Comorbidity between Communicable and Non-communicable Diseases:

The Example of the Dual Burden of Tuberculosis and Diabetes in Dar es Salaam, Tanzania

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Summary

Background

Although recognized for centuries, the association between tuberculosis (TB) and diabetes mellitus (DM) was forgotten with the discovery of efficient treatments. In the last decade, the prevalence of DM has dramatically increased, particularly in low- and middle-income countries experiencing a high burden of TB, leading to a new interest in this association. DM increases TB risk while TB, as an infectious disease, leads to hyperglycemia. The relationship between TB and DM has been poorly studied in Sub-Saharan Africa, where the high incidence of TB is associated with HIV infection. Concentration of vitamin D is inversely associated with TB and DM, and it has been suggested that low vitamin D could mediate some of the association between TB and DM. DM affects the immune response to TB, but the precise mechanisms underlying this association are not clear.

To address this issue of high public health relevance, we undertook a project on the association between TB, DM and HIV in Tanzania. The project had three major components:

(1) Assessing the association of TB and its outcome with the presence and persistence of hyperglycemia in Tanzania, using three different DM screening tests.
(2) Describing the association between vitamin D, TB and DM.
(3) Studying the immunological features underlying TB and DM comorbidity in sub-Saharan Africa and testing the hypothesis of delayed adaptive immune response with increasing glycemia.

The overall aim of the project was to improve knowledge on the dynamic interaction between TB and DM in an African setting with high HIV prevalence by integrating a longitudinal component into the case-control study.

Methods

A case-control study with longitudinal follow-up of cases was conducted in Dar es Salaam. Consecutive adults with new active TB were included and followed up for five months after the start of anti-TB treatment. Healthy controls, matched by age and sex

to TB cases, were recruited among volunteering adults accompanying patients to the outpatient departments of the same hospitals. Exclusion criteria were a biological relationship to TB case, TB history, symptoms or signs of TB, other acute infection or major trauma within the last three months. All underwent 25-hydroxyvitamin D (25(OH)D) measurement and DM screening tests (fasting glucose (FCG), 2-hour capillary glucose after standard oral glucose tolerance test (2h-CG) and glycated hemoglobin (HbA1c)) at enrolment and TB patients were again tested after five months of TB treatment. Data on the outcome of TB (treatment failure, death, lost to follow-up) were collected.

For the nested immunological study, four groups of HIV negative patients were included: i) active TB without DM, ii) active TB with DM, iii) latent TB patients without DM and iv) latent TB patients with DM. Latent TB patients were selected among the healthy volunteering adults, as well as among diabetic patients attending the DM clinic in the participating hospitals. Exclusion criteria for groups iii and iv were past TB history and symptoms or signs of active TB. Peripheral blood mononuclear cells were stimulated with *Mycobacterium tuberculosis* (*Mtb*)-specific peptide pools and live *Mycobacterium bovis* BCG and then analysed by polychromatic flow cytometry for Th1, Th2, Th9 and Th17 cytokine production. Cell culture supernatants were analysed by Luminex® for 34 cytokines and chemokines.

Findings

At enrolement, DM prevalence was significantly higher among TB patients (N=539; FCG>7mmol/L: 4.5%, 2-hCG>11mmol/L: 6.8% and HbA1c>6.5%: 9.3%) compared to controls (N=496; 1.2%, 3.1% and 2.2%). However, the association between hyperglycemia and TB disappeared after TB treatment (aOR(95% CI) at enrolment vs follow-up: FCG 9.6(3.7-24.7) vs 2.4(0.7-8.7); 2-hCG 6.6(4.0-11.1) vs 1.6(0.8-2.9); HbA1c 4.2(2.9-6.0) vs 1.4(0.9-2.0)). FCG hyperglycemia at enrolment was associated with TB treatment failure or death (aOR(95%CI) 3.3(1.2-9.3).

The prevalence of 25(OH)D insufficiency (25(OH)D<75nmol/l) was not statistically different between TB patients and controls (25.8% versus 31.0%; p=0.22). But the association between 25(OH)D insufficiency and TB was modified by hyperglycemia ($p_{interaction}$=0.01). Patient with vitamin D insufficiency were only at higher risk for TB in

the presence of underlying hyperglycemia. The OR (95%CI) for TB risk in patients with vitamin D insufficiency and hyperglycemia was 4.94(1.16-21.0) versus 0.68(0.39-1.17) for patients with vitamin D insufficiency and normoglycemia where normoglycemia and normal vitamin D were the reference category.

Patients with active TB and DM had a lower frequency of INF-γ $CD4^+$ T cells and a lower proportion of $CD4^+$ T cells producing both TNF-α and IFN-γ after live *M. bovis* BCG but not after *Mtb*-specific peptide pool stimulation, compared to normoglycemic TB patients. A negative correlation between INF-γ or TNF-α $CD4^+$ T cell frequency and increasing glycemia was observed in the context of live *M. bovis* BCG stimulation only.

Conclusions

Transient hyperglycemia is frequent during TB, and DM needs confirmation after TB treatment. However, DM screening at TB diagnosis gives the opportunity to detect patients at risk of adverse outcome.

25(OH)D insufficiency seams to increase the risk of TB only if associated with hyperglycemia. DM patients living in high TB burden settings might benefit from preventive vitamin D supplementation.

The immunological findings suggest that DM might affect *Mtb*-specific $CD4^+$ T cell immune responses at the level of reduced antigen processing and presentation, a defect that could be compensated by metformin.

The results of the study are of public health and clinical utility. First, they lend support to the integration of care between TB and DM programs. Second, they imply that, at the time of TB diagnosis, patients should be screened for hyperglycemia using cost-effective fasting glucose tests. Treatment of hyperglycemia should be initiated to improve TB outcome. Third, before initiation of long-term DM treatment, DM diagnosis must be confirmed after the resolution of TB. Finally, in the absence of evidence for a strong contribution of DM to TB risk in this African setting with high HIV prevalence, DM patients should not be screened for TB with expensive test. DM physicians and patients should rather be trained for recognizing TB symptoms and signs as a cost-effective way to recognize TB early.

Zusammenfassung

Hintergrund

Obwohl die Verknüpfung zwischen Tuberkulose (TB) und Diabetes mellitus (DM) seit langem bekannt ist, wurde sie doch mit der Entdeckung von wirksamen Therapien vergessen. In Ländern mit hohen Anzahlen von Tuberkulosefällen, also vor allem in den Ländern mittleren und niedrigen Einkommens, stieg die Prävalenz von DM in den letzten Jahren stark an und das Interesse an der vergessenen Verknüpfung wurde neu geweckt. DM erhöht das Risiko für Erkrankung an TB, und TB wiederum, als Infektionskrankheit, führt zu hohen Blutzuckerwerten. Das Verhältnis zwischen TB und DM wurde bisher wenig in Afrika südlich der Sahara erforscht. In dieser Region beruht die hohe Prävalenz von TB vor allem auf HIV Infektion. Die Plasmakonzentration von Vitamin D ist invers mit TB und DM korreliert; es wurde also stipuliert, dass Vitamin-D Mangel teilweise für die Verbindung von TB und DM verantwortlich sein könnte. DM beeinflusst die Immunantwort auf TB Infektion, aber der genaue Mechanismus ist bisher unklar. Um diesen wichtigen Fragen nachzugehen, erforschte unser Projekt den Zusammenhang zwischen TB, DM, und HIV in Tansania. Das Projekt bestand aus den folgenden drei Hauptteilen:

(1) Die Erforschung des Zusammenhangs zwischen TB-Infektion /-Outcome und erhöhten Blutzuckerwerten (einmalig oder anhaltend) mit Hilfe von drei verschiedenen DM Screening Tests.
(2) Die Beschreibung des Zusammenhangs zwischen Vitamin D, TB und DM
(3) Die Untersuchung der immunologischen Merkmale, die der TB-DM-Komorbidität in Afrika südlich der Sahara unterliegen. Dabei testeten wir auch die Hypothese, dass zu hohe Blutzuckerwerte zu einer verzögerten angepassten Immunreaktion (*delayed adapative immune response*) führen.

Das Hauptziel dieses Projektes war, im afrikanischen Kontext mit hoher HIV-Prävalenz die dynamische Interaktion zwischen TB und DM, durch die Integration einer Längsschnitt-Studien Komponente in eine Fall-Kontroll-Studie, weiter zu beleuchten.

