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Research Paper

Patients with Concurrent Tuberculosis and Diabetes Have a Pro-Atherogenic Plasma Lipid Profile

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ABSTRACT

Background: Type 2 diabetes mellitus (DM) is a major risk factor for development of tuberculosis (TB), however the underlying molecular foundations are unclear. Since lipids play a central role in the development of both DM and TB, lipid metabolism may be important for TB-DM pathophysiology.
Methods: A ^{1}H NMR spectroscopy-based platform was used to determine 225 lipid and other metabolic intermediates in plasma samples of healthy controls ($n = 50$) and patients with TB ($n = 50$), DM ($n = 50$) or TB-DM ($n = 27$).
Results: TB patients presented with wasting disease, represented by decreased amino acid levels including histidine and alanine. Conversely, DM patients were dyslipidemic as evidenced by high levels of very low-density lipoprotein triglycerides and low high-density lipoprotein cholesterol. TB-DM patients displayed metabolic characteristics of both wasting and dyslipidemia combined with disease interaction-specific increases in phospholipid metabolites (e.g. sphingomyelins) and atherogenic remnant-like lipoprotein particles. Biomarker analysis identified the ratios of phenylalanine/histidine and esterified cholesterol/sphingomyelin as markers for TB classification regardless of DM-status.
Conclusions: TB-DM patients possess a distinctive plasma lipid profile with pro-atherogenic properties. These findings support further research on the benefits of improved blood lipid control in the treatment of TB-DM.

1. Introduction

Type 2 diabetes mellitus (DM) is a major risk factor for tuberculosis (TB) and triples the risk of developing active TB disease [1]. At present approximately 15% of global TB cases can be attributed to DM comorbidity [2]. Clinically, DM increases TB severity and impairs TB treatment [3], while conversely TB hampers glycemic control [4]. DM impacts both susceptibility to infection and progression towards active disease [1,5], however the immunological processes involved are unclear [6]. The number of DM patients in TB-endemic regions of Africa and Asia is predicted to rise significantly during the coming decades [7], and TB-DM comorbidity is estimated to seriously affect TB and consequently general global health. Therefore the TANDEM project seeks to optimize treatment and diagnosis of comorbid TB-DM and to understand its causal mechanisms [8].

While DM is primarily characterized by hyperglycemia and insulin resistance, it is often also associated with severe dyslipidemia as a result of high dietary fat intake and deregulated hepatic lipid metabolism [9]. DM-associated high insulin levels stimulate de novo lipogenesis in hepatocytes while failing to suppress lipolysis in insulin-resistant adipocytes of DM patients, leading to increased free fatty acid flux to the liver and overproduction of large triglyceride-rich very low-density lipoprotein (VLDL) particles [10]. Diabetic dyslipidemia is defined as having high levels of plasma triglycerides and/or cholesterol in combination with low levels of high-density lipoprotein (HDL) cholesterol and is a major risk factor for cardiovascular disease and atherosclerosis, which often complicate DM.

In contrast to DM, TB is often associated with malnutrition and wasting syndrome [11], and a low bodyweight is a risk factor for TB disease [12,13]. Additionally, TB leads to decreased body fat mass and

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levels of the adipocyte hormone leptin [14]. Interestingly, the causative agent of TB, *Mycobacterium tuberculosis* (*Mtb*), has been shown to rely heavily on host-derived lipids for its survival [15–17]. *Mtb* induces the formation of lipid-loaded foamy macrophages, similar to the ones found in atherosclerotic lesions, and exploits these cells as its primary niche for replication. Several studies have identified high cholesterol levels as risk factor for TB [18–20], and reducing cholesterol levels using statins was beneficial in *Mtb*-infected macrophages, mice and patients through enhancing the bactericidal effect of first-line antibiotics and phagosome maturation [21–25].

To identify potential differences in lipid metabolism, we compared plasma lipid profiles of patients with TB-DM to those of patients with TB or DM. To this end we determined plasma metabolic profiles [26] in healthy controls (HC) and patients with TB, DM or TB-DM. We hypothesized that the combination of these two diseases on seemingly opposite sides of the metabolic spectrum would result in distinctive plasma lipid profiles, as well as novel biomarkers.

2. Materials & Methods

2.1. Ethics Statement/Patient Inclusion

This study was undertaken as part of a EU-funded collaborative project (TANDEM) [8]. Patients (18–70 years) were enrolled in Cape Town, South-Africa from six public health care clinics around Tygerberg Academic Hospital (Elsies River, Ravensmead, Uitsig, Adriaanse, Durbanville, Fisantekraal). In total, 177 participants were included: 50 healthy community controls, 50 DM patients, 50 TB patients and 27 TB-DM patients. DM patients without TB were recruited from community health centers/day hospitals in Elsies River and Durbanville and previously diagnosed with DM according to WHO-criteria [27]. TB patients were screened for DM and classification was based on hyperglycaemia (random plasma glucose ≥ 200 mg/dL), HbA1c ≥ 6.5% and/or self-reported DM, in which case previous determination of random plasma glucose levels was not repeated. From the patients included in this study there is clinical evidence suggesting that one participant has type 1 diabetes, whereas all other patients suffered from type 2 diabetes. TB patients were identified based on positive Xpert *Mtb*/RIF assay (Cephaid Inc., Sunnyvale, CA, USA), MGIT culture and *Mtb* confirmation. Participants were excluded if they were HIV-positive, pregnant, on steroid therapy (in the last 6 months), had a hemoglobin <10 g/L, presented with emphysema, chronic bronchitis, asthma, steroid-induced DM, cancer or known alcohol abuse. The study was approved by the Health Research Ethics Committee of the University of Stellenbosch, and conducted according to the Helsinki Declaration and International Conference of Harmonization guidelines. Written informed consent was obtained from all participants.

