matched to trehalose-6-mycolate, PI, and the D-series resolvins within study subject plasma (TB disease patients with drug-sensitive TB, MDR-TB, and household contacts) is shown in **Table 5.3**. Notably, in all 13 patients with drug sensitive TB with the detectable trehalose-6-mycolate metabolite in plasma, the PI metabolite was concomitantly detected. Also, in all 12 patients with drug-sensitive TB with detectable plasma D-series resolvin(s), both the trehalose-6-mycolate and the PI metabolites were detected. One of three patients with MDR-TB had concomitantly detectable D-series resolvin(s) and trehalose-6-mycolate and PI metabolites. In another MDR-TB patient, the D-series resolvin(s) were demonstrated, but neither of the two *Mtb* cell wall-related metabolites were detected. The D-series resolvin(s) metabolite was detected in the plasma of two household contacts, but it was not linked to detection of either of the putative *Mtb* cell wall-related metabolites. Only one of the 17 household contacts demonstrated the trehalose-6-mycolate metabolite, which was detected together with the PI metabolite in this same individual.

Discussion

Our study demonstrates that this information-rich metabolic profiling method can differentiate adults with pulmonary TB disease from asymptomatic household contacts. The metabolite profile includes specific resolvins, L-glutamate and putative *Mtb* cell wall metabolites. A unique feature is our use of high-resolution LC-MS metabolomics to profile > 23,000 metabolites in plasma from humans with TB disease and their aymptomatic household contacts. Patients with active TB disease likely had similar exposures (e.g. specific dietary foods, environmental chemicals, household microbes, etc.) as the individual control subjects living in the same dwelling. Thus, metabolite differences between the two groups were likely due to the presence or absence of TB disease itself, and not to other differences in the "exposome" of the respective groups.[78]

Among the differentiating metabolic features, were known anti-TB drugs as expected and several metabolites not previously identified in the plasma of patients with TB disease. Most notably, these included endogenously produced lipid mediators involved in the resolution of inflammation,[119] and metabolites from the *Mtb* cell wall components including mycobacterium specific cell wall glycolipid trehalose-6-mycolate and the non-specific phospholipid PI, that could represent potential biomarkers of TB disease.[168,169,172,173] In addition, many unidentified metabolites were either increased or decreased in the plasma of the pulmonary TB subjects relative to controls, and may represent future targets for investigation.[78] This large proportion of unidentified metabolites detected is consistent with our previous studies using an earlier adaptation of this LC-MS method in several eukaryotic species, including man.[153]

In our study, dietary intake does not seem to explain the overall metabolite profile that discriminated the two groups. It is possible, however, that the increased triglyceride metabolite (**Table 5.2**) was related to higher recent fat or carbohydrate intake and the increased choline metabolite from increased intake per kilogram body weight of choline-

rich foods such as eggs and meats. It is highly unlikely that modestly higher essential and non-essential amino acid intake derived from dietary protein in those with TB disease versus household contacts (**Table 5.1**) accounts for a single amino acid (glutamate) to be within the discriminating metabolite profile.

Despite the challenges in TB research, there is a considerable need to extend metabolomic analyses to advance understanding of TB and its pathogenesis. Previous metabolomic studies in human TB have sometimes lacked culture confirmation in all subjects, lacked information on the course of disease after TB samples were collected, or lacked dietary intake data or other relevant health behavior data.[84,85,87,93] Also, prior to the present study, the analytic platforms measured a relatively small number of total metabolites (total metabolites = 30 to 498).[84,85,87,93]

Despite the limited coverage, considerable information has been gained from metabolomic analyses. Weiner et al, using low-resolution MS and gas chromatography MS (GC-MS) methods, explored the metabolome of 428 distinct small molecules in a cross-sectional study in healthy controls, asymptomatic patients with latent TB, and patients with active TB disease.[84] A total of 20 metabolites differentiated subjects with TB disease (n=44) compared to those with latent TB (n=46) and healthy individuals (n=46) combined. In subjects with TB disease, these included altered abundance of several amino acids, and increased N-acetylneuraminate (involved in aminosugar metabolism), pyroglutamine (glutamate metabolism), inosine (purine metabolism) and mannose (protein glycosylation).[84]

Zhou et al used NMR-based methods to compare metabolites (n=30 metabolites) in serum of subjects with TB disease versus healthy controls.[93] The primary finding was an increase in several amino acids (including glutamate), ketones, lactate and pyruvate, with a decrease in low-density lipoproteins, glycerolphosphocholine, alanine and glycine in those patients with TB disease.[93] Che et al reported serum data from patients with

"active" TB versus controls, using a GC-MS method; 5-oxoproline (pyroglutamic acid, a metabolite of glutaminase-mediated glutamine hydrolysis), lactate, inositol, ornithine, phenylalanine and glycerol-3-phosphate, and cholesterol were decreased, while galactose, cholesterol, malonate and several unidentified metabolites were increased in TB disease.[87] du Preez and Loots used NMR-based methods to analyze sputum from *Mtb* culture-positive versus culture-negative patients to detect 498 metabolites.[85] *Mtb* culture-positive sputum samples demonstrated upregulated *Mtb*-associated cell wall metabolites (e.g. L-mannopyranose and D-mannopyranoside), D-citramalate (involved in C_5-branched dibasic acid metabolism of the *Mtb* organism),[161] and certain host-response markers to TB infection, including γ-aminobutarate (GABA).[85]

Our data show, for the first time, *m/z* matches for two putative *Mtb* cell wall components in the plasma of patients with TB disease—phosphatidylinositol and trehalose-6-mycolate. Also, these were detected concurrently in 14 of 17 of the TB disease subjects at a similar time point in the course of their disease (**Table 5.3**). Mannose, which was upregulated in TB disease in the Weiner report,[84] is a critical component of the *Mtb* cell wall glycan lipoarabinomannan (LAM)[168,169,174,175] and is required for mycobacterial growth.[176] In the *Mtb* cell wall, LAM is anchored to both the mycolic acid layer and the cell membrane by PI.[168,169] Therefore, it is possible that the increased mannose identified by Weiner et al[84] was derived from cell walls of the infecting *Mtb*, consistent with our observations of the elevated metabolites matching to PI and trehalose-6-mycolate.

Previous metabolomic studies noted a variety of amino acid alterations that distinguished TB disease from controls.[84,87,93] We identified glutamate to be elevated in TB disease as the only discriminating amino acid (**Table 5.2**), consistent with the upregulation of serum glutamate in TB disease noted by Zhou et al[93] and metabolically with the decreased glutamine and elevated pyroglutamine noted by Weiner et al.[84]

Glutamate is a major amino acid utilized by *Mtb* during the growth phase[177] and this amino acid is a critical constituent for components of the *Mtb* cell wall.[161,178] After granuloma formation in the host, *Mtb* shifts from a predominantly aerobic energy metabolism to anaerobic metabolism in the oxygen-limiting environment of the granuloma. Previous biochemical and targeted metabolomic studies have shown that varient tricarboxylic acid (TCA) cycle pathways are employed by *Mtb* under hypoxic conditions, including a half-cycle to generate succinate from GABA that utilizes increased glutamate availability as a substrate.[160,163] The C^5 branched dibasic acid metabolic pathway is also utilized by *Mtb* to provide alternative carbon and nitrogen sources for energy, including glutamate.[161,179] (**Figure 5.5**) The upregulated glutamate in the differentiating plasma profile of TB disease subjects may thus be a result of increased *Mtb* glutamate synthesis and an example of a potential host-pathogen metabolic interaction.

This is the first study, to our knowledge to identify a systemic increase of specific resolvins in TB in humans. Resolvins are lipid mediators derived from endogenous omega-3 fatty acids (docosahexaenoic acid;DHA) from a genus of anti-inflammatory and specialized pro-resolving lipid mediators (SPM): resolvins, protectins, maresins, and lipoxins.[119,180] Increased local production of D-series resolvins occurs during infection at sites of macrophage recruitment, particularly when macrophages are clearing apoptotic polymorphonuclear neutrophils (PMN).[117,119,181,182] D-series resolvins act as autocrine factors and facilitate phagocytosis and microbial killing by a variety of mechanisms. Apoptotic PMNs themselves produce SPMs and their uptake by macrophages (efferocytosis) during bacterial infection also stimulates macrophage synthesis of D-series resolvins, which, in turn, induce local anti-inflammatory effects and enhance microbial clearance.[117,119,180] RvD1 has been shown to block neutrophil inflammatory responses and trans-endothelial migration in inflammatory lung diseases in mice.[183] In

macrophages, RvD1 regulates phagocytosis utilizing receptors in humans and increases clearance of apoptotic neutrophils *in vitro.*[181,183] In a murine microbial sepsis model, RvD2 significantly dampened the local and systemic bacterial burden by preventing excessive leukocyte infiltration and enhancing clearance of microbes.[182] Although the role of resolvins in *Mtb* infection is currently little understood, the data shown in **Table 5.3** suggest the possibility of a common pathophysiologic pathway in TB disease involving events that result in local generation of D-series resolvins by PMN and macrophages in the lung granuloma that are subsequently detectable in plasma, concomitant with the presence of specific *Mtb* cell wall-related metabolites that are potentially a result of *Mtb* killing.

Limitations of this study include the cross-sectional nature, the relatively small sample size and some patients with MDR-TB. In addition, while the household contacts did not have active TB disease, no studies were carried out to assess whether they had latent TB infection. Further metabolomic studies are needed among patients with documented latent TB infection to detect potential biomarkers for transition to active disease. Another major limitation to this ancillary study is the larger trial was not designed specifically for metabolomics analysis. We had no basis for a power calculation, thus this is a pilot study with a limited number of samples. Also we did not obtain absolute identity of most metabolites in the discriminating metabolites by tandem MS/MS studies. Metabolites in plasma are an indirect measure of *Mtb* metabolism and local tissue response to infection and we have no data from patient sputum or the *Mtb* organism itself.

In summary, these data show that adults with active TB disease can be differentiated from persons without active TB disease by high-resolution metabolic profiling of plasma. We were able to identify multiple metabolites relevant to *Mtb* disease and its unique metabolism, as well as D-series resolvins that may reflect a

pathophysiologic response to TB disease in humans. Prospective and confirmatory studies are needed to gain pathophysiologic insight before and after initiation of conventional anti-TB drug therapy and during stages of TB disease and with clinical recovery and ultimate cure. Studies are needed to determine whether plasma metabolic profiles can be used to predict development of TB disease in individuals with latent TB, and whether metabolite profiling can identify subjects with drug-sensitive versus MDR-TB and whether the responses to new anti-TB therapies can be predicted.

Table 5.1: Demographic characteristics of subjects with TB disease and their sputum smear-negative household contacts

Characteristic*	TB Disease Patients (n=17)	Household Contacts (n=17)	P-value
Age [years; mean (SD)]	35 (12)	42 (11)	0.0944
Male gender, n (%)	10 (59%)	6 (35%)	0.1673
Current smoker, n (%)	13 (76%)	7 (41%)	0.0365
Currently employed, n (%)	8 (47%)	11 (64%)	0.0238
Total calorie intake [kcal/kg/day; mean (SD)]	56.6 (17.8)	42.1 (11.7)	0.0092
Total protein intake [g/kg/day; mean (SD)]	1.7 (0.8)	1.2 (0.5)	0.0582
Total fat intake [g/kg/day; mean (SD)]	2.0 (0.8)	1.5 (0.5)	0.0745
Total carbohydrate intake [g/kg/day; mean (SD)]	8.3 (2.4)	5.5 (1.4)	0.0006
BMI [kg/m^2; mean (SD)]	20.7 (2.0)	25.6 (4.0)	<0.0001

* Annual income and educational level were similar between the two groups (data not shown).