Methoden

Wir führten eine Fall-Kontroll-Studie mit Längsschnitt Follow-Up in Dar es Salaam durch. Erwachsene mit TB-Neuinfektion wurden konsekutiv in eine fünf-monatige Langzeitstudie aufgenommen. Anfangspunkt war hierbei der Start der TB-Behandlung. Gleichalterige und gleichgeschlechtliche, gesunde Kontrollen wurden unter freiwilligen Angehörigen rekrutiert, die PatientInnen zur Krankenhausambulanz begleiteten. Ausschlusskriterien für Kontrollen waren: Blutsverwandtschaft zu den Fällen, TB-Krankengeschichte, Anzeichen von TB–Infektion sowie andere Infektionen oder ein schwerwiegender Unfall in den letzten drei Monaten. Bei allen Teilnehmern wurde bei Studienaufnahme 25(OH)Vitamin D_3 Messung und Screening für DM durchgeführt (Nüchtern-Blutzucker (FCG), Zweistunden-Blutzucker nach Standard Glukosetoleranz-Test (2h-CG) und Glykohämoglobin (HbA1c)). Bei den TB PatientInnen wurden die gleichen Messungen nach 5 Monaten TB-Behandlung wiederholt, sowie Daten zum TB-Behandlungsergebnis erhoben (Therapieversagen, Tod, „lost to follow-up“).

Für die immunologische, „Nested“ Fall-Kontroll-Studie, schlossen wir HIV-negative PatientInnengruppen mit vier verschiedenen Merkmalskombinationen ein: i) aktive TB ohne DM, ii) aktive TB mit DM, iii) latente TB ohne DM und iv) latente TB mit DM. Die latenten TB-PatientInnen rekrutierten unter gesunden Freiwilligen und unter DiabetespatientInnen, die die Diabetesklinik der teilnehmenden Krankenhäuser besuchten. Ausschlusskriterien für die Gruppen iii und iv waren: TB-Infektion in der Krankengschichte, und Anzeichen einer aktiven TB-Infektion. Mononukleäre Zellen des peripheren Blutes wurden dann mit *Mycobacterium tuberculosis(Mtb)*-spezifischen Peptid-Pools sowie lebend *Mycobacterium bovis*-BCG stimuliert und dann auf Th1-, Th2-, Th9- and Th17- Zytokinproduktion mittels polychromatischer Durchflusszytometrie untersucht. Desweiteren wurden in Zellkultur-Überstände 34 Zytokine und Chemokine mittels Luminex® bestimmt.

Ergebnisse

Zum Zeitpunkt der Rekrutierung war die Prävalenz von DM signifikant höher bei TB-PatientInnen (N=539; FCG>7mmol/L: 4.5%, 2-hCG>11mmol/L: 6.8% and HbA1c>6.5%: 9.3%) verglichen mit Kontrollen (N=496; 1.2%, 3.1% and 2.2%). In der Langzeitstudie bei TB-PatientInnen, verschwand diese Verbindung zwischen erhöhten

Blutzuckerwerten und nach fünfmonatiger TB-Therapie: FCG 9.6(3.7-24.7) vs 2.4(0.7-8.7); 2-hCG 6.6(4.0-11.1) vs 1.6(0.8-2.9); HbA1c 4.2(2.9-6.0) vs 1.4(0.9-2.0)). Erhöhter FCG zum Zeitpunkt der Rekrutierung war hier auch mit Therapieversagen und Tod assoziiert (aOR(95%CI) 3.3(1.2-9.3).

Die Prävalenz von 25(OH)Vitamin D_3-Mangel (25(OH)D<75nmol/l) war nicht signifikant unterschiedlich zwischen TB-PatientInnen und Kontrollen (25.8 versus 31.0%; p=0.22). Jedoch wurde die Assoziation zwischen 25(OH)Vitamin D_3-Mangel und TB durch Hyperglykämie modifiziert ($p_{interaction}$=0.01): PatientInnen mit 25(OH)Vitamin D_3-Mangel hatten nur dann ein erhöhtes TB-Infektionsrisiko, wenn gleichzeitig Hyperglykämie vorlag: Die OR (95% CI) für TB-Infektion waren: 4.94(1.16-21.0) für PatientInnen Hyperglykämie und Vitamin-D Mangel, 0.68 (0.39-1.17) für PatientInnen mit Normoglykämie und Vitamin-D Mangel, jeweils verglichen zu PatientInnen mit Normoglykämie als und normalen Vitamin-D Werten. Nach Stimulierung mit lebend *M. bovis*- BCG, aber nicht mit *Mtb*-spezifischen Peptid-Pool, hatten PatientInnen mit aktiver TB und DM, im Vergleich zu PatientIennen ohne TB und Normoglykämie, weniger INF-γ $CD4^+$ T-Zellen und einen niedrigeres Prozent an TNF-α- und IFN-γ-produzierenden $CD4^+$ T-Zellen. Nach Stimulierung mit lebend *M. bovis*- BCG stellten wir eine negative Korrelation zwischen INF-γ- TNF-α $CD4^+$ T-Zell Häufigkeit und ansteigenden Blutzuckerwerten.

Schlussfolgerung

Vorübergehend erhöhte Blutzuckerwerte kommen häufig bei TB-Infektion vor. Eine DM-Diagnose muss daher nach Therapiebeginn nochmals bestätigt werden. Jedoch bietet DM-Screening bei TB Diagnosestellung die Möglichkeit, PatientInnen mit erhöhtem Riskiko für Komplikationen zu identifizieren.

25(OH)Vitamin D_3-Mangel, wenn mit Hyperglykämie kombiniert, erscheint das Risiko für TB-Infektion zu erhöhen. DM PatientInnen, in deren Umfeld ein hohes TB-Übertragungsrisiko herrscht, könnten also eine Vit-D Nahrungsergänzung zugutekommen.

Die immunologischen Ergebnisse legen nahe, dass DM die *Mtb*-spezifische $CD4^+$ T-Zell Immunantwort beeinflusst, nämlich über eine verminderte Antigenprozessierung und –präsentation. Dieser Defekt könnte durch Metformin kompensiert werden.

Die Ergebnisse dieser Studie sind von klinischer Bedeutung und von Relevanz für das öffentliche Gesundheitswesen. Zum ersten befürworten sie die Vernetzung von TB- und DM-Therapieprogrammen. Des Weiteren sollten TB PatientInnen bei Therapiebeginn, mit Hilfe der kosteneffizienten Nüchtenblutzuckermessung, auf Hyperglykämie untersucht werden. Eventuelle Hyperglykämie sollte dann behandelt werden, um die TB-Therapieergebnisse zu verbessern. Darüber hinaus sollte eine definitive DM Diagnose, und somit die Indikation zur Langzeitbehandlung, erst nach Abschluss der TB-Behandlung gestellt werden. Schliesslich, da es keine Evidenz dafür gibt, dass DM signifikant das TB-Ansteckungsrisiko im afrikanischen Kontext mit hoher HIV-Prävalenz erhöht, sollten DM PatientInnen nicht systematisch mit teuren Testvefahren auf TB untersucht werden. Als kosteneffizientere Massnahme sollten vielmehr DM behandelnde Ärzte in der Erkennung von klinischen TB Anzeichen ausgebildet werden.

Muhtasari

Historia

Ingawa unajulikana kwa karne nyingi, uhusiano kati ya kifua kikuu na kisukari ulisahaulika tangu ugunduzi wa tiba fanisi. Kwa kipindi cha karne iliopita, kiwango cha ugonjwa wa kisukari kimeongezeka kwa kasi, hususani katika nchi zenye kipato cha chini na kati zikikumbwa na kiwango kikubwa cha kifua kikuu, na kuleta fikra mpya katika uhusiano huu. Kisukari huongeza hatari ya kupata kifua kikuu, huku kifua kikuu kama ugonjwa ambukizi , huleta hali ya kuongezeka kiwango cha sukari katika damu. Uhusiano kati ya kifua kikuu na kisukari haujachunguzwa vizuri katika nchi za kusini mwa Afrika, ambapo kiwango kikubwa cha kifua kikuu kinahusiana na maambukizi ya VVU. Kiwango cha vitamin D kimehusishwa na kifua kikuu na kisukari, na imefikiriwa kuwa kiwango kidogo cha vitamin D kunaweza elezea baadhi ya uhusiano kati ya kifua kikuu na kisukari. Kisukari kinaathiri kinga dhidi ya kifua kikuu, lakini njia haswa inayosababisha hili haijajulikana vizuri bado.