2.2. Metabolic Profile Quantification by ^{1}H-Nuclear Magnetic Resonance (NMR) Spectroscopy

A high-throughput ^{1}H NMR spectroscopy platform was used to determine plasma metabolic profiles consisting of 225 parameters (Nightingale Health, Helsinki, Finland), including detailed concentrations and compositions of 14 lipoprotein subclasses, fatty acids & glycerides, amino acids and glycolytic molecules [28]. Methods regarding sample preparation and measurement procedures were described previously [26].

2.3. Statistical Analysis

For multivariate analysis, metabolite ratios were excluded. Metabolites were log-transformed to correct for skewed distributions, with the exception of lipoprotein particle concentrations for regression analysis due to a substantial amount of zero measurements. Partial least squares discriminant analysis (PLS-DA) modelling and hierarchical clustering was used to visualize metabolic differences between the groups. Only samples and measurements with ≤10% missing or zero values were considered for PLS-DA modelling. The optimal number of components was determined based on estimated classification error rates calculated by fivefold cross validation, which was repeated ten times. To illustrate the differences between our individual groups (HC, TB, DM and TB-DM patients), separate linear regression models were fitted for each pairwise combination of groups while adjusting for age and sex. 98 measures of specific particle concentrations and compositions of 14 lipoprotein subclasses were analyzed distinctly from the remaining parameters (44 metabolite subset, Supplementary Table S1). Next, interaction-specific effects of TB-DM comorbidity were investigated by fitting the following linear model:

$$\text{Metabolite} : \beta_0 + \beta_1 \text{TB} + \beta_2 \text{DM} + \beta_3 \text{TB}^{*}\text{DM} + \beta_4 \text{Age} + \beta_5 \text{Sex} + \varepsilon$$

where TB = TB-status (true/false), DM = DM-status (true/false), TB*DM = disease interaction effect, Age = age (years) and Sex = sex (male/female).

Univariate biomarker analysis was performed to identify metabolic measures with potential for TB diagnosis. Analysis was stratified by DM-status (HC vs. TB, DM vs. TB-DM). Log-transformed data of the 44 metabolite subset was mean-centred, scaled to standard deviation (SD) units and top 20 metabolite ratios based on *p*-values were calculated and added to the analysis. For each biomarker receiver operating characteristic (ROC) curves were plotted and area under the curve (AUC) values with 95% confidence interval (CI) determined. Biomarker analysis was performed using the online tool MetaboAnalyst 3.5 and methodological details were published previously [29].

Statistical analysis of clinical characteristics was performed in SPSS 23 (IBM) by one-way ANOVA (reported *p*-values are the outcome of the *F*-test), independent samples *t*-test or chi-squared test. Univariate analysis of absolute metabolite concentrations was done in Graphpad Prism 7 by Kruskal-Wallis test with post-hoc Dunn's test. PLS-DA and multiple linear regression analysis were performed using R version 3.3.2. including the following packages: mixOmics [30] version 6.3.0, limma [31] version 3.30.13 and phenotypicForest [32] version 0.3.

3. Results

3.1. Clinical and Metabolic Characteristics of the Study Population

Patient characteristics are shown in Table 1. On average, DM patients were older and had a higher BMI compared to the other groups. TB patients had a relatively low BMI, while this was comparable for TB-DM patients and HC. All (non-TB) DM patients were on anti-diabetic drugs, while this was the case for 55.6% of TB-DM patients. TB-DM patients not on treatment were newly diagnosed DM cases. In total, 177 participants were included in the study and their plasma metabolic profiles were determined using ^{1}H NMR spectroscopy. Metabolite ratios were excluded in the multivariate analysis to limit parameter interdependence, resulting in a total of 142 variables.

Partial least square discriminant analysis (PLS-DA) (Fig. 1a) was performed to visualize the metabolic differences between the four groups based on the complete metabolic signature. The score plot of the first two principal components (explaining 42% and 16% of total variance, respectively) is depicted in Fig. 1a. TB-DM patients appeared largely scattered over both single disease groups, implying significant metabolic heterogeneity. To explore this further we compared TB, DM and TB-DM patients by hierarchical clustering analysis (Fig. 1b). While the majority of TB and DM patients each clustered together, TB-DM patients were again dispersed throughout the two single disease groups, further illustrating high inter-individual variation.

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