Table 5.2: Clusters of metabolites that discriminate between patients with TB disease and their household contacts

Cluster	*m/z*	RT (sec)	Metlin Matches
1	988.8253	37	Unidentified
	869.8372	38	Triglyceride
	484.9452	47	Quercetin
	446.8292	51	Unidentified
	444.8320	52	Unidentified (Cl^-)
	270.9566	56	Environmental exposure (cosmetic) (C12)
	212.9984	53	Pesticide (Linuron)
2	621.9278	51	Unidentified
	192.0231	50	Plant hormone
	273.9602	50	Unidentified
	553.9406	50	Unidentified
	485.9535	51	Thyroid hormone mimetic
	214.0050	48	Drug
	148.0594	71	Glutamate
	104.1063	52	Choline
3	1321.9177	427	Unidentified
	825.5768	497	Unidentified
	399.2116	329	D-series Resolvin
	850.5915	467	Phosphatidylserine (C12)
	800.5743	422	Phosphatidylserine (C12)
	851.5953	471	Phosphatidylinositol

			(C13)
	801.5767	446	*Mtb* cell wall compound (trehalose-6-mycolate) (C13)
4	459.8740	51	Herbicide (Bromofenoxim)
	271.9629	50	Dichlorophenolindophenol (C13)
	574.9516	51	Unidentified
	305.1028	53	Chiral alcohol; acrylonitrile
	306.9330	53	Unidentified
	564.4350	402	Unidentified
	495.2198	248	Unidentified Drug
5	227.1718	48	Ethambutol (Na^+)
	206.1934	47	Sphingosine
	228.1751	47	Unidentified Drug
	125.0531	142	Unidentified
	107.0232	64	Unidentified
	205.1898	48	Ethambutol (H^+)
	823.4076	304	Rifampin (H^+)
	791.3806	304	Unidentified (C12)
	792.3868	304	Unidentified (C13)
	824.4129	303	Unidentified
	789.3655	272	Unidentified Drug

	845.3897	304	Rifampin (Na^+)
6	464.1383	129	Unidentified
	309.1807	132	Androstenedione
	431.1446	84	Unidentified Drug
	1591.6414	38	Unidentified
7	880.7371	45	Unidentified
	237.0191	39	Protein kinase inhibitor
	478.6161	57	Unidentified
	284.0310	79	Phosphotyrosine
	158.8735	62	Unidentified
	558.7576	53	Unidentified
8	536.2780	378	Vignatic acid A
	435.2326	376	Plant toxin
	271.2319	548	Unidentified
	1329.4225	297	Unidentified
	1117.1021	294	Unidentified
	1544.5580	289	Unidentified
	307.2149	320	Ruscopine
	1453.0526	296	Unidentified

Elements in parentheses represent different adducts of the same metabolite (e.g. rifampin). *m/z* = mass/charge ratio; RT= retention time; sec=seconds

Table 5.3: Linkage of likely *M. tuberculosis* cell wall-derived metabolites and the pro-resolving D-series resolvin metabolite in plasma of patients without or with MDR-TB

Metabolite feature	Non-MDR-TB patients*†	MDR-TB patients**	Household contacts***
Trehalose-6-mycolate	13/14	1/3	1/17
Phosphatidylinositol (PI)	14/14	1/3	1/17
D-series resolvin	12/14	2/3	2/17

MDR-TB= multidrug resistant TB (resistance to both isoniazid and rifampin)

* In all 13 non-MDR-TB patients with detectable trehalose-6-mycolate metabolite in plasma, the phosphatidylinositol metabolite was also detected.

† In all 12 non-MDR-TB patients with the D-series resolvin metabolite in plasma, the trehalose-6-mycolate and PI metabolites were concomitantly detected.

** One of 3 patients with MDR-TB demonstrated the D-series resolvin(s), trehalose-6-mycolate and PI metabolites in plasma. In another MDR-TB patient, the D-series resolvin was demonstrated but neither of the putative *Mtb* cell wall-related metabolites were detected.

*** One of the 17 smear-negative household contacts demonstrated both trehalose-6-mycolate and the PI metabolites in plasma, but these metabolites were undetectable the other household contacts. The D-series resolvin metabolite(s) was detected in plasma of 2 other household contacts.

Table 5.S1. Metabolites Statistically Different Between TB Disease and HC Subjects*

m/z	RT (sec)	Relative Log Intensities		Median CV	-log *P*
		HC	TB		
104.1063	52	-0.25	0.35	8.087	4.0585
107.0232	64	0.55	10.85	23.15	5.4717
125.0531	142	0.77	11.87	12.82	6.1267
148.0594	71	-0.76	1.52	114	4.4395
158.8735	62	4.70	-5.87	23.25	3.7418
192.0231	50	-8.61	0.51	19	4.5457
205.1898	48	-8.78	3.54	10.69	5.3422
206.1934	47	0.71	11.71	7.518	5.3353
212.9984	53	-0.41	0.66	6.846	5.3677
214.0050	48	-0.89	1.36	10.94	3.9180
227.1718	48	1.86	14.02	11.94	5.0526
228.1751	47	1.04	10.72	16.46	4.4315
237.0191	39	10.80	1.91	39.47	4.5193
270.9566	56	-0.59	0.45	6.427	4.1726
271.2319	548	0.23	-6.06	36.81	3.8250
271.9629	50	-7.41	0.92	22.02	3.6356
273.9602	50	1.07	11.50	18.85	4.6567
284.0310	79	10.46	1.55	17.88	4.0773
305.1028	53	-9.61	-0.92	30.9	4.1687
306.9330	53	-7.94	-0.41	42.47	3.7474
307.2149	320	0.75	-5.91	37.09	3.7628

309.1807	132	-5.45	3.72	18.23	4.7786
399.2116	329	1.20	10.24	13.3	4.1745
431.1446	84	-3.79	5.99	8.974	3.6972
435.2326	376	1.55	-8.29	8.301	4.8942
444.8320	52	-7.79	0.36	26.85	4.3731
446.8292	51	-8.28	0.15	13.79	4.0070
459.8740	51	-8.32	0.37	42.63	4.6951
464.1383	129	1.27	9.62	21.66	4.2564
478.6161	57	12.19	2.41	11.62	3.6752
484.9452	47	-8.73	0.37	44.04	3.9977
485.9535	51	-11.08	-1.46	19.36	3.7046
495.2198	248	-5.75	1.16	16.7	4.0494
536.2780	378	0.38	-9.01	5.93	4.5813
553.9406	50	-13.32	0.01	17.62	8.6632
558.7576	53	-0.52	-9.86	47.99	4.0936
564.4350	402	-10.61	0.33	41.6	4.6393
574.9516	51	-9.44	-0.63	35.12	4.0346
621.9278	51	-3.96	6.72	19.19	5.1978
789.3655	272	0.47	11.67	9.716	6.2066
791.3806	304	0.84	14.35	3.981	7.3652
792.3868	304	0.67	12.84	7.124	6.4730
800.5743	422	2.03	16.14	10.84	6.0803
801.5767	446	1.20	13.30	39.19	5.4153
823.4076	304	0.98	15.68	6.108	7.4054
824.4129	303	1.04	13.06	4.121	4.9367

825.5768	497	0.78	13.42	16.56	6.3607
845.3897	304	0.02	10.37	5.492	5.8864
850.5915	467	-3.39	10.02	24.2	4.7266
851.5953	471	1.13	15.14	10.86	7.0192
869.8372	38	-13.11	-1.47	19.61	4.6992
880.7371	45	11.63	1.64	21.14	4.1317
988.8253	37	-10.87	-1.18	44	3.8264
1117.1021	294	0.50	-8.10	43.42	4.0558
1321.9177	427	1.42	12.45	26.34	5.3731
1329.4225	297	1.03	-6.72	39.7	3.8532
1453.0526	296	0.64	-9.51	16.46	4.4895
1544.5580	289	2.05	-6.27	44.43	3.7544
1591.6414	38	-7.43	1.37	24.84	3.8262

CV= coefficient of variation; HC= asymptomatic household contact of subject with TB disease;

m/z= mass/charge; RT= retention time; sec=seconds, TB = tuberculosis

* False discovery rate (q=0.05)

Figure 5.1A

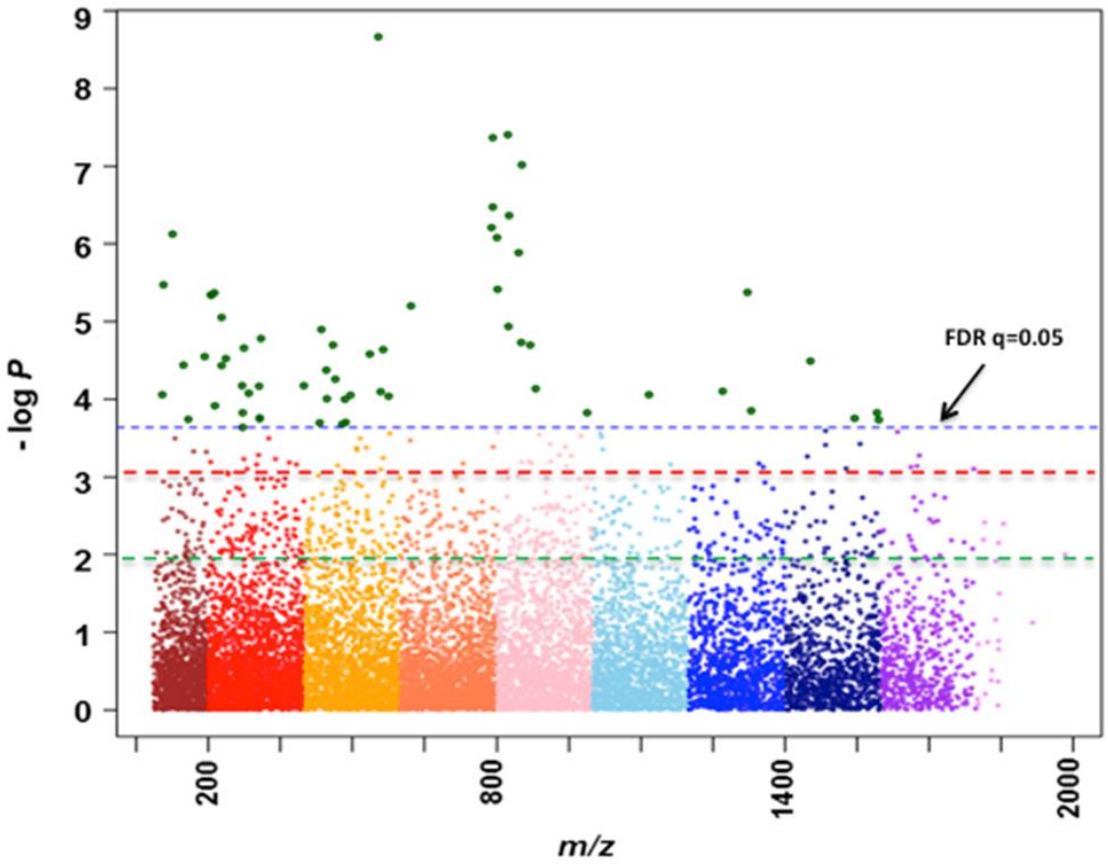

Figure 5.1A: Plasma metabolome-wide association study (MWAS) of pulmonary tuberculosis (TB) disease in adults. The Manhattan plot depicts the -log *P* analysis of 23,241 metabolites comparing 17 adults with recently diagnosed pulmonary TB and 17 adult asymptomatic household contacts who were sputum smear and culture negative for acid-fast bacilli (HC). The x-axis represents the *m/z* of the metabolites, ordered in increasing value from left (85) to right (2000). A total of 61 metabolites significantly differed (q = 0.05) between the two groups following FDR analysis (above horizontal dashed blue line). Metabolites above the dashed red line (n=122) were different between those the TB disease and their household at q= 0.10, while metabolites above the dashed green line (n=711) were different between the two groups at q= 0.20.