Ili kukabiliana na suala hili lenye umuhimu wa juu katika afya ya umma, tulifanya uchunguzi kuangalia uhusiano kati ya kifua kikuu, kisukari na maambukizi ya VVU Tanzania. Uchunguzi ulikuwa una maeneo makuu matatu:

Kutathmini uhusiano wa kifua kikuu na matokeo yake katika uwepo na usugu wa kiwango cha juu cha sukari katika damu Tanzania, tukitumia vipimo vitatu tofauti vya kuchunguza kisukari

Kuelezea uhusiano kati ya vitamini D, kifua kikuu na kisukari

Kuchunguza misingi ya kinga pale ambapo kifua kikuu na kisukari hutokea kwa pamoja katika nchi za kusini mwa Sahara na kupima dhana ya kuchelewa kujitokeza kwa kinga kiwango cha sukari kiongezekapo katika damu

Kwa ujumla, lengo la utafiti huu lilikuwa ni kuboresha maarifa juu ya muingiliano wa nguvu kati ya kifua kikuu na kisukari katika mazingira ya Africa yenye kiwango kikubwa cha maambukizi ya Ukimwi kwa kuingiza mfumo endelevu katika tafiti za kulinganisha mgonjwa (kesi) na mtu mwenye sifa zote sawa na mgonjwa isipokuwa ugonjwa wenyewe (kontrol).

Mbinu

Uchunguzi wa kesi na kontrol, uliokuwa ukiwafuatilia kesi ulifanyika Dar es Salaam. Wagonjwa wapya ambao ni watu wazima waliogundulika wana ugonjwa wa kifua kikuu waliingizwa katika utafiti na kufuatiliwa kwa kipindi cha miezi mitano baada ya kuanza matibabu ya kifua kikuu. Kontrol wenye afya nzuri waliofanana kwa umri na jinsia na kesi za kifua kikuu na waliojitolea kuingia katika utafiti, walichaguliwa miongoni mwa watu wazima waliowasindikiza wagonjwa wasiolazwa kupata huduma za hospitali.

Wafuato hawakuingizwa katika utafiti; waliokuwa na uhusiano wa kibiolojia na kesi (mgonjwa wa kifua kikuu), waliowahi kuugua ugonjwa wa kifua kikuu, wenye dalili au viashiria vya ugonjwa wa kifua kikuu, na wenye magonjwa mengine ya dharura au waliohusika na ajali kubwa katika kipindi cha miezi mitatu iliopita. Wote walifanyiwa vipimo vha 25-hydroxyvitamini (25(OH)D) na uchunguzi wa kisukari (kipimo cha sukari wakiwa wamefunga kula – fasting glucose FCG), kipimo cha kuangalia kiwango cha sukari masaa mawili baada ya kunywa sukari iliyopimwa kwa kiwango maalum (2h-CG) na kipimo cha kuangalia kiwango cha sukari katika chembe chembe nyekundu za damu (HbA1c) katika hudhurio la kwanza, huku wagonjwa wa kifua kikuu wakipimwa tena miezi 5 baada ya kuanza tiba ya kifua kikuu. Matokea ya ugonjwa wa kifua kikuu (waliofeli tiba, waliokufa, waliopotea) yalikusanywa.

Katika utafiti wa kuangalia kinga, uliofanyika ndani ya utafiti huu, wagonjwa wa kifua kikuu wasiokuwa na maambukizi ya virusi vya ukimwi waligawanywa kulingana na kuwepo au kutokuwepo kwa kisukari na maambukizi ya kifua kikuu. Hawa waliochaguliwa miongoni mwa watu wazima wenye afya nzuri waliojitolea kushiriki katika utafiti pamoja na wagonjwa wa kisukari waliokuwa wakihudhuria kliniki ya kisukari miongoni mwa hospitali zilizohusika na utafiti. Wafuato hawakuingizwa katika utafiti; waliokuwa na maambukizi ya ukimwi, waliowahi kuugua kifua kikuu na wenye dalili au viashiria vya ugonjwa wa kifua kikuu. Seli za damu (PBMCs) zilifanyiwa uchunguzi kwa kuzichochea na vijidudu vya *Mycobacterium tuberculosis* (Mtb) na vijidudu hai vya *Mycobacterium bovis* BCG na kuchunguzwa kwa kutumia kipimo cha polymorphic flow cytometry kuangalia uzalishaji wa cytokini Th1, Th2, Th9 na Th17. Seli zilizooteshwa zilichunguzwa kwa kutumia kipimo cha Luminex kuangalia cytokini 34 na kemokini.

Matokeo

Wakati wa ugunduzi wa ugonjwa wa kifua kikuu, uwingi wa kisukari ulikuwa juu zaidi kati ya wagonjwa wa kifua kikuu (N=539; FCG>7mmol/L:4.5%, 2-hCG>11mmol/L:6.8% na HbA1c>6.5%:9.3%) ukilinganisha na kontroli (N=496; 1.2%, 3.1% na 2.2%). Hata hivyo, uhusiano kati ya kiwango cha juu cha sukari na kifua kikuu ulitoweka baada ya matibabu ya kifua kikuu (aOR(95% CI) wakati wa kujiandikisha katika utafiti huu na kipindi cha mahudhurio ya ufuatiliaji FCG 9.6(3.7-24.7) vs 2.4(0.7-8.7); 2-hCG 6.6(4.0-11.1) vs 1.6(0.8-2.9); HbA1c 4.2(2.9-6.0) vs 1.4(0.9-2.0). Kiwango cha juu cha sukari wakati wa kujiandikisha katika utafiti huu kulihusiana na kufeli kwa matibabu ya kikua kikuu au kifo (aOR(95%CI) 3.3(1.2-9.3).

Kiwango cha upungufu wa vitamini D (25(OH)D<75nmol/l) haukua tofauti kitakwimu kati ya wagonjwa wa kifua kikuu na wasio na kifua kikuu (25.8 versus 31.0%; p=0.22). Lakini uhusiano kati ya upungufu wa vitamini D na kifua kikuu ulibadilika kiwango cha sukari kilipokuwa juu ($p_{interaction}$=0.01). Upungufu wa vitamini D ulihusiana na hatari ya kifua kikuu pale ambapo kiwango cha sukari kilikuwa juu katika damu (OR(95%CI): 4.94(1.16-21.0)), lakini si wakati kiwango cha sukari katika damu kiwapo kawaida (OR(95%CI): 0.68(0.39-1.17)) ukifananisha na washiriki wasiokuwa na kiwango cha juu cha sukari na upungufu wa vitamini D.

Wagonjwa wenye ugonjwa wa kifua kikuu walikuwa na uwingi mdogo zaidi wa seli za INF-γ $CD4^+$T na kiasi kidogo zaidi cha seli za $CD4^+$ T zinazotengeneza TNF-α na IFN-γ baada ya kuchochea na vijidudu hai vya *M. bovis* BCG lakini sio baada ya kuchochea na *Mtb*. Uusiano hasi kati ya uwingi wa seli za INF-γ au TNF-α $CD4^+$T na kuongezeka kwa kiwango cha sukari katika damu ulionekana tu wakati wa kuchochea na vijidudu hai *M. bovis*BCG.

Hitimisho

Kuongezeka kwa kisuakari katika damu kwa muda mfupi kunajitokeza mara kwa mara wakati wa kifua kikuu, na ugonjwa wa kisukari unahitaji uthibitisho baada ya tiba ya kifua kikuu. Hata hivyo, uchunguzi wa kisukari wakati wa ugunduzi wa kifua kikuu unatoa fursa ya kugundua wagonjwa wenye hatari ya kupata matokeo mabaya.

Upungufu wa 25(OH)D unaelekea kuongeza hatari ya kifua kikuu kama tu ukihusiana na kiwango cha juu cha sukari katika damu. Wagonjwa wa kisukari wanaoishi katika

mazingira yenye kiwango cha juu cha maambukizi ya kifua kikuu wanaweza pata faida kwa kupatiwa nyongeza ya vitamini D. Matokeo ya utafiti wa kinga unaashiria kwamba kisukari kinaweza athiri kinga ya $CD4^+$ T inayojitokeza kwa *Mtb* tu katika kiwango cha kupungua kwa usindikaji na uonekanaji wa antijeni, kasoro inayoweza fidiwa na dawa ya metformin.