Figure 5.1B

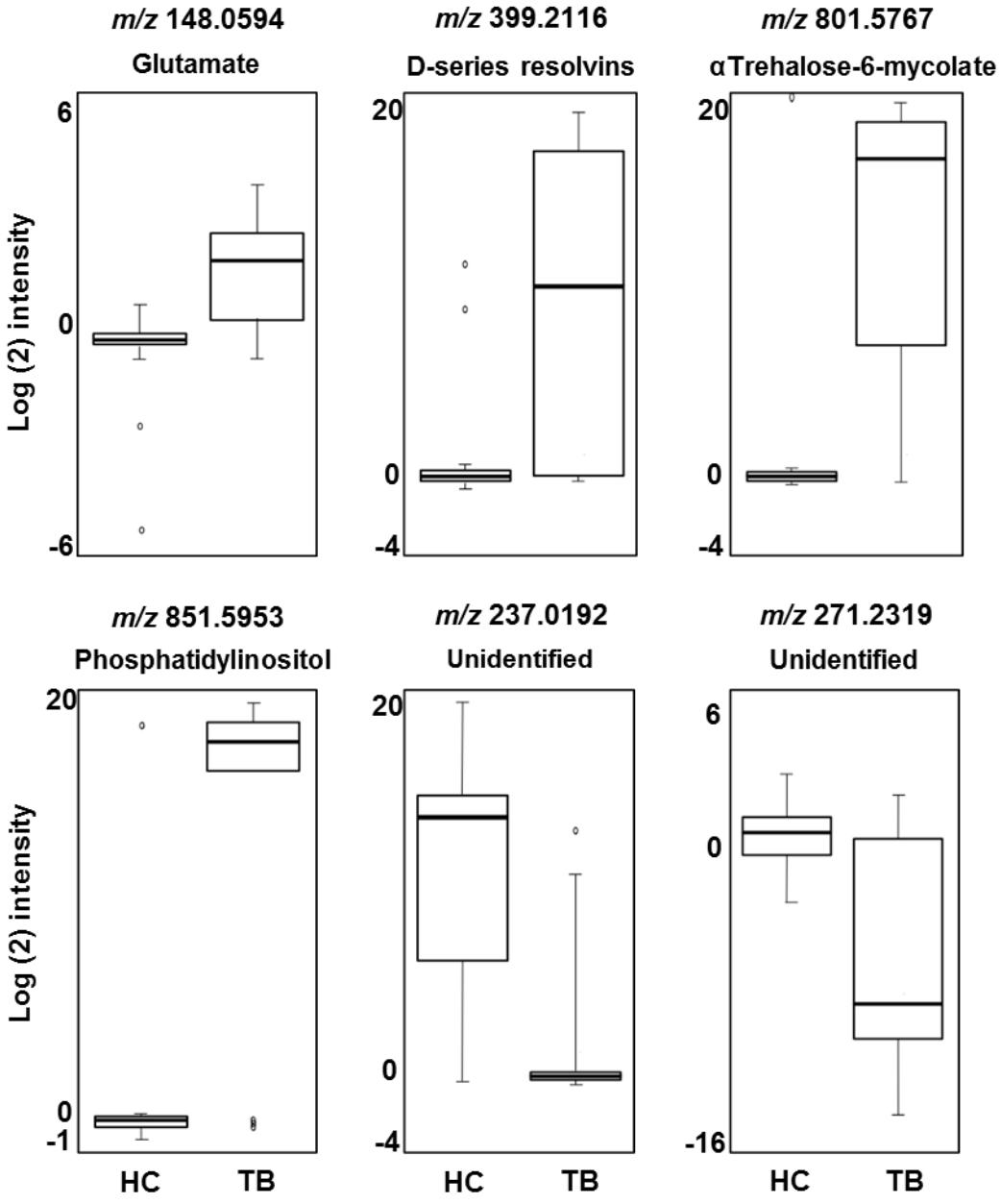
m/z 148.0594
Glutamate
m/z 399.2116
D-series resolvins
m/z 801.5767
αTrehalose-6-mycolate
m/z 851.5953
Phosphatidylinositol
m/z 237.0192
Unidentified
m/z 271.2319
Unidentified
Log (2) intensity
HC
TB

Figure 5.1B: Box-and-whisker plots of log (2) intensities comparing patients with TB and household contacts for selected metabolites, with *m/z* and putative metabolite identification from Metlin and KEGG (left to right upper panel: glutamate, D-series resolvin, and trehalose-6-mycolate, respectively; left to right lower panel: phosphatidylinositol, and two unidentified metabolites, respectively).

Figure 5.2A

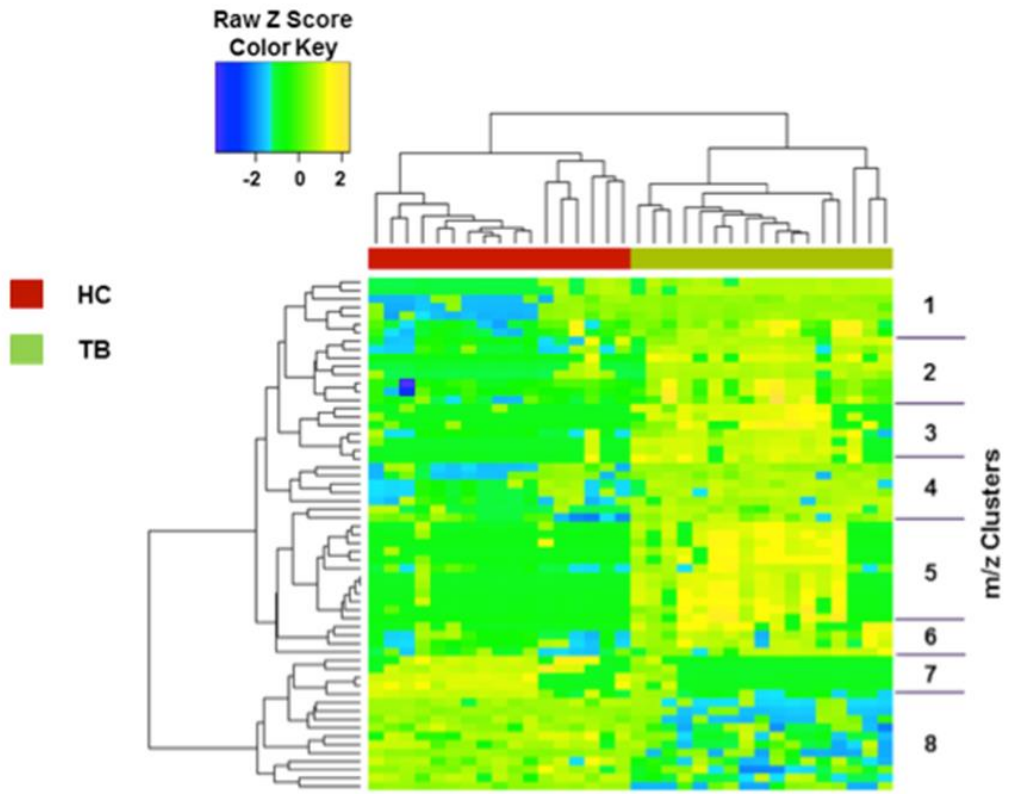

Figure 5.2A: Two-way hierarchical cluster analysis (HCA) using C18 chromatography shows 8 clusters of metabolites from human plasma and illustrates the patterns distinguishing those with active TB from household contacts without TB. The 17 TB subjects with TB (shown in green) and the 17 household contacts (without evidence of TB disease (shown in red) are shown along the x-axis.

Figure 5.2B

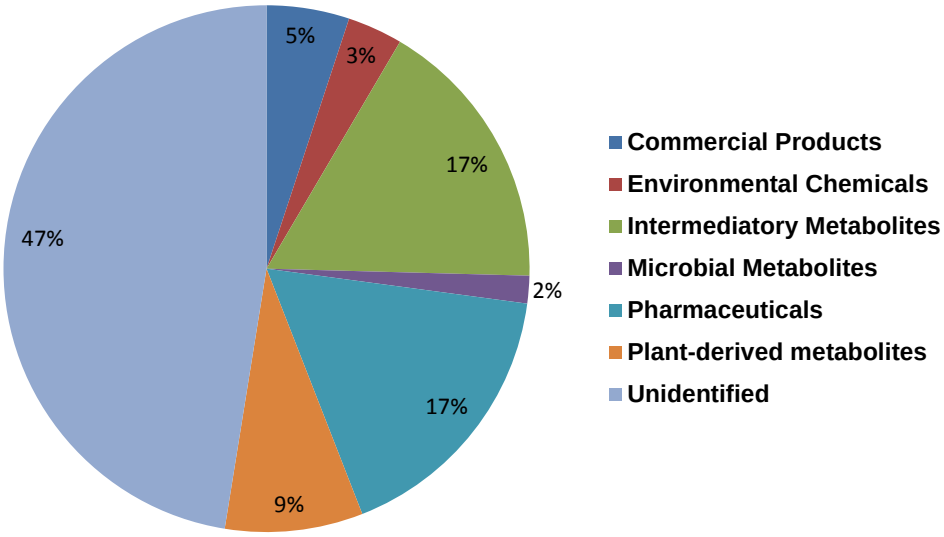

Figure 5.2B: Pie chart depicts chemical classes of the 61 significant metabolites from panel 2A according to high-resolution matches to metabolite databases.[78]

Figure 5.3

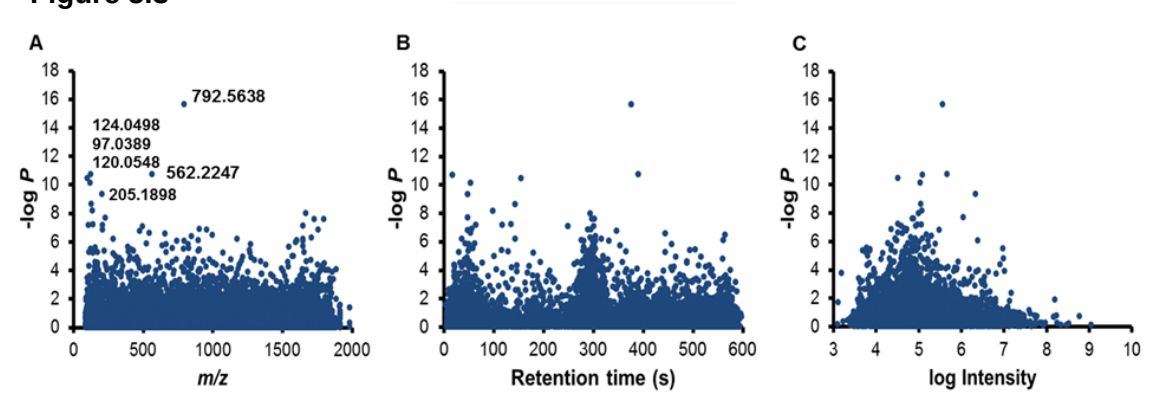

Figure 5.3. Metabolome-wide association study (MWAS) of pulmonary tuberculosis in humans. This Manhattan plot depicts FDR analysis testing associations of 23,241 metabolites to pyrazinamide (*m/z* 124.0498) ion intensities from 17 persons with pulmonary tuberculosis and 17 household contacts. (A) The negative log *P* value is plotted against the *m/z*. (B) The negative log *P* value is plotted against the retention time of the metabolites. (C) The negative log *P* value is plotted against the log ion intensity of the metabolites. The top five significant metabolite correlations to the pyrazinamide *m/z* were phosphatidylethanolamine (*m/z* 792.5638), acetyl-hydrazine; a known isoniazid metabolite[171,172] (*m/z* 97.0389), the anti-TB drug isoniazid (*m/z* 120.0548), an unidentified metabolite (*m/z* 562.2247) and the anti-TB drug ethanbutol (*m/z* 205.1898). The m/z to another known isoniazid metabolite, isoniazid pyruvate (*m/z* 208.0682),[171] was also significantly correlated with the pyrazinamide metabolite.