Matokeo ya utafiti huu ni katika matumizi ya afya ya umma na tiba. Kwanza, yanatoa msaada katika ushirikiano wa programu za huduma za kifua kikuu na kisukari. Pili, huashiria kwamba, wakati wa ugunduzi wa kifua kikuu, wagonjwa hupaswa kufanyiwa uchunguzi wa kiwango cha sukari kwa kutumia vipimo vha gharama nafuu vha kupima sukari baada ya kufunga kula. Matibabu ya kiwango cha juu cha sukari yanatakiwa yaanzishwe ili kuboresha matokea ya kifua kikuu. Tatu, kabla ya kuanzisha matibabu ya muda mrefu ya kisukari, ugunduzi wa kisukari lazima uhakikiwe baada ya kupona ugonjwa wa kifua kikuu. Na mwisho, kukosekana kwa uhusiano wa nguvu kati ya kisukari na hatari ya kifua kikuu katika mazingira haya ya Africa yenye uwingi wa juu wa maambukizi ya virusi vya ukimwi, wagonjwa wa kisukari hawapaswi kufanyiwa uchunguzi wa kisukari kwa kutumia vipimo vya gharama ya juu. Madaktari wa kisukari pamoja na wagonjwa wapatiwe mafunzo ya kugundua dalili na viashiria vya kifua kikuu kama njia ya gharama nafuu ya kugundua kifua kikuu mapema.

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List of Abbreviations

$1,25(OH)_2D$	1,25-Dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
2h-CG	2-hour Capillary Glucose after standard Oral Glucose Tolerance Test
2h-PG	2-h Plasma Glucose after standard Oral Glucose Tolerance Test
95% CI	95% Confidence Interval
ADA	American Diabetes Association
aOR	adjusted Odds Ratio
BCG	Bacillus Calmette–Guérin
BMI	Body Mass Index
BP	Blood Pressure
cART	Combined Antiretroviral Therapy
CD	Communicable Disease
CFP10	10-kDa Culture Filtrate Antigen
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Hemorrhagic Fever
DM	Diabetes Mellitus
DSS	Dengue Shock Syndrome
ELISPOT	Enzyme-Linked Immunospot Assay
ESAT6	6-kDa Early Secretory Antigen Target
FCG	Fasting Capillary Glucose
FPG	Fasting Plasma Glucose
HbA1c	Glycated haemoglobin
IFN-γ	Interferon-gamma
IL	Interleukin
IQR	Interquartile Range
LMIC	Low- and Middle-Income Countries
MDR-TB	Multidrug-resistant Tuberculosis
Mtb	*Mycobacterium tuberculosis*
NCD	Non-communicable Disease
NTD	Neglected Tropical Disease

NTLP	National Tuberculosis and Leprosy Control Programme
ODK	Open Data Kit
OGTT	Oral Glucose Tolerance Testing
PBMC	Peripheral Blood Mononuclear Cells
PPD	purified protein derivative
pre-DM	Pre-diabetes
SD	Standard Deviation
SEB	Staphylococcal Enterotoxin B
SES	Socio-economic Status
TB	Tuberculosis
Th1	T Helper 1
The Union	International Union against Tuberculosis and Lung Disease
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor-alfa
WHO	World Health Organization

1 Introduction

Non-communicable diseases (NCD) are chronic diseases that are not passed from person to person contrary to communicable diseases which are acute or chronic infections. There are four main groups of NCDs: cardiovascular diseases, cancers, chronic respiratory diseases and diabetes mellitus (DM). As a result of economic transition and resulting increase in life expectancy, globalization and rapid urbanization leading to lifestyle changes, NCDs are increasing worldwide and are the leading cause of death globally (68%) with three quarter of them occurring in low- and middle-income countries. The rising burden of these NCDs is one of the major health and development challenges of the 21st century, particularly for low- and middle-income countries. At the same time, socioeconomic and environmental factors, international travel and migration increase the spread of communicable infectious diseases. Non-communicable and communicable diseases increasingly co-occur geographically and interact with each other. Interactions between non-communicable and communicable diseases have been recognized for centuries but the interest in this association disappeared with the discovery of effective treatment for both types of diseases such as antibiotic and antidiabetic drugs. The importance of these interactions is reemerging as a consequence of the NCD increase, particularly in low- and middle-income countries. The reciprocal link between infections and DM is well known. On the one hand, stress related to inflammation as a result of infection induces hyperglycemia and on the other hand, DM with chronic hyperglycemia weakens the immune system and increases susceptibility to infections and also worsens their outcome. Most data are from developed countries which do not experience the same variety of infectious diseases than developing countries. There is a need to improve knowledge on the consequences of the NCD increase in low- and middle-income countries.

This PhD work focused on two diseases which depict well how a convergence of different type of illnesses can become a threat beyond the effect of each of them separately. DM is one of the most common NCDs, it is on the rise all over the world and is emerging in Africa. We studied its interrelation with tuberculosis (TB) in a longitudinal manner.

1.1 Diabetes Mellitus

DM is a chronic metabolic illness mainly characterized by hyperglycemia which leads to long-term complications (microvascular disease, such as nephropathy, retinopathy, neuropathy, and macrovascular disease, such as cardiovascular disease and stroke). Different types of DM have been described. Type 2 DM accounts for over 90% of DM cases in the United States, Canada, Europe as well as in sub-Saharan Africa (ADA, 2014, Hall et al., 2011). Type 2 DM results from a progressive insulin secretion defect on the background of insulin resistance and more commonly occurs with adult-onset and in association with obesity and physical inactivity. Type 1 DM accounts for another 5 to 10% in high-income countries and usually affects children or young adults. It results from autoimmune pancreatic β-cell destruction, leading to absolute insulin deficiency and, therefore, to an absolute requirement for insulin therapy. Type 1 DM is rare in sub-Saharan Africa, probably because these people die as a consequence of lack of insulin access (Hall et al., 2011, Swai et al., 1993). Gestational DM occurs during pregnancy and can lead to serious health risks to the mother and her infant (IDF, 2013). The remainder DM are due to other causes such as genetic defects, pancreas diseases, endocrinopathies and drugs side effects (e.g. Lamivudine, protease inhibitors and efavirenz, currently used in HIV treatment) (Paula et al., 2013).

Type 2 DM is increasing rapidly worldwide as a consequence of energy- and carbohydrate-rich diets and increasingly sedentary lifestyles (2014). A higher prevalence of dysglycemia is observed in the HIV-infected population. On top of antiretroviral drugs side effects, HIV-specific mechanisms include immune dysfunction with increased inflammatory response leading to a higher prevalence of metabolic syndrome, dyslipidemia and dysglycemia (Paula et al., 2013).

Currently, the global DM prevalence is estimated at 8.3% (387 million people, most of them aged between 40 and 59) and is expected to rise beyond 592 million in less than 25 years. The regional DM prevalence in Africa is estimated at 5.1%, and is expected to double by 2035. The African region has the highest proportion of undiagnosed DM (63%), and in this region DM accounts for 8.6% of all deaths (IDF, 2013, Shaw et al., 2010). In sub-Saharan Africa, differences in DM prevalence have been reported between urban and rural areas and between ethnic communities, with the highest prevalence reported within the Indian community in Tanzania (7.1-9.1%) and the

lowest prevalence in indigenous black populations (1.1-5.3%) (Idemyor, 2010, Ramaiya et al., 1995, Stevenson et al., 2007b, Unwin et al., 2010).

1.2 Tuberculosis

TB is caused by an intracellular bacteria belonging to the *Mycobacterium tuberculosis* (*Mtb*) complex and is transmitted from person to person though infectious particles which are aerosolized by coughing or talking. In the majority of cases, the infection is either eradicated by the host response or contained as a latent infection with a 10% lifetime risk of reactivation and development of clinical TB (13 per 1000 person-years) (Frieden et al., 2003). Factors associated with progression to active TB comprise extremes of age, malnutrition, smoking, DM and immunosuppression, HIV infection being the strongest risk factor (20-fold to 40-fold increase). The most common clinical presentation of active TB is lung infection, extrapulmonary disease (lymph node, central nervous system, bone, joint, genitourinary system) accounting for around 20% of disease in HIV-negative patients (Frieden et al., 2003).

In 2000, the stop TB partnership, an international body involved in the fight against TB, set a strategic plan linked to the millennium development goals with the targets to reduce the global burden of TB disease by 50% relative to 1990 levels by 2015 and to eliminate TB by 2050. As a consequence, the global burden of disease is decreasing, but not fast enough. TB elimination is challenged by the duet TB/HIV and by multidrug-resistant TB (MDR-TB, caused by *Mtb* strains resistant to at least two of the most powerful first-line anti-TB drugs, isoniazide and rifampicine). TB remains a global health problem with 9.6 million of people who developed TB in 2014, 12% of them being HIV infected Almost three-quarters of these HIV-positive cases are in Africa, where 32% of TB patients are screened positive for HIV (WHO, 2015). Resistant TB is arising due to improper use of antibiotics during TB treatment, particularly in areas with week TB control programs. Globally, MDR-TB is detected in 3.3% of new TB cases and 20% of those previously treated for TB (WHO, 2015). In most African countries, levels of drug resistance among new cases remain low (<3%).

1.3 Association between tuberculosis and diabetes

The last decade has experienced a global increase of DM, particularly in developing countries with a high TB burden. The convergence of these two ongoing epidemics has a potentially strong impact on TB surveillance and treatment (Stevenson et al., 2007b). Two systematic reviews of the medical literature alerted the scientific community in 2007 and 2008, showing that DM doubles or triples the risk of TB compared with people without DM (Jeon and Murray, 2008, Stevenson et al., 2007a). The World Health Organization (WHO) and the International Union against TB and Lung Disease (The Union) established a framework which aims to guide national programs to establish a coordinated response to DM and TB in 2011 (Maurice, 2011). However, the prevalence of DM among patients with TB varies according to the setting. The highest proportion of people with DM among TB patients was observed in the Western Pacific (37%) and in India (25-32%) (Gupta et al., 2011, Viney et al., 2015, Viswanathan et al., 2012). In Africa, DM prevalence is generally lower than in other continents, to some degree also as a result of underdiagnosis. In contrast, both TB and HIV are very common on the African continent (Figure 1-1).

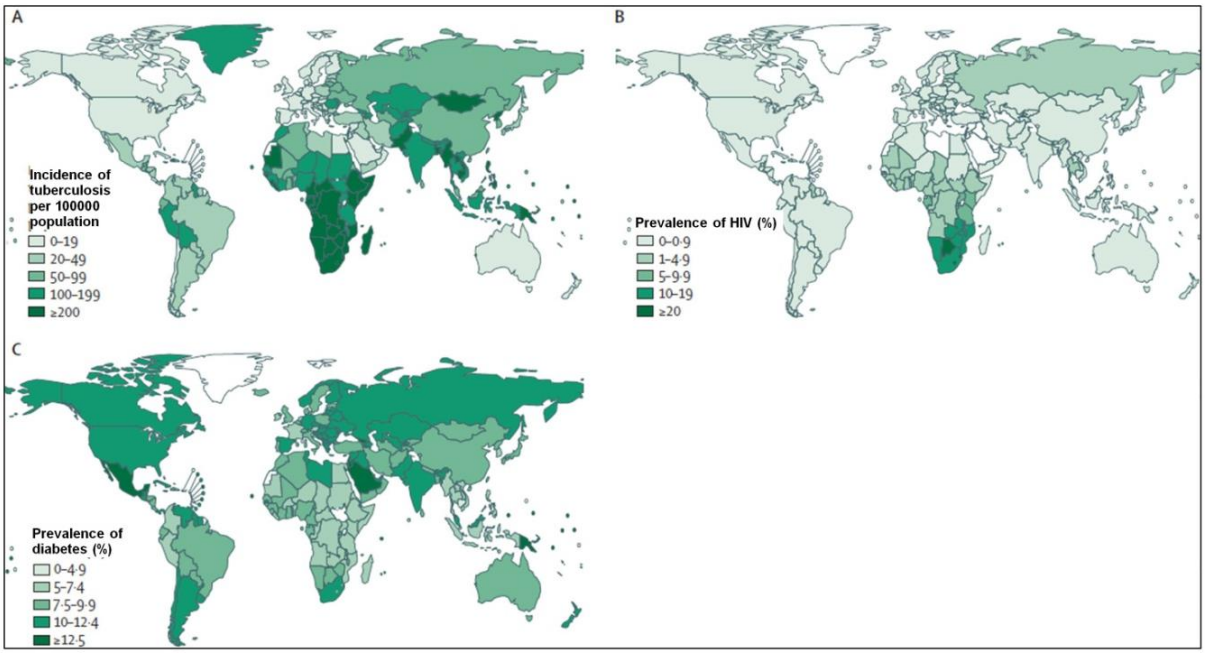

Figure 1-1. Worldwide incidence of TB in 2011 (A), prevalence of HIV in 2011 (B) and DM in adults in 2008 (C) (Adapted from (Marais et al., 2013)).

Up to date, only four studies examined the association between the two diseases in sub-Saharan Africa. In Mwanza, Tanzania, DM prevalence was 16.7% among TB patients versus 9.4% in the control group. Interestingly, the association depended on

HIV status with an association only among HIV uninfected patients (Faurholt-Jepsen et al., 2011). In Uganda, DM prevalence was 8.5% among admitted TB patients and HIV infection was a protective factor against DM (Kibirige et al., 2013). Two additional studies, one in Benin and the other in Guinea-Bissau found a very low DM prevalence among patients with TB (1.9-2.8%) but did not analyze the interaction with HIV (Ade et al., 2015, Haraldsdottir et al., 2015). No study observed a synergistic immunosuppressive effect of HIV and DM on the incidence of active TB and the results point to the predominant effect of HIV on TB risk in African settings. However, the complex interplay between immunosuppression linked to HIV and DM cannot be analyzed cross-sectionally and needs further investigations.

DM was also associated with an increased risk of failure and death during TB treatment (Baker et al., 2011). Sputum culture conversion rate after two to three months of TB therapy has been analyzed in different settings among diabetics and showed conflicting results (Alisjahbana et al., 2007, Dooley et al., 2009, Tatar et al., 2009). Several cross-sectional studies conducted in different settings showed an association between MDR-TB and DM but none of them was conducted in Africa (Fisher-Hoch et al., 2008, Gomez-Gomez et al., 2015, Magee et al., 2015, Rifat et al., 2014).

For decades, DM screening has been based on plasma glucose criteria. As an infectious disease, TB can increase insulin resistance and lead to stress-induced hyperglycemia and to an overdiagnosis of DM during the acute phase of the disease (Gearhart and Parbhoo, 2006). The American Diabetes Association (ADA) and WHO recommend now the use of glycated haemoglobin (Hba_{1c}) for diagnosing DM (ADA, 2014, WHO, 2011a). Hba_{1c} has several advantages over plasma glucose including greater convenience, since fasting is not required, and less day-to-day perturbations during periods of stress and illness. The half-life of Hba_{1c} is also much longer than that of glucose, and reflects blood glucose concentrations over the course of about 3 months. Hba_{1c} measurement could therefore be a more reliable tool for diagnosing preexisting DM during acute TB and to assess the impact of DM on the risk and prognosis of active TB. Indeed, the normalization of glycemic status during TB treatment has already been described in small study samples (n=20-50) using oral glucose tolerance tests (Oluboyo and Erasmus, 1990, Singh et al., 1984).

1.4 Vitamin D as a potential link between tuberculosis and diabetes

Vitamin D is generated in the skin after sun exposure and the dietary source of vitamin D is limited. Vitamin D deficiency was first described as the cause of rickets and osteomalacia. The importance of sufficient vitamin D level for musculoskeletal health is now well recognized. However, the vitamin D receptor is expressed in most cells in the body and a wide variety of vitamin D associated health outcomes have been described (Rosen, 2011). An inverse association between type 2 DM and poor vitamin D status has been described in several cross-sectional and prospective studies and in a meta-analysis of 21 prospective studies (Song et al., 2013). Several studies have also shown an association between low level of vitamin D and TB (Martineau, 2012). Indeed, vitamin D boosts the antimicrobial activity of human macrophages against *Mtb*, modulates adaptive response and affects pancreatic β-cell function (Hawn et al., 2015). In monocytes, activation of Toll-like receptors (TLRs; pathogen recognition receptors) by mycobacteria induces expression of an enzyme which converts vitamin D into the bioactive form $1,25(OH)_2D$ and upregulates vitamin D receptor. Then, vitamin D stimulates the receptor to induce cathelicidin expression which has both immunoregulatory and direct antimicrobial activity (Hawn et al., 2015, Schauber et al., 2007). The association of vitamin D with both, TB and DM, suggests that vitamin D deficiency could mediate some of the association between TB and DM (Handel and Ramagopalan, 2010). A cross-sectional Indian study confirmed the association between vitamin D deficiency, TB and DM while another study conducted in China did not show any association (Chaudhary et al., 2013, Zhan and Jiang, 2015).