Figure 5.4

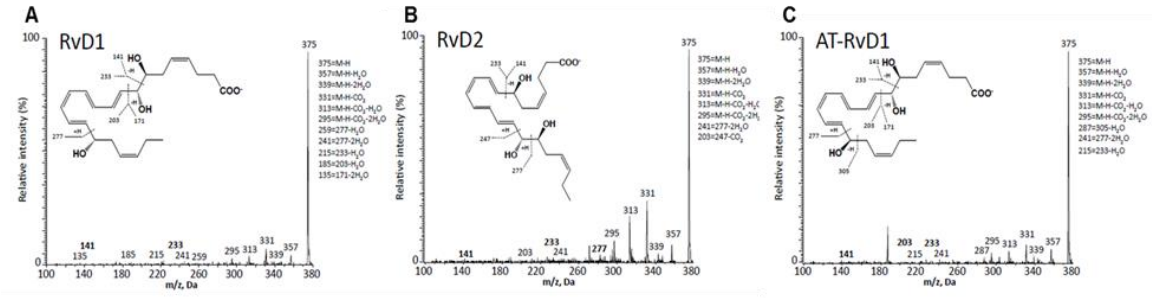

Figure 5.4. MS/MS fragmentation spectra show positive identification of resolvin D1 (RvD1), resolvin D2 (RvD2), and aspirin-triggered resolvin D1 (AT-RvD1) in plasma from subjects with TB disease. Metabololipidomics analytical methods that incorporated high-resolution liquid chromatography-ultraviolet (UV) coupled with tandem mass spectroscopy (LC-MS/MS, ABI 5500, see methods) were used to verify these DHA-derived specialized pro-resolving lipid mediators.[116,117]

Figure 5.5

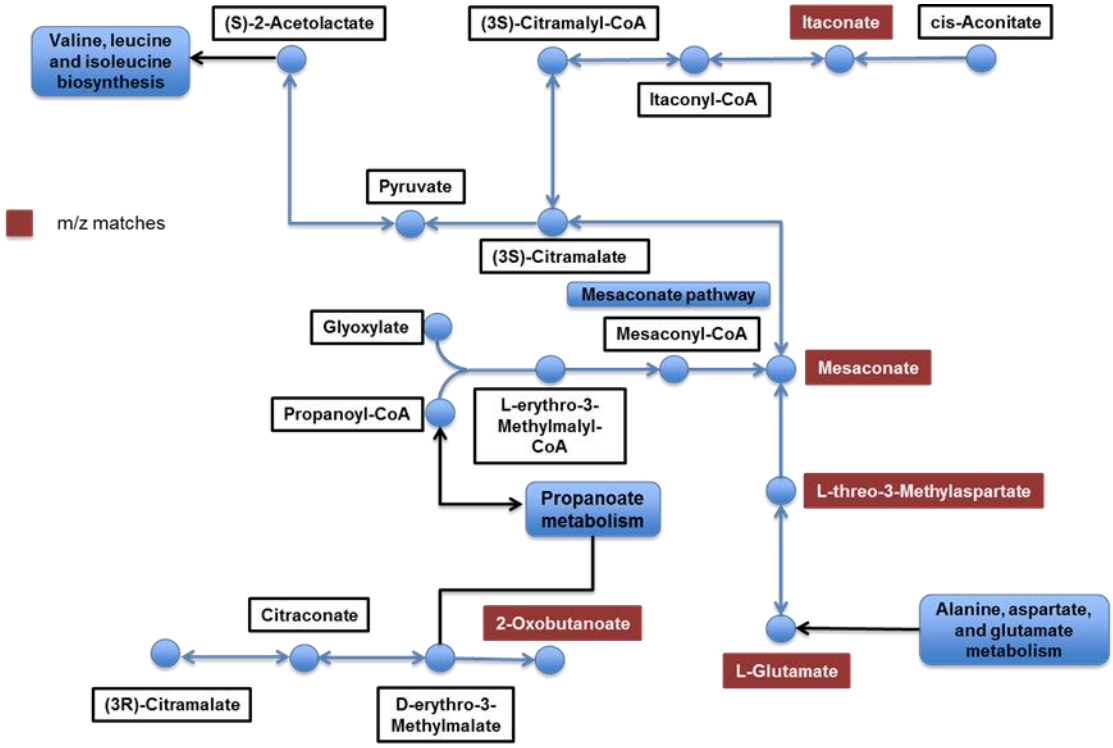

Figure 5.5. Schematic of C_5-branched dibasic acid metabolism of *Mtb* based on the KEGG reference pathway. Red boxes depict *m/z* matches found in plasma of patients with TB disease.

Chapter 6: SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Key findings

There is a need to develop culturally-sensitive food intake assessment instruments that more accurately estimate intake of specific nutrient in low and medium resource countries. In support of the randomized clinical trial of high-dose vitamin D outlined in this dissertation, we developed and validated such a tool. In order to capture serial macro- and micronutrient intake over time, a new instrument was required, as there was no previous country-wide food or nutrient intake data or dietary intake assessment tool used in the country of Georgia. Our hybrid design incorporated an interviewer/interviewee-based three-day food intake record and included specific prompts to capture common Georgian foods. It was designed to take advantage of the daily visits of the TB disease subjects for their DOTS therapy in the study clinic, and to account for the fact, that traditional dietary records and 24-hour recalls were not practical given lack of prior training of our co-investigators in nutritional assessment and the absence of dietitian health professions and nutrition-related research in Georgia. Training the Georgian research team utilized a variety of methods to ensure complete understanding of both nutrition assessment in general as well as conducting interviews using the new instrument. Our web-based CRF provided the ability for real-time quality control assessment of the dietary intake record data.

The published validation study provides confidence in the tool for estimation of the macronutrients and most micronutrients compared to conventional 24-hour recall data. The mean difference between assessment methods for most nutrients was small and the interclass correlation coefficient and Pearson R estimates were congruent with other such validation studies in the literature. The nutrient intake assessment instrument was instrumental in obtaining the chapter 4 data on macronutrient intake and body composition changes over the initial 16-week period after diagnosis of pulmonary TB

disease. We were able to obtain novel serial data on macronutrient intake coupled with body composition in MDR-TB patients. This added to the very limited literature on serial dietary intake and body composition in TB disease in our relatively large cohort of 191 TB subjects. A small group of household contacts was used as a relative comparison group. In addition, we tested for the first time whether a regimen of high-dose vitamin D influences either macronutrient intake or body composition.

Adjunctive high-dose vitamin D therapy did not affect changes in macronutrient intake or body composition over the course of treatment. However, total calorie, protein and fat intake increased over the 16-week period in TB patients overall. Body composition also improved over time with concurrent increases in body weight, BMI, fat mass and fat-free mass. The data show that despite TB subjects overall being predominantly male there was still a difference within TB status for body composition indexes, likely due to the catabolic nature of TB disease. In addition, for the first time, MDR-TB subjects were compared to drug-sensitive TB subjects in terms of nutrient intake and body composition over time. There were no differences in terms of age, gender, income, employment or marital status between MDR-TB and drug-sensitive TB, but differences in current smoking was observed. Despite the fact that MDR-TB patients consumed similar amounts of macronutrients per kilogram body weight, they did exhibit concomitantly lower body weight and fat-free mass over time compared to their drug-sensitive TB counterparts, even when adjusted for smoking status. Further analysis provided information on the relationship between calorie and protein intake and body composition. These data showed no nutritionally relevant change in body weight, BMI or fat-free mass as a function of either calorie or protein intake over time.

Chapter 5 outlines our novel high-resolution LC-MS plasma metabolomics data in a sample of 17 patients with TB disease and their matched household contacts without apparent TB disease. This pilot study was designed to explore the utility of plasma

metabolomics analysis to differentiate subjects with active TB disease from reference subjects and to determine metabolic features that reflect possible host-*Mtb* metabolic interactions. Our pilot study builds on the very limited human data to date in metabolomics research in patients with TB and is, to our knowledge, the first study to use high-resolution metabolomics methods in plasma from these individuals. We found that 61 plasma metabolites conclusively distinguished subjects with or without TB disease.

In this study, mean three-day daily total calorie and carbohydrate intake was significantly higher per kilogram body weight in TB disease subjects than their household contacts, and total fat and protein intake tended to be higher. Dietary intake may not explain the overall metabolite profile that discriminated the two groups, but it is possible that the increased triglyceride match found in TB disease subjects was related to their higher recent fat and/or carbohydrate intake. Also, the increased choline metabolite in TB disease subjects could have been due, in part, to increased intake/kg body weight of choline-rich foods. We identified glutamate as being one of the 61 differentiating metabolites between subjects with TB disease and controls. However, the modestly higher amino acid intake derived from increased overall dietary protein in the subjects with TB disease versus the healthy household contacts is highly unlikely to account for a single amino acid such as glutamate. It is possible that higher glutamate concentrations in TB disease patients are due to increased production and use alternative energy-generating metabolic pathways in the *Mtb* organism itself. Novel findings from this study should inform future research in TB disease given that high-resolution plasma metabolomics distinguished patients with active TB disease from household contacts without TB disease. In addition, in the plasma of TB patients relative to household controls, we detected an upregulation of confirmed specific resolvins (likely a

pathophysiologic response to TB disease) and two *Mtb* cell wall components (either a function of *Mtb* killing by the anti-TB drugs or a marker of TB infectious disease burden).

The phospholipid metabolites (phosphatidylserine and phosphatidylinositol), which increased in TB disease, are general cell membrane constituents and their elevation may reflect, in part, a lipolytic catabolic response to acute TB disease. However, the phosphatidylinositol (PI) was linked to other *Mtb* cell wall components (trehalose-6-mycolate) in most TB subjects, showing that the PI is associated with *Mtb* and not general cell membranes. Although further analysis would be needed for absolute determination, our metabolomics study results did not appear to be a function of individual diets of subjects with TB or the healthy controls. The major conclusion is that this proof of principle study suggests the utility of high-resolution LC-MS-based plasma metabolomics to differentiate adults with TB disease and apparently healthy controls. Multiple metabolites relevant to *Mtb* disease and its unique metabolism were identified, as well as D-series resolvins that may reflect a pathophysiologic response to TB disease in humans.