Large trials of adjunctive vitamin D therapy during TB treatment have failed to show a significant acceleration of sputum culture conversion despite demonstration of enhanced resolution of inflammatory markers (Coussens et al., 2012, Daley et al., 2015, Martineau et al., 2011b). Particular sub-groups of patients with lower levels of vitamin D, such as diabetic patients, may have a clinical benefit of vitamin D supplementation for TB treatment and prevention (Davies and Martineau, 2015).

Up to date, no longitudinal study has described the interplay between vitamin D concentrations, DM status and TB (Figure 1-2).

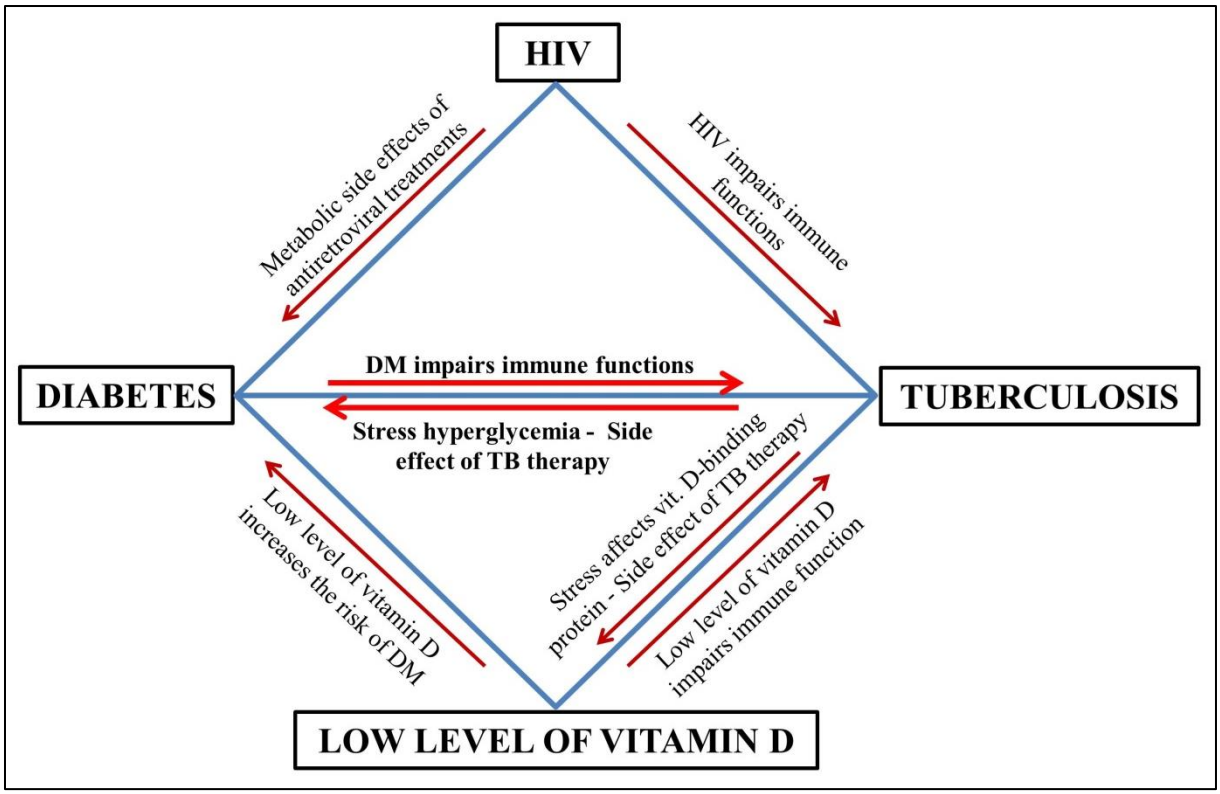

Figure 1-2. Complex interaction between TB, DM, HIV and vitamin D deficiency.

Figure 1-2 underlines the importance of a longitudinal analysis of these comorbidities as the causal relationship can be bidirectional. Our longitudinal study will explore the interplay of DM and TB in Tanzania, a setting with high HIV and TB prevalence. The present study will evaluate the role of low vitamin D level in the association between TB and DM. It will also assess the optimal method and timing of screening for DM in TB patients. By this way, it could improve the management of both, TB and DM and lead to the development of new recommendations regarding DM screening and management in this population.

1.5 Diabetes and immune response to Tuberculosis

DM is known to increase susceptibility to bacterial, fungal and viral infections through reduced innate and adaptive immune response, particularly in patients suffering from chronic hyperglycemia. Regarding innate response, polymorphonuclear cells and macrophages have impaired performance in the presence of hyperglycemia with decreased mobilization, chemotaxis, phagocytic activity and cytokine expression (Schuetz et al., 2011). Some studies demonstrated impaired T-cell functions characterized both by an aberrant regulation in the interplay between anti-inflammatory

and pro-inflammatory cytokines, as well as functional defects in the antigen-presenting cell compartment (Geerlings and Hoepelman, 1999, Schuetz et al., 2011). The negative effect of DM on immune responses could explain the higher co-morbidity with TB since it is well established that macrophages and $CD4^+$ T helper type 1 lymphocytes play a major role in the immune defense mechanisms against TB.

The pathophysiological mechanisms linking the two diseases are poorly understood. The effect of DM on immunity has been primarily attributed to hyperglycemia, but there is also a potential contribution of other factors associated to DM such as older age, obesity, vitamin D deficiency and other co-morbidities (Restrepo and Schlesinger, 2013).

The main port of entry of TB is the lung where alveolar macrophages phagocyte the bacilli. Dissemination of the bacteria to the draining lymph nodes activates naive T-cells which proliferate and become effector cells. These effector cells migrate from the lymph node to the lung in the granuloma. They mediate immune protection by activating infected macrophages with the induction of oxygen radicals and nitric oxide to combat the intra-cellular localized *Mtb*. A dynamic balance between bacterial persistence and host defence develops within an inflammatory lesion, the granuloma (Cooper, 2009).

$CD4^+$ T helper type 1 (Th1) lymphocytes largely mediate resistance to mycobacterial infections through cytokine production (e.g. interleukin-2 [IL-2], interferon-γ [IFN-γ], tumor necrosis factor-alfa [TNF-α]), whereas $CD4^+$ T helper type 2 (Th2) lymphocytes and their cytokines (interleukin-4 [IL-4], interleukin-10 [IL-10]) correlate with disease susceptibility in TB (Dooley and Chaisson, 2009, Flynn and Chan, 2001, Al-Attiyah and Mustafa, 2009). $CD4^+$ T helper type 17 (Th17) lymphocytes, producing e.g. Il-17a, are critical for the enhancement of memory responses again *Mtb* and excessive activation of this pathway has been associated with metabolic diseases such as DM (Pappu et al., 2011). Specific chemokines (e.g. MIP-1beta/CCL4) produced during *Mtb* infection and recruiting $CD4^+$ Th1 cells for granuloma formation, are present at higher levels in diabetic patients (Bala et al., 2011, Vesosky et al., 2010).

The immunological consequences of DM co-morbidity on TB specific adaptive immunity have been evaluated in different human studies but a defect in protective immunity has not yet been identified (Martinez and Kornfeld, 2014). A higher

production of Th1 cytokines following tuberculin-purified protein derivative stimulation among patients with active TB with DM compared to patients without DM has been reported but not reproduced in an Indonesian setting (Kumar et al., 2013b, Restrepo et al., 2008, Stalenhoef et al., 2008). Mice studies provided interesting data showing a delayed but unimpaired cellular immune response to *Mtb* among mice with DM (Martinez et al., 2014, Vallerskog et al., 2010).

The present study will describe the immunological features underlying TB and DM comorbidity in sub-Saharan Africa and test the hypothesis of delayed adaptive immune response in humans. We performed a broad phenotypic and functional characterisation of T cells in subjects with active or latent *Mtb* infection stratified by their diabetic status following stimulation with either live *M. bovis* BCG or *Mtb*-specific peptide pools that respectively requires or not antigen processing or presentation.

2 Goal and Objectives

2.1 Goal

The goal of the present project was to improve knowledge on the interplay between communicable and non-communicable diseases focusing on DM and TB comorbidity in an African setting with high HIV prevalence.

2.2 Objectives

2.2.1 Objective 1

To assess the association of TB and its outcome with the presence and persistence of hyperglycemia in Tanzania, using three different DM screening tests.

2.2.2 Objective 2

To analyse the modifying effect of a low level of vitamin D on the association between DM and TB.

2.2.3 Objective 3

To study the immunological features underlying TB and DM comorbidity in sub-Saharan Africa and to test the hypothesis of delayed adaptive immune response with increasing glycemia.

3 Study setting and Methods

3.1 Study site and Setting

3.1.1 Study site

The field work of the present project took place in Dar es Salaam, the economic capital of the United Republic of Tanzania (Figure 3-1).

Figure 3-1. Study site. (Adapted from: http://www.lib.utexas.edu/maps/africa/tanzania_pol_2003.pdf).

Located in East Africa, Tanzania is a low income country with a Gross National Income per capita of 930$ (World-Bank, 2015). In 2014, the population of Tanzania was 51.82 million and more than 70% of the population lived in rural areas. Life expectancy at birth has been constantly increasing during the last decade and was 61 years in 2013 (World-Bank, 2015) (Figure 3-2).

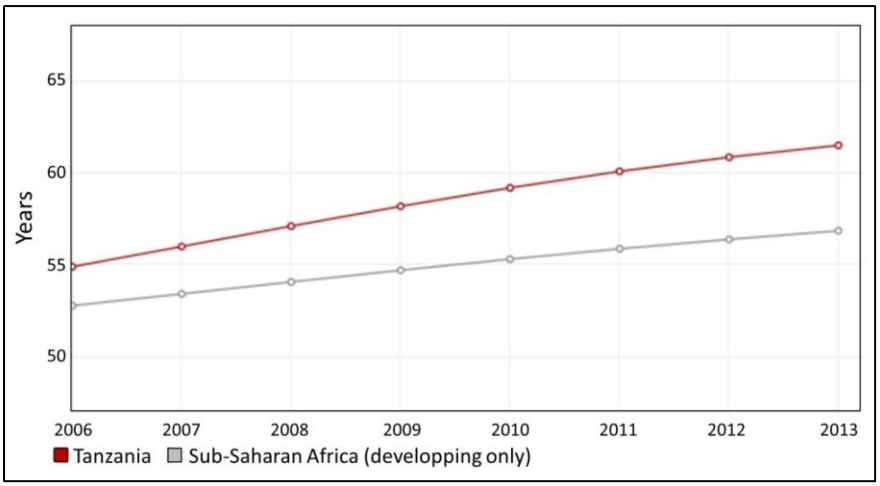

Figure 3-2. Life expectancy at birth in Tanzania and Sub-Saharan Africa (developing countries only). (Adapted from: http://data.worldbank.org/indicator/SP.DYN.LE00.IN/countries/TZ-ZF?display=graph).

Dar es Salaam is the economic capital of the country, located on the East coast. It is the largest city in Tanzania with 4.4 million inhabitants in 2012 (PHC, 2012). It is among the ten fastest growing cities in the world with an annual growth rate of 5.6% and 70% of its population living in informal habitations (Figure 3-3).

Figure 3-3. Rapidly growing Dar es Salaam. (Adapted from: Wikipedia)

The administrative structure divides Dar es Salaam into three districts or municipalities, Kinondoni, Ilala, and Temeke, from North to South. Each district is further divided in wards and subwards. Three District Medical Offices are in charge of the management of the health facilities (one district hospital, health centers and dispensaries) that are located in their district.

Tanzania has a high burden of TB with an incidence of 327:100'000 population in 2014. The number of estimated new TB cases in 2014 according to WHO was 63'000. The HIV prevalence in incident TB cases was estimated to be 35% (WHO, 2015). Dar es Salaam is the major contributor to TB incidence with 21.8% of the new TB patients diagnosed here (NTLP, 2013a).

The prevalence rate of DM in Tanzania is estimated at being between 1 and 9% depending on setting, with the highest rates reported within the Indian community and in urban settings (Aspray et al., 2000, Kolling et al., 2010, McLarty et al., 1989, Ramaiya et al., 1995, Swai et al., 1990, Unwin et al., 2010). The last WHO report reported a prevalence of 5% (WHO, 2014a).

3.1.2 Study setting

The study took place in Kinondoni district, the biggest district of Dar es Salaam. Patient recruitment was located in different health facilities from this district: Mwananyamala hospital (referral hospital), Sinza hospital (district hospital), Magomeni healthcare center and Tandale dispensary (Figure 3-4).

Figure 3-4. Participating Healthcare centers in Kinondoni District, Dar es Salaam.

3.2 Methods

3.2.1 Evaluation of the association of TB and its outcome with the presence and persistence of hyperglycemia

Study design

Case-control study with longitudinal follow-up of cases.

Study population

Consecutive adults with new active TB diagnosed in the participating hospitals in Dar es Salaam were included and followed up for five months after the start of anti-TB treatment. TB diagnosis was based on sputum smear microscopy, chest X-ray findings, clinical evidence of TB and decision by the clinician to treat with a full-course of anti-TB therapy (2013b). Healthy controls were recruited among volunteering adults accompanying patients, other than the one included in the study, to the outpatient departments of the same hospitals and living in Kinondoni District. We used frequency matching on sex and age (10-year age groups) to select the controls. Exclusion criteria were a biological relationship to TB case, TB history, symptoms or signs of TB, other acute infection or major trauma within the last three months.

Measurements

All underwent DM screening tests (fasting capillary glucose (FCG), 2-hour capillary glucose after standard oral glucose tolerance test (2h-CG) and glycated hemoglobin (HbA1c)) at enrolment and TB patients were again tested after 5-month of TB treatment. Every abnormal glycemic (FCG ≥5.6 mmol/l; 2-hCG ≥7.8 mmol/l) or HbA1c value (HbA1c ≥5.7% (39 mmol/mol)) was confirmed by repeat testing two to five days later. Data on the outcome of TB (treatment failure, death, lost to follow-up) were collected.

Outcomes

Description of the longitudinal course of hyperglycemia in TB patients.

Assessment of the association of TB with hyperglycemia at baseline and 5-months follow-up.

Assessment of the association between TB outcome and hyperglycemia at baseline.

3.2.2 Description of the association between low vitamin D and TB and its dependence on DM

Study design

Case-control study with longitudinal follow-up of cases.

This study was part of the study on the association between TB and DM described previously.

Study population

Consecutive adults with active TB and healthy volunteers were included as described above. TB patients were followed up for five months after the start of anti-TB treatment.

Measurements

All underwent DM screening tests (FCG, 2-hCG and HbA1c) and total 25 hydroxyvitamin D (25(OH)D) measurement at enrolment and after TB treatment for TB patients. Data on the outcome of TB were collected.

Outcomes

Assessment of the association of TB with 25(OH)D level at baseline and 5-months follow-up and its dependence on DM.

Assessment of the association between TB outcome and 25(OH)D level at baseline.

3.2.3 Phenotypic and functional characterisation of T cells in subjects with active or latent TB stratified by their diabetic status following stimulation with either whole-mycobacteria or peptide pools to test the hypothesis of delayed adaptive immune response

Study design

Ex-vivo immunological TB case control study.

Study population

Active TB patients (Xpert MTB/RIF positive in sputum) HIV negative were recruited in the participating hospitals in Dar es Salaam and stratified according to their DM status.

Latent TB patients. Healthy volunteering adults and diabetic patients attending the DM clinic in the participating hospitals were included if they were latently infected with *Mtb*, HIV negative, had no past TB history and no symptoms or signs of active TB.

Exclusion criteria were pregnancy and first week of lactation, any kind of immunosuppression during the last 6 months and severe anemia.