Strengths and limitations

Collectively, this series of investigations provide novel contributions and methodologies to the study of human TB derived from well-characterized cohorts from a randomized controlled trial of high-dose vitamin D_3 supplementation in Georgia, a country with a moderately high burden of TB and a high burden of MDR-TB. These findings can inform future studies in this patient population on nutritional interventions and ultimately better nutritional guidelines for individuals with active pulmonary TB. In addition, the metabolomics study identifies potentially useful plasma biomarkers that could be tested in future validation studies in subjects with both latent TB and TB disease. The culturally-sensitive nutrient intake assessment tool provides a picture of the habitual diet in a previously little-studied culture in terms of dietary intake of patients with TB. In addition, the research contained a cohort of patients with MDR-TB where serial dietary intake and body composition has not been previously studied in any country. To compliment this, metabolomics studies were performed in patients with concomitant available recent dietary intake data. Future analyses will include exploration via metabolome wide association studies of various nutrients in a larger sample.

There are several limitations of these dissertation studies. The published data on the nutrient intake assessment instrument are limited by our small sample size (albeit similar to previous such food/nutrient intake instrument comparison/validation studies), known measurement error in dietary recalls, assumptions made about content of certain food items and the fact that 72-hour dietary recall is a novel method of assessment. Also the validation strategy could have been simplified by use of three conventional 24-hour recalls and reproducibility was not measured. Although the instrument appears to be an accurate method to estimate nutrient intake in TB disease patients in the Tbilisi clinical setting, it is unknown whether the tool is valid among healthy or rural Georgians

or those with other diseases. Results from the validation studies showed the instrument likely overestimates total calorie intake. However, an unpublished pre and post knowledge intervention study conducted in 16 healthy physicians and nurses in Tbilisi showed a lower mean calorie intake (~1400 kcal/day). This warrants further investigation of this instrument in a healthy Georgian population.

The subjects studied for both the validation of the nutrient assessment tool and the metabolic profiling were small convenience samples. Due to the novelty of these methods in this particular patient population, I was unable to perform power calculations. Bioelectrical impedance analysis may be seen as a limitation of my body composition data although well-validated against gold-standard methods of body composition measures such as DXA in malnourished patients.[184] Although a multi-compartmental model would be the most accurate way of determining body composition in humans, this was not practical in the medium resource country in which I performed these studies due to financial and practical restrictions.[185] Limitations of the metabolomics study include the cross-sectional nature, the small cohort size overall, and the low number of patients studied with MDR-TB; thus, the results are subject to bias and/or random enrichment of metabolites in the TB disease group. In addition, while the household contacts did not have apparent active TB disease and were sputum culture-negative, no testing was carried out to assess whether these individuals harbored latent TB infection. Nonetheless, based on my hypothesis-generating data, high-resolution plasma metabolomics would be of interest to study in patients with presumed latent TB infection and to predict relapse after anti-TB drug therapy. Another limitation of the metabolomics pilot study is that absolute identity of most metabolites in the discriminating biosignature was not confirmed by tandem MS/MS studies. In addition, metabolites in plasma should be considered an indirect measure of *Mtb*, host metabolism and local tissue response to infection and I have no data from patient sputum or the *Mtb* organism itself.

Implications of this research

The developmental strategies and methodologies for the Georgian nutrient assessment instrument may be a potentially useful model for investigators in other countries requiring dietary intake data, particularly in those settings that lack food composition tables or other means of collecting these data. In addition, the utilization of the robust NDS-R software for nutrient analysis in Georgia, where habitual consumption of fresh, minimally processed foods is routine, appears an advantage, especially when there is minimal or no food fortification policy in-country.

Findings regarding increased dietary macronutrient intake and changes in body composition over time in patients with TB disease may provide direction for future studies to define nutrition guidelines during treatment of TB where none currently exist. Due to the catabolic nature of the disease, increased intake of certain nutrients (e.g. high-quality protein for amino acids and fat sources for energy) have the potential to improve body composition toward greater lean body mass, and potentially improve both short and long-term outcomes, but such hypotheses need to be tested in a rigorous manner. Unfortunately, the nutrient intervention studies in TB patients to date have been of lower quality in general and have not conclusively identified specific effective macronutrient or micronutrient interventions for individuals with pulmonary TB disease.[16,20,26,81] Future nutrient intervention studies ideally should be double-blind, well-controlled with clearly defined, consistent outcomes such as TB clearance or TB cure. Interventions of interest would include the impact of longitudinal increases in overall macronutrient intake beyond what is required for normal weight maintenance and increased amounts of high-biological value complete protein sources during the entire course of TB disease through to recovery/cure/relapse in individuals diagnosed with MDR-TB. My approach in developing culture-sensitive nutrient intake assessment tools, comprehensively following serial nutrient intake and body composition in TB disease

subjects from diagnosis over time and use of high-resolution plasma metabolomics could serve as models for development of such studies in populations of TB disease patients in other countries, including those with a high burden of HIV co-infection.[7]

Prospective and confirmatory studies are needed to determine whether metabolomics profiling is a useful method for new biomarker discovery and/or to gain pathophysiologic insight before and after initiation of conventional anti-TB drug therapy and during the various stages of TB disease, that may include recovery, cure and relapse. Studies to determine whether plasma metabolic profiling is useful to predict development of TB disease in individuals with latent TB, and whether verified single metabolites or metabolite biosignatures can identify subjects with drug-sensitive versus MDR-TB and predict the responses to new anti-TB therapies will also be of interest.

Future directions

A variety of new research questions stem from this dissertation research. One example is to determine whether my methods for developing a culturally-sensitive nutrition assessment tool for a developing country with limited nutrition-related resources and expertise can be applied to similar countries. Further studies on dietary intake and TB are required in other countries with a high TB burden and this method could be useful in obtaining habitual dietary intake over time in these populations. We have collected, but have yet to analyze, dietary micronutrient intake over time in this TB disease cohort, as well as plasma concentrations of zinc, copper, iron, manganese, minerals, retinol and tocopherols in a subset of subjects. Such data, when published will also be of interest to inform future studies in TB disease.

My findings from Chapter 4, coupled with data from other studies, suggest that supplying nutrient-rich whole food interventions in areas with food insecurity and improving the quality of dietary macronutrients (e.g. protein) in populations with adequate food availability may be fruitful areas of research to potentially improve TB clearance, recovery and prevention of TB relapse. However, such rigorous nutritional intervention studies have largely not been performed. In the country of Georgia, I could not address questions related to food insecurity, which was not prevalent. The metabolomics data generate hypotheses that can be addressed using the serial plasma biobank from the 191 subjects with TB disease studied in the RCT. We have developed a case control study from the RCT to answer the following aims:

> **Aim 1**: To determine the utility of metabolomic analysis to differentiate drug-sensitive TB vs MDR-TB, we will perform metabolomic analysis on baseline plasma samples from 23 documented MDR-TB patients and 46 drug-sensitive TB patients, clinically matched to the MDR-TB cohort (2:1 case control design).

Aim 2: To determine the utility of metabolomics analysis to predict TB clearance responses to conventional anti-TB therapy (sputum culture clearance over time), we will study serial metabolic profiles in both drug-sensitive TB and MDR-TB patients.

Aim 3: To determine the impact of high-dose vitamin D_3 on metabolism, we will study serial plasma metabolomic profiles in subjects receiving vitamin D supplementation versus placebo.

By utilizing the robust completed RCT with multiple plasma samples over the first 16 weeks of treatment I can determine metabolic profile differences between MDR-TB and drug-susceptible TB, differences in metabolic profiles as a function of time to culture conversion, and the impact of high-dose vitamin D therapy in this sample.

References

1. Zumla A, Raviglione M, Hafner R, von Reyn CF. Tuberculosis. *N. Engl. J. Med.* 2013;368(8):745-755.

2. *Nobel Lectures Physiology or Medicine 1901-1921.* Amsterdam: Elsevier Publishing Company; 1967.

3. Hippocrates. Aphorisms. 460 BC.

4. Haas F, Haas SS. The origins of *Mycobacterium tuberculosis* and the notion of its contagiousness. *Tuberculosis*. Boston: Little Brown and Co; 1996.

5. McCarthy OR. The key to the sanatoria. *J. R. Soc. Med.* 2001;94(8):413-417.

6. Comstock G. The International Tuberculosis Campaign: a pioneering venture in mass vaccination and research. *Clin. Infect. Dis.* 1994;19(3):528-540.

7. World Health Organization. *Global tuberculosis report 2013.* Geneva2013.

8. Lawn SD, Zumla AI. Tuberculosis. *The Lancet.* 2011;378:57-72.

9. UNAIDS. AIDSinfo, country profiles. 2012; http://www.unaids.org/en/dataanalysis/datatools/aidsinfo/. Accessed January 16, 2014, 2014.

10. World Health Organization. WHO Country profile. 2012; http://www.who.int/countries/geo/en/ Accessed May 3, 2012.

11. Khan A, Sterling TR, Reves R, Vernon A, Horsburgh CR, Tuberculosis Trials Consortium. Lack of weight gain and relapse risk in a large tuberculosis treatment trial. *Am. J. Respir. Crit. Care Med.* 2006;174:344-348.

12. Bhargava A, Chatterjee M, Jain Y, et al. Nutritional status of adult patients with pulmonary tuberculosis in rural central India and its association with mortality. *PLoS One.* 2013;8(10):e77979.

13. Benova L, Fielding K, Greig J, et al. Association of BMI category change with TB treatment mortality in HIV-positive smear-negative and extrapulmonary TB patients in Myanmar and Zimbabwe. *PLoS One.* 2012;7(4):e35948.

14. PrayGod G, Range N, Faurholt-Jepsen D, et al. Weight, body composition and handgrip strength among pulmonary tuberculosis patients: a matched cross-sectional study in Mwanza, Tanzania. *Trans. R. Soc. Trop. Med. Hyg.* 2011;105:140-147.

15. Paton NI, Ng Y. Body composition studies in patients with wasting associated with tuberculosis. *Nutrition.* 2006;22:245-251.

16. Sudarsanam TD, John J, Kang G, et al. Pilot randomized trial of nutritional supplementation in patients with tuberculosis and HIV-tuberculosis coinfection receiving directly observed short-course chemotherapy for tuberculosis. *Trop. Med. Int. Health.* 2011;16(6):699-706.

17. Schaible UE, Kaufmann SH. Malnutrition and infection: complex mechanisms and global impacts. *PLoS Med.* 2007;4(5):e115.

18. Lonnroth K, Williams BG, Cegielski JP, Dye C. A consistent log-linear relationship between tuberculosis incidence and body mass index. *Int. J. Epidemiol.* 2010;39(1):149-155.

19. Macallan DC. Malnutrition in tuberculosis. *Diagn. Microbiol. Infect. Dis.* 1999;34(2):153-157.

20. Mupere E, Parraga IM, Tisch DJ, Mayanja HK, Whalen CC. Low nutrient intake among adult women and patients with severe tuberculosis disease in Uganda: a cross-sectional study. *BMC Public Health.* 2012;12:1050.

21. Gler MT, Guilatco R, Caoili JC, Ershova J, Cegielski JP, Johnson JL. Weight gain and response to treatment for multidrug-resistant tuberculosis. *Am. J. Trop. Med. Hyg.* 2013;89(5):943-949.

22. Nansera D, Bajunirwe F, Elyanu P, Asiimwe C, Amanyire G, Graziano FM. Mortality and loss to follow-up among tuberculoissi and HIV co-infected patients in rural soutwestern Uganda. *Int. J. Tuberc. Lung Dis.* 2012;16(10):1371-1376.

23. Zachariah R, Spielmann MP, Harries AD, Salaniponi FM. Moderate to severe malnutrition in patients with tuberculosis is a risk factor associated with early death. *Trans. R. Soc. Trop. Med. Hyg.* 2002;96(3):291-294.

24. Jubulis J, Kinikar A, Ithape M, et al. Modifiable risk factors assocatied with tuberculosis disease in children in Pune, India. *Int. J. Tuberc. Lung Dis.* 2014;18(2):198-204.

25. Harling G, Castro MC. A spatial analysis of social and economic determinents of tuberculosis in Brazil. *Health Place.* 2014;25:56-67.

26. Sinclair D, Abba K, Grobler L, Sudarsanam TD. Nutritional supplements for people being treated for active tuberculosis. *The Cochrane Library.* 2011;Nov 9(11).

27. Koyanagi A, Kuffo D, Gresely L, Shenkin A, Cuevas LE. Relationships between serum concentrations of C-reactive protein and micronutrients, in patients with tuberculosis. . *Ann. Trop. Med. Parasitol.* 2004;98(4):391-399.

28. Range N, Changalucha J, Krarup H, Magnussen P, Andersen AB, Friis H. The effect of multi-vitamin/mineral supplementation on mortality during treatment of pulmonary tuberculosis: a randomised two-by-two factorial trial in Mwanza, Tanzania. *J. Nutr.* 2006;95(4):762-770.

29. Ross AC. Vitamin A and retinoic acid in T cell-related immunity. *American Journal of Clinical Nutrition.* 2012;96(5):1166S-1172S.

30. Greenstein RJ, Su L, Brown ST. Vitamins A & D inhibit the growth of mycobacteria in radiometric culture. *PLoS One.* 2012;7(1).

31. Saper RB, Rash R. Zinc: an essential micronutrient. *Am. Fam. Physician.* 2009;79(9):768-772.

32. Desai NS, Tukvadze N, Frediani JK, et al. Effects of sunlight and diet on vitamin D status of pulmonary tuberculosis patients in Tbilisi, Georgia. *Nutrition.* 2012;28(4):362-366.

33. Karyadi E, West CE, Schultink W, et al. A double-blind, placebo-controlled study of vitamin A and zinc supplementation in persons with tuberculosis in Indonesia: effects on clinical response and nutritional status. *American Journal of Clinical Nutrition.* 2002;75(4):720-727.

34. Lawson L, Thacher TD, Yassin MA, et al. Randomized controlled trial of zinc and vitamin A as co-adjuvants for the treatment of pulmonary tuberculosis. *Trop. Med. Int. Health.* 2010;15(12):1481-1490.

35. Kassu A YT, Mahmud ZH, Mohammad A, Nguyen N, Huong BT, Hailemariam G, Diro E, Ayele B, Wondmikun Y, Motonaka J, Ota F. Alterations in serum levels of trace elements in tuberculosis and HIV infections. *Eur. J. Clin. Nutr.* 2006;60(5):580-586.

36. Cernat RI, Mihaescu T, Vornicu M, Vione D, Olariu RI, Arsene C. Serum trace metal and ceruloplasmin variability in individuals treated for pulmonary tuberculosis. *Int. J. Tuberc. Lung Dis.* 2011;15(9):1239-1245.

37. Wolschendorfa F, Ackartb D, Shresthac TB, et al. Copper resistance is essential for virulence of Mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U. S. A.* 2011;108(4):1621-1626.

38. Beck MA. Selenium and host defence towards viruses. *Proc. Nutr. Soc.* 1999;58(3):707-711.

39. van Lettow M, West CE, van der Meer JWM, Wieringa FT, Semba RD. Low plasma selenium concentrations, high plasma human immunodeficiency virus load and high interleukin-6 concentrations are risk factors associated with anemia in adults presenting with pulmonary tuberculosis in Zomba district, Malawi *Eur. J. Clin. Nutr.* 2005;59(4):526-532.

40. Seyedrezazadeh E, Ostadrahimi A, Mahboob S, Assadi Y, Ghaemmagami J, Pourmogaddam M. Effect of vitamin E and selenium supplementation on oxidative stress status in pulmonary tuberculosis patients. *Respirology.* 2008;13(2):294-298.

41. Hemila H, Kaprio J. Vitamin E supplementation may transiently increase tuberculosis risk in males who smoke heavily and have high dietary vitamin C intake. *Br. J. Nutr.* 2008;100(4):896-902.

42. Centers for Disease Control and Prevention. Basic TB Facts. [web page]. 2012; http://www.cdc.gov/TB/TOPIC/basics/default.htm. Accessed January 15, 2014, 2014.

43. Frieden RT, Sterling TR, Munsiff SS, Watt CJ, Dye C. Tuberculosis. *Lancet.* 2003;362(9387):887-899.

44. Middlekoop K, Bekker LG, Shashkina E, Kreiswirth B, Wood R. Retreatment tuberculosis in a South African community: the role of re-infection, HIV and antiretroviral treatment. *Int. J. Tuberc. Lung Dis.* 2012;16(11):1510-1516.

45. Gonzalez-Curiel I, Castañeda-Delgado J, Lopez-Lopez N, et al. Differential expression of antimicrobial peptides in active and latent tuberculosis and its relationship with diabetes mellitus. *Hum. Immunol.* 2011;72(8):656-662.

46. Mack U, Migliori GB, Sester M, et al. LTBI: latent tuberculosis infection or lasting immune responses to M. tuberculosis? A TBNET consensus statement. *Eur. Respir. J.* 2009;33(5):956-973.

47. Malech HL, Deleo FR, Quinn MT. The role of neutrophils in the immune system: an overview. *Methods Mol. Biol.* 2014;1124:3-10.

48. Holick MF. Vitamin D deficiency. *N. Engl. J. Med.* 2007;357:266-281.

49. Liu PT, Stenger S, Li H, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science.* 2006;311:1770-1773.

50. Liu PT, Krutzik SR, Modlin RL. Therapeutic implications of the TLR and VDR partnership. *Trends Mol. Med.* 2007;13:117-124.

51. Durr UHN, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochim. Biophys. Acta.* 2006;1758:1408-1425.

52. Liu PT, Stenger S, Tang DH, Modlin RL. Cutting edge: vitamin D-mediated human antimicrobial activity against Mycobacterium tuberculosis is dependent on the induction of cathelicidin. *The Journal of Immunology.* 2007;179:2060-2063.

53. Tangpricha V PE, Chen TC, Holick MF. Vitamin D insufficiency among free-living healthy young adults. *Am. J. Med.* 2002;112:659-662.

54. Mithal A, Wahl DA, Bonjour JP, et al. Global vitamin D status and determinants of hypovitaminosis D. *Osteoporos. Int.* 2009;20(11):1807-1820.

55. Kim JH, Park J, Cho Y, et al. Low serum 25-hydroxyvitamin D level: An independent risk factor for tuberculosis? *Clin. Nutr.* 2013.

56. Mehta S, Mugusi FM, Bosch RJ, et al. Vitamin D status and TB treatment outcomes in adult patients in Tanzania: a cohort study. *BMJ Open.* 2013;3:e003703.

57. Rathored J, Sharma B, Singh JN, et al. Risk and outcome of multidrug-resistant tuberculosis: vitamin D receptor polymorphisms and serum 25(OH)D. *Int. J. Tuberc. Lung Dis.* 2012;16(11):1522-1528.

58. Salahuddin N, Ali F, Hasan Z, Rao N, Aqeel M, Mahmood F. Vitamin D accelerates clinical recovery from tuberculosis: results fo the SUCCINCT Study [Supplementary Cholecalciferol in recovery from tuberculosis]. A randomized, placebo-controlled, clinical trial of vitamind D supplementation in patients with pulmonary tuberculosis. *BMC Infect. Dis.* 2013;13(22):1-11.

59. Yu X, Li C, Hong W, Pan W, Xie J. Autophagy during mycobacterium tuberculosis infection and implications for future tuberculosis medications. *Cell. Signal.* 2013;epub ahead of print.

60. Waters WR, Palmer MV, Nonnecke BJ, Whipple DL, Horst RL. Mycobacterium bovis infection of vitamin D-deficient NOS2-/- mice. *Microb. Pathog.* 2004;36:11-17.

61. Martineau AR, Honecker FU, Wilkinson RJ, Griffiths CJ. Vitamin D in the treatment of pulmonary tuberculosis. *J. Steroid Biochem. Mol. Biol.* 2007;103:793-798.

62. Grange JM, Davies PO, Brown RC, Woodhead JS, Kardjito T. A study of vitamin D levels in Indonesian patients with untreated pulmonary tuberculosis. *Tubercle.* 1985;66:187-191.

63. Stead WW, Senner JW, Reddick WT, Lofgren JP. Racial differences in susceptibility to infection by *Mycobacterium tuberculosis*. *N. Engl. J. Med.* 1990;322:422-427.

64. Cegielski JP, McMurray ON. The relationship between malnutrition and tuberculosis: evidence from studies in humans and experimental animals. *Int. J. Tuberc. Lung Dis.* 2004;8:286-298.

65. Ustianowski A, Shaffer R, Collin S, Wilkinson RJ, Davidson RN. Prevalence and associations of vitamin D deficiency in foreign-born persons with tuberculosis in London. *J. Infect.* 2005;50:432-437.

66. Wejse C, Olesen R, Rabna P, et al. Serum 25-hydroxyvitamin D in a West African population of tuberculosis patients and unmatched healthy controls. *American Journal of Clinical Nutrition.* 2007;86:1376--1383.

67. Ong PY, Ohtake T, Brandt C, et al. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* 2002;347:1151-1160.

68. Ramanathan B, Davis EG, Ross CR, Blecha F. Cathelicidins: microbicidal activity, mechanisms of action, and roles in innate immunity. *Microbes and Infection.* 2002;4:361-372.

69. Schauber J, Dorschner RA, Coda AB, et al. lnjury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J. Clin. Invest.* 2007;117:803-901.

70. Zasloff M. Fighting infections with vitamin D. *Nat. Med.* 2006;12:388-390.

71. Klug-Micu GM, Stenger S, Sommer A, et al. CD40 ligand and interferon gamma induce an antimicrobial response against Mycobacterium tuberculosis in human monocytes. *Immunology.* 2013;139(1):121-128.

72. Nursyam EW, Amin Z, Rumende CM. The effect of vitamin D as supplementary treatment in patients with moderately advanced pulmonary tuberculous lesion. *Acta Medica Indonesia.* 2006;38:3-5.

73. Wejse C, Gomes VF, Rabna P, et al. Vitamin D as supplementary treatment for tuberculosis. *Am. J. Respir. Crit. Care Med.* 2009;179:843-850.

74. Frediani JK, Tukvadze N, Sanikidze E, et al. A culture-specific nutrient intake assessment instrument in patients with pulmonary tuberculosis. *Clin. Nutr.* 2013.