DM status. The presence of DM was defined as repeated measurements of the three DM screening tests: ≥7.0 mmol/l for FCG, ≥11.1 mmol/l for 2-hCG and ≥6.5% for HbA1c and/or in the presence of history and treatment for DM. The three tests had to be in the DM range in order to avoid as much as possible patients with stress hyperglycemia. The absence of DM was defined as FCG<6.1 mmol/l and 2-hCG<7.8 mmol/l. Patients with pre-DM were excluded. The capillary glucose values were confirmed by plasma testing (Cobas Integra 400 plus).

Measurements

All underwent DM screening tests (FCG, 2h-CG and HbA1c) at enrolment. Peripheral blood mononuclear cells were stimulated with peptide pools and live *Mycobacterium bovis* BCG and analysed by polychromatic flow cytometry for Th1, Th2, Th9 and Th17 cytokine production. Cell culture supernatants were analysed by Luminex for 34 cytokines and chemokines.

Outcomes

Comparison of the frequencies of $CD4^+$ T cells producing cytokines after live *M. bovis* BCG vaccine and *Mtb*-specific peptide pool stimulation between the four study groups (active TB or latent TB patients stratified by their DM status).

Comparison of frequencies of $CD4^+$ T cells producing cytokines according to fasting glycemia.

4 Transient Hyperglycemia in Patients with Tuberculosis in Tanzania: Implications for Diabetes Screening Algorithms

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4.1 Abstract

Background

Diabetes mellitus (DM) increases tuberculosis (TB) risk while TB, as an infectious disease, leads to hyperglycemia. We compared hyperglycemia screening strategies in controls and TB patients in Dar es Salaam, Tanzania.

Methods

Consecutive adults with TB and sex and age-matched volunteers were included in a case-control study between July 2012-June 2014. All underwent DM screening tests (fasting (FCG), 2-hour capillary glucose (2-hCG) and glycated hemoglobin (HbA1c) at enrolment and cases again after TB treatment. Association of TB and its outcome with hyperglycemia were assessed using logistic regression adjusted for sex-age-body mass index-HIV-socioeconomic status. TB patients with newly-diagnosed DM were not treated for hyperglycemia.

Results

At enrolment, DM prevalence was significantly higher among TB patients (N=539; FCG>7mmol/L:4.5%, 2-hCG>11mmol/L:6.8% and HbA1c>6.5%:9.3%) compared to controls (N=496; 1.2%, 3.1% and 2.2%). Association between hyperglycemia and TB disappeared after TB treatment (aOR(95% CI) at enrolment vs follow-up: FCG 9.6(3.7-24.7) vs 2.4(0.7-8.7); 2-hCG 6.6(4.0-11.1) vs 1.6(0.8-2.9); HbA1c 4.2(2.9-6.0) vs 1.4(0.9-2.0)). FCG hyperglycemia at enrolment was associated with TB treatment failure or death (aOR(95%CI) 3.3(1.2-9.3).

Conclusions

Transient hyperglycemia is frequent during TB and DM needs confirmation after TB treatment. DM screening at TB diagnosis gives the opportunity to detect patients at risk of adverse outcome.

4.2 Background

Interest in the comorbidity between tuberculosis (TB) and diabetes mellitus (DM) has reemerged due to the global increase of DM and its potential impact on TB control. DM doubles or triples the incidence of active TB and increases the risk of TB treatment failure and death (Baker et al., 2011, Jeon and Murray, 2008, Stevenson et al., 2007b). A framework was developed to guide national programs in their response to DM and TB (2011b). The prevalence of DM among TB patients differs between countries (16-45%). Sub-Saharan Africa has the highest rate of undiagnosed DM, is expecting the sharpest increase in DM by 2035, has a high TB burden, and the highest rate of TB and HIV coinfection (Shaw et al., 2010, WHO, 2014b). Two studies in Tanzania and Uganda confirmed the cross-sectional TB-DM association in the presence of high HIV prevalence (Faurholt-Jepsen et al., 2011, Kibirige et al., 2013). Unexpectedly, HIV seamed to protect against DM. The impact of HIV infection on the TB-DM association needs further investigations as HIV increases metabolic risk, but can also lower body mass index (Paula et al., 2013).

When and how best diagnose DM in the presence of infections is challenging. As an infectious disease, TB increases insulin resistance and stress-induced hyperglycemia which may lead to over-diagnosis of DM during the acute phase of TB (Dungan et al., 2009). Screening of DM is based on blood glucose criteria, and, lately, on the use of glycated hemoglobin (HbA_{1c}) with its advantage of not requiring fasting and exhibiting less day-to-day variations during periods of stress and illness due to its half-life of 120 days (2014, WHO, 2006, WHO, 2011a). Few small studies have examined the longitudinal course of glycemia during TB treatment, but none was conducted on the African continent or compared the performance of different DM screening tests over time within the same study population (Jawad et al., 1995, Oluboyo and Erasmus, 1990, Singh et al., 1984, Tabarsi et al., 2014).

We examined the association of TB and its outcome with the presence and persistence of hyperglycemia in Tanzania, using three different DM screening tests.

4.3 Methods

Study design and setting

TB patients were recruited between June 2012 and December 2013 and followed up for a median time of five months (IQR 4.7-6.1) after the start of anti-TB treatment. Controls were recruited between December 2012 and September 2013 and did not have follow-up visits. The study was carried out in Kinondoni, the most populated District of Dar es Salaam. Patients were recruited in Mwananyamala Regional Hospital and connected health facilities (Sinza Hospital, Magomeni Health Centre, Tandale Dispensary).

Study participants

Cases

Consecutive adult patients (age ≥ 18 years; living in Kinondoni District) presenting in the participating hospitals with new active TB diagnosed by the National TB and Leprosy Control Programme (NTLP) were screened for study inclusion.

TB diagnosis was based on sputum smear microscopy, chest X-ray findings, clinical evidence of TB and decision by the clinician to treat with a full-course of anti-TB therapy (2013). TB patients were classified as pulmonary smear-positive, smear-negative or extrapulmonary. TB patients were treated for 6 months with a standard regimen or longer if necessary (2013, WHO, 2010). Sputum microscopy was repeated at months two-three and five by NTLP to evaluate the response to treatment in pulmonary smear-positive TB patients (2013, WHO, 2010). TB treatment outcome was defined according to WHO guidelines as treatment success (cure, treatment completed) or adverse outcome (failure, death, lost to follow-up) (WHO, 2010).

Controls

We used frequency matching on sex and age (10-year age groups) to select controls among adults accompanying patients, other than the one included in the study, to the outpatient departments and living in Kinondoni District. Exclusion criteria were a biological relationship to case, TB history, symptoms or signs of TB, other acute infection or major trauma within the last three months.

Study procedures

Data collection

Information on demographic characteristics, health history (DM, high blood pressure (BP), TB and HIV) and symptoms (type and duration), SES (indicators of education, occupation, wealth using factor analysis), smoking (ever daily smoking), and alcohol misuse (≥three drinks per day or ≥six drinks per occasion (WHO, 2001a)) was obtained during an in-person interview. Clinical examination and screening for hyperglycemia were performed on the day of the interview. Body mass index (BMI) was calculated as measured weight divided by measured height squared (kg/m^2). BP was measured and defined according to WHO (WHO, 2013). Data were entered directly into an open data kit (ODK) in a personal digital assistant with real-time error, range and consistency checks (Hartung et al., December 2010).

Laboratory and chest X-ray investigations

Chest X-rays, performed in every TB patient, were read by an experienced radiologist. Participants were screened for HIV infection according to the national algorithm using first a rapid immune-chromatographic test (Alere Determine™ HIV-1/2) and for confirmation a second rapid test (Trinity Biotech Uni-gold™ Recombigen® HIV-1/2). In case of HIV diagnosis, cART was started by NTLP according to national guidelines (2013). Complete blood count was performed (Sysmex XS-800i automated hematology analyzer). Anemia was defined as hemoglobin <13 g/dl in men and <12 g/dl in women (WHO, 2001b).

Hyperglycemia screening and DM diagnosis

Blood glucose testing was conducted between 8 and 11 am after an overnight fast of ≥8 hours (fasting capillary glucose-FCG; GlucoPlus™, Diabcare); two-hours capillary glucose (2-hCG; standard 75-gram OGTT); glycated hemoglobin