75. Martineau AR, Nhamoyebonde S, Oni T, et al. Reciprocal seasonal variation in vitamin D status and tuberculosis notifications in Cape Town, South Africa. *Proc. Natl. Acad. Sci. U. S. A.* 2011;108(47):19013-19017.

76. Martineau AR, Timms PM, Bothamley GH, et al. High-dose vitamin D3 during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomized controlled trial. *Lancet.* 2011;377:242-250.

77. Martineau AR, Wilkinson RJ, Wilkinson KA, et al. A single dose of vitamin D enhances immunity to mycobacteria. *Am. J. Respir. Crit. Care Med.* 2007;176:208-213.

78. Jones DP, Park Y, Ziegler TR. Nutritional metabolomics: progress in addressing complexity in diet and health. *Annu. Rev. Nutr.* 2012;32:183-202.

79. Teixeira JA, Baggio ML, Giuliano AR, Fisberg RM, Marchioni DM. Performance of the quantitative food frequency questionnaire used in the Brazilian center of the prospective study Natural History of Human Papillomavirus Infection in Men: The HIM Study. *Journal of the American Dietetic Association.* 2011;111(7):1045-1051.

80. Cade JE, Burley VJ, Warm DL, Thompson RL, Margetts BM. Food-frequency questionnaires: a review of their design, validation, and utilisation. *Nutrition Research Reviews.* 2004;17:5-22.

81. Paton NI, Chua Y, Earnest A, Chee CBE. Randomized controlled trial of nutritional supplementation in patients with newly diagnosed tuberculosis and wasting. *American Journal of Clinical Nutrition.* 2004;80(2):460-465.

82. Masson LF, McNeill G, Tomany JO, et al. Statistical approaches for assessing the relative validity of a food-frequency questionnaire: use of correlation coefficients and the kappa statistic. *Public Health Nutr.* 2003;6(3):313-321.

83. Bisson GP, Mehaffy C, Broeckling C, et al. Upregulation of the phthiocerol dimycocerosate biosynthetic pathway by rifampin-resistant, rpoB mutant Mycobacterium tuberculosis. *J. Bacteriol.* 2012;194(23):6441-6452.

84. Weiner J, Parida SK, Maertzdorf J, et al. Biomarkers of inflammation, immunosuppression and stress with active disease are revealed by metabolomic profiling of tuberculosis patients. *PLoS One.* 2012;7(7):e40221.

85. du Preez I, Loots DT. New sputum metabolite markers implicating adaptations of the host to Mycobacterium tuberculosis, and vice versa. *Tuberculosis (Edinb).* 2013;93(9):330-337.

86. Somashekar BS, Amin AG, Tripathi P, et al. Metabolomic signatures in guinea pigs infected with epidemic-associated W-Beijing strains of Mycobacterium tuberculosis. *J. Proteome Res.* 2012;11(10):4876-4884.

87. Che N, Cheng J, Li H, et al. Decreased serum 5-oxoproline in TB patients is associated with pathological damage of the lung. *Clin. Chim. Acta.* 2013;423:5-9.

88. Gonzalez-Martin J, Garcia-Garcia JM, Anibarro L, et al. Consensus document on the diagnosis, treatment and prevention of tuberculosis. *Arch. Bronconeumol.* 2010;45(5):255-274.

89. Lonnroth K, Castro KG, Chakaya JM, et al. Tuberculosis control and elimination 2010-50: cure, care, and social development. *Lancet.* 2010;375:1814-1829.

90. Wallis RS, Pai M, Menzies D, et al. Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. *Lancet.* 2010;375(1920-1937).

91. Jacobsen M, Repsilber D, Gutschmidt A, et al. Candidate biomarkers ofr discrimination between infection and disease caused by *Mycobacterium tuberculosis*. *J. Mol. Med.* 2007;85(6):613-621.

92. Maertzdorf J, Repsilber D, Parida SK, et al. Human gene expression profiles of susceptibility and resistance in tuberculosis. *Genes Immun.* 2011;12(1):15-22.

93. Zhou A, Ni J, Xu Z, et al. Application of ^{1}H NMR spectroscopy-based metabolomics to sera of tuberculosis patients. *J. Proteome Res.* 2013;12:4642-4649.

94. du Preez I, Loots DT. Altered fatty acid metabolism due to rifampicin-resistance conferring mutations in the *rpoB* gene of *Mycobacterium tuberculosis*: mapping the potential of pharmaco-metabolomics for global health and personalized medicine. *OMICS.* 2012;16(11):596-603.

95. Uppal K, Soltow QA, Strobel FH, et al. xMSanalyzer: automated pipline for improved feature detection and downstream analyisis of large-scale, non-targeted metabolomics data. *BMC Bioinformatics.* 2013;14(15).

96. Matts JP, Lachin JM. Properties of permuted-block randomization in clinical trials. *Control. Clin. Trials.* 1988;9:327-344.

97. Khazai NB, Judd SE, Jeng L, et al. Treatment and prevention of vitamin D insufficiency in cystic fibrosis patients: comparative efficacy of ergocalciferol, cholecalciferol, and UV light. *J. Clin. Endocrinol. Metab.* 2009;94(6):2037-2043.

98. Trang HM, Cole DE, Rubin LA, Pierratos A, Siu S, Vieth R. Evidence that vitamin D3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D2. *American Journal of Clinical Nutrition.* 1998;68:854-858.

99. Armas LA, Hollis BW, Heaney RP. Vitamin D2 is much less effective than vitamin D3 in humans. *J. Clin. Endocrinol. Metab.* 2004;89:5387-5391.

100. Nilsson SF, Ostberg L, Peterson PA. Binding of vitamin D to its human carrier plasma protein. *Biochem. Biophys. Res. Commun.* 1972;46:1380-1387.

101. Parsons LM, Somoskovi A, Gutierrez C, et al. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. *Clin. Microbiol. Rev.* 2011;24(2):314-350.

102. Tukvadze N, Kempker RR, Kalandadze I, et al. Use of a molecular diagnostic test in AFB smear positive tuberculosis suspects greatly reduces time to detection of multidrug resistant tuberculosis. *PLoS One.* 2012;7.

103. Harnack L, Stevens M, van Heel N, Schakel S, Dwyer JT, Himes J. A computer-based approach for assessing dietary supplement use in conjuction with dietary recalls. *Journal of Food Composition and Analysis.* 2008;21:S78-82.

104. Willett W. *Nutritional Epidemiology.* New York: Oxford University Press; 1998.

105. Martin GS, Tapsell LC, Denmeade S, Batterham MJ. Relative validity of a diet history interview in an intervention trial manipulating dietary fat in the management of Type II diabetes mellitus. *Preventive Medicine.* 2003;36(4):420-428.

106. Schroder H, Covas MI, Marrugat J, Vila J, Pena A, Alcantara MR. Use of a three-day estimated food record, a 72-hour recall and a food-frequency questionnaire for dietary assessment in a Mediterranean Spanish population. *Clin. Nutr.* 2001;20(5):429-437.

107. Shal I, Rosner BA, Shahar DR, et al. Dietary evaluation and attenuation of relative risk: Multiple comparisons between blood and urinary biomarkers,food frequency, and 24-hour recall questionnaires: the DEARR Study. *J. Nutr.* 2005;135(3):573-579.

108. Cardoso MA, Kida AA, Tomita LY, Stocco PR. Reproducibility and validity of a food frequency questionnaire among women of Japanese ancestry living in Brazil. *Nutrition Research.* 2001:725-733.

109. Bland JM, Altman DG. Measuring agreement in method comparison studies. *Stat. Methods Med. Res.* Jun 1999;8(2):135-160.

110. Bartko JJ. The intraclass correlation coefficient as a measure of reliability. *Psychol. Rep.* 1966;19(1):3-11.

111. Hoaglin D, Mosteller F, Tukey J. *Understanding robust and exploratory data analysis.* New York: John Wiley & Sons; 1983.

112. Soltow QA, Strobel FH, Mansfield KG, Wachtman L, Park Y, Jones DP. High-performance metabolic profiling with dual chromatography-Fourier-transform mass spectrometry (DC-FTMS) for study of the exposome. *Metabolomics.* 2013;9(1):132-143.

113. Johnson JM, Yu T, Strobel FH, Jones DP. A practical approach to detect unique metabolic patterns for personalized medicine. *Analyst.* 2010;135:2864-2870.

114. Hodel EM, Zanolari B, Mercier T, et al. A single LC-tandem mass spectrometry method for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma. *Journal of chromatography B: Analytical technologies in the biomedical and life sciences.* 2009;877(10):867-886.

115. Aronson KJ, Wilson JW, Hamel M, et al. Plasma organochlorine levels and prostate cancer risk. *Journal of Exposure Science and Environmental Epidemiology.* 2010;20(5):434-445.

116. Yang R, Chiang N, Oh SF, Serhan CN. Metabolomics-lipidomics of eicosanoids and docosanoids generated by phagocytes. *Curr. Protoc. Immunol.* 2011;Chapter 14(Unit 14.26).

117. Dalli J, Serhan CN. Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators. *Blood.* 2012;120:e60-e72.

118. Levy BD, Serhan CN. Resolution of acute inflammation in the lung. *Annu. Rev. Physiol.* 2014.

119. Spite M, Claria J, Serhan CN. Resolvins, specialized pro-resolving lipid mediators, and their potential roles in metabolic disease. *Cell Metabolism.* 2014;19(1):21-36.

120. Yu T, Park Y, Li S, Jones DP. Hybrid feature detection and information accumulation using high-resolution LC-MS metabolomics data. *J. Proteome Res.* 2013;12:1419-1427.

121. Smyth GK. Limma: linear models for microarray data. In: Gentleman VCR, Dudoit S, Irizarry R, Huber W, eds. *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. New York2005:397-420.

122. Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. *Stat. Med.* 1990;9:811-818.

123. Scalbert A, Brennan L, Fiehn O, et al. Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. *Metabolomics.* 2009;5(4):435-458.

124. Roede JR, Uppal K, Park Y, et al. Serum metabolomics of slow vs. rapid motor progression Parkinson's disease: a pilot study. *PLoS One.* 2013;8(10):377629.

125. Osborn MP, Park Y, Parks MB, et al. Metabolome-wide association study of neovascular age-related macular degeneration. *PLoS One.* 2013;8(8):e72737.

126. Smith CA, O-Maille G, Want EJ, et al. METLIN: a metabolite mass spectral database. *Ther. Drug Monit.* 2005;27(6):747-751.

127. Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* 1999;1(27):29-34.

128. Wishart DS, Knox C, Guo AC, et al. HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res.* 2009;37(Database):D603-610.

129. Godfrey AR, Brenton AG. Accurate mass measurements and their appropriate use for reliable analyte identification. *Analytical and Bioanalytical Chemistry.* 2012;404:1159-1166.

130. Khan NC, Mai LB, Hien VTT, et al. Development and validation of food frequency questionnaire to assess calcium intake in postmenopausal Vietnamese women. *J. Nutr. Sci. Vitaminol. (Tokyo).* 2008;54(2):124-129.

131. Dixon LB, Subar AF, Wideroff L, Thompson FE, Kahle LL, Potischman N. Carotenoid and Tocopherol Estimates from the NCI Diet History Questionnaire are valid comared with multiple recalls and serum biomarkers. *J. Nutr.* 2006;136(12):3054-3061.

132. Ogawa K, Tsubono Y, Nishino Y, et al. Validation of a food-frequency questionnaire for cohort studies in rural Japan. *Public Health Nutr.* 2003;6(2):147-157.

133. Pandey D, Bhatia V, Boddula R, Singh HK, Bhatia E. Validation and reproducibility of a food frequency questionnaire to assess energy and fat intake in affluent north Indians. *Natl. Med. J. India.* 2005;18(5):230-235.

134. Biltoft-Jensen A, Matthiessen J, Rasmussen LB, Faqt S, Groth MV, Hels O. Validation of the Danish 7-day pre-coded food diary among adults: energy intake v. energy expeniture and recording length. *Br. J. Nutr.* 2009;102(12):1838-1846.

135. Lomtadze N, Aspindzelashvili R, Janjgava M, et al. Prevalence and risk factors for multidrug-resistant tuberculosis in the Republic of Georgia: a population-based study. *Int. J. Tuberc. Lung Dis.* 2009;13(1):68-73.

136. Lorente Ramos RM, Azpeitia Armán J, Arévalo Galeano N, Muñoz Hernández A, García Gómez JM, Gredilla Molinero J. Dual energy X-ray absorptimetry: fundamentals, methodology, and clinical applications. *Radiologia (Roma).* 2012;54(5):410-423.

137. Norman K, Stobäus N, Gonzalez MC, Schulzke JD, Pirlich M. Hand grip strength: outcome predictor and marker of nutritional status. *Clin. Nutr.* 2011;30(2):135-142.

138. Deutz NE, Wolfe RR. Is there a maximal anabolic response to protein intake with a meal? *Clin. Nutr.* 2013;32(2):309-313.

139. Richards DC, Mikiashvili T, Parris JJ, et al. High prevalence of hepatitis C virus but not HIV coinfection among patients with tuberculosis in Georgia. *Int. J. Tuberc. Lung Dis.* 2006;10:396-401.

140. Villamor E, Saathoff E, Mugusi F, Bosch RJ, Urassa W, Fawzi WW. Wasting and body composition of adults with pulmonary tuberculosis in relation to HIV-1 coinfection, socioeconomic status, and severity of tuberculosis. *Eur. J. Clin. Nutr.* 2006;60(2):163-171.

141. Mdivani N, Zangaladze E, Volkova N, et al. High Prevalence of Multidrug-Resistant Tuberculosis in Georgia. *Int. J. Infect. Dis.* 2008;12(6):635-644.

142. Tang Y, Hua SC, Qin GX, Xu LJ, Jiang YF. Different subsets of macrophages in patients with new onset tuberculous pleural effusion. *PLoS One.* 2014;9(2):e88343.

143. MacPherson P, Houben RMGJ, Glynn JR, Corbett EL, Kranzer K. Pre-treatment loss to follow-up in tuberculosis patients in low- and lower-middle-income countries and high-burden countries: a systematic review and meta-analysis *Bull. World Health Organ.* 2013.

144. World Health Organization. WHO Global Report. 2013; http://www.who.int/tb/publications/global_report/en/. Accessed February 25, 2013, 2013.

145. Zumla A, George A, Sharma V, Herbert N, Baroness Masham of Ilton. WHO's 2013 global report on tuberculosis: successes, threats, and opportunities. *Lancet.* 2013;382(9907):1765-1767.

146. Zumla A, Nahid P, Cole ST. Advances in the development of new tuberculosis drugs and treatment regimens. *Nature Reviews Drug Discovery.* 2013;12(5):388-404.

147. Brennan PJ. Structure, function, and biogenesis of the cell wall of Mycobacterium *tuberculosis*. *Tuberculosis.* 2003;83:91-97.

148. Bekmurzayeva A, Sypabekova M, Kanayeva D. Tuberculosis diagnosis using immunodominant, secreted antigens of Mycobacterium tuberculosis. *Tuberculosis.* 2013;93(4):381-388.

149. Lawn SD. Diagnosis of pulmonary tuberculosis. *Curr. Opin. Pulm. Med.* 2013;19(3):280-288.

150. North EJ, Jackson M, Lee RE. New approaches to target the mycolic acid biosynthesis pathway for the development of tuberculosis therapeutics. *Curr. Pharm. Des.* 2013.

151. Maertzdorf J, Weiner Jr, Kaufmann SH. Enabling biomarkers for tuberculosis control. *Int. J. Tuberc. Lung Dis.* 2012;16(9):1140-1148.

152. Wallis RS, Kim PC, S., Hanna D, et al. Tuberculosis biomarkers discovery: developments, needs, and challenges. *Lancet Infectious Disease.* 2013;13(4):362-372.

153. Park YH, Lee K, Soltow QA, et al. High-performance metabolic profiling of plasma from seven mammalian species for simultaneous environmental chemical surveillance and bioeffect monitoring. *Toxicology.* 2012;295(1-3):47-55.

154. Menni C, Fauman E, Erte I, et al. Biomarkers for type 2 diabetes and impaired fasting glucose using a nontargeted metabolomics approach. *Diabetes.* 2013;62(12):4270-4276.

155. Sonawat HM, Sharma S. Host responses in malaria disease evaluated through nuclear magnetic resonance-based metabonomics. *Clin. Lab. Med.* 2012;32(2):129-142.

156. Sana TR, Gordon DB, Fischer SM, et al. Global mass spectrometry based metabolomics profiling of erythrocytes infected with Plasmodium falciparum. *PLoS One.* 2013;8(4):e60840.

157. Behrends V, Ryall B, Zlosnik JE, Speert DP, Bundy JG, Williams HD. Metabolic adaptations of Pseudomonas aeruginosa during cystic fibrosis chronic lung infections. *Environmental Microbiology.* 2013;15(2):398-408.

158. Cribbs SK, Park Y, Guidot DM, et al. Metabolomics of Bronchoalveolar Lavage Differentiate Healthy HIV-1-Infected Subjects from Controls. *AIDS Res. Hum. Retroviruses.* 2014.

159. Seymour CW, Yende S, Scott MJ, et al. Metabolomics in pneumonia and sepsis: an analysis of the GenIMS cohort study. *Intensive Care Med.* 2013;39(8):1423-1434.

160. Tian J, Bryk R, Itoh M, Suematsu M, Nathan C. Variant tricarboxylic acid cycle in Mycobacterium tuberculosis: identification of alpha-ketoglutarate decarboxylase. *Proc. Natl. Acad. Sci. U. S. A.* 2005;102(30):10670-10675.

161. Anishetty S, Pulimi M, Pennathur G. Potential drug targets in Mycobacterium tuberculosis through metabolic pathway analysis. *Comput. Biol. Chem.* 2005;29(5):368-378.

162. Marrero J, Rhee KY, Schnappinger D, Pethe K, Ehrt S. Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for Mycobacterium tuberculosis to establish and maintain infection. *Proc. Natl. Acad. Sci. U. S. A.* 2010;107(21):9819-9824.

163. Eoh H, Rhee KY. Multifunctional essentiality of succinate metabolism in adaptation to hypoxia in Mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U. S. A.* 2013;110(16):6554-6559.

164. Loots DT. An altered M. tuberculosis metabolome induced by katG mutations resulting in isoniazid-resistance. *Antimicrob. Agents Chemother.* 2014.

165. Shin JH, Yang JY, Jeon BY, et al. (1)H NMR-based metabolomic profiling in mice infected with Mycobacterium tuberculosis. *J. Proteome Res.* 2011;10(5):2238-2247.

166. Li S, Park Y, Duraisingham S, et al. Predicting network activity from high throughput metabolomics. *PLoS Comput. Biol.* 2013;9(7):e1003123.

167. Tukvadze N, Bablishvili N, Apsindzelashvili R, Blumberg HM, Kempker RR. Performance of the MTBDRsl assay in Georgia. *Int. J. Tuberc. Lung Dis.* 2014;18(2):233-239.

168. Brennan PJ, Crick DC. The cell-wall core of *Mycobacterium tuberculosis* in the context of drug discovery. *Curr. Top. Med. Chem.* 2007;7(5):475-488.

169. Esko JC, Doering TL, Raetz CRH. Eubacteria and Archaea. In: Varki A, Cummings RD, Esko JD, et al., eds. *Essentials of Glycobiology*. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 2009.

170. Li FM, Y., Zhang L, Neuenswander SA, Douglas JT, Ma X. Metabolomic analysis reveals novel isoniazid metabolites and hydrazones in human urine. *Drug Metab. Pharmacokinet.* 2011;26(6):569-576.

171. Vuilleumier N, Rossier MF, Chiappe A, et al. CYP2E1 genotype and isoniazid-induced hepatotoxicity in patients treated for latent tuberculosis. *Eur. J. Clin. Pharmacol.* 2006;62(6):423-429.

172. Rivera-Betancourt OE, Karls R, Grosse-Siestrup B, Helms S, Quinn F, Dluhy RA. Identification of mycobacteria based on spectroscopic analyses of mycolic acid profiles. *Analyst.* 2013;138(22):6774-6785.

173. Marrakchi H, Lanéelle MA, Daffé M. Mycolic acids: structures, biosynthesis, and beyond. *Chem. Biol.* 2014;21(1):67-85.

174. Dobson CM, Hempel SJ, Stalnaker SH, Stuart R, Wells L. O-Mannosylation and human disease. *Cell. Mol. Life Sci.* 2013;70(16):2849-2857.

175. Rivera-Marrero CA, Ritzenthaler JD, Newburn SA, Roman J, Cummings RD. Molecular cloning and expression of a novel glycolipid sulfotransferase in Mycobacterium tuberculosis. *Microbiology.* 2002;148(pt 3):783-792.

176. Patterson JH, Waller RF, Jeevarajah D, Billman-Jacobe H, McConville MJ. Mannose metabolism is required for mycobacterial growth. *Biochem. J.* 2003;372(Pt 1):77-86.

177. Lyon RH, Hall WH, Costas-Martinez C. Utilization of amino acids during growth of Mycobacterium tuberculosis in rotary cultures. *Infect. Immun.* 1970;1(6):513-520.

178. Wietzerbin J, Lederer F, Petit JF. Structural study of the poly-l-Glutamic acid of the cell wall of Mycobacterium tuberculosis var hominis, strain Brevannes. *Biochem. Biophys. Res. Commun.* 1975;62(2):246-252.

179. Peregrín-Alvarez JM, Sanford C, Parkinson J. The conservation and evolutionary modularity of metabolism. *Genome Biol.* 2009;10(6):R63.

180. Serhan CN, Hong S, Gronert K, et al. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J. Exp. Med.* 2002;196(8):1025-1037.

181. Krishnamoorthy S, Recchiuti A, Chiang N, et al. Resolvin D1 binds human phagocytes with evidence for proresolving receptors. *Proc. Natl. Acad. Sci. U. S. A.* 2010;107(4):1660-1665.

182. Spite M, Norling LV, Summers L, et al. Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature.* 2009;461:1287-1291.

183. Tobin DM, Roca FJ, Oh SF, et al. Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. *Cell.* 2012;148(3):434-446.

184. Pérez-Matute P, Pérez-Martínez L, Blanco JR, et al. Multiple frequency bioimpedance is an adequate tool to assess total and regional fat mass in HIV-positive patients but not to diagnose HIV-associated lipoatrophy: a pilot study. *Journal of the International AIDS Society.* 2013;16(1):18609.

185. Wilson JP, Strauss BJ, Fan B, Duewer FW, Shepherd JA. Improved 4-compartment body-composition model for a clinically accessible measure of total body protein. *American Journal of Clinical Nutrition.* 2013;97(3):497-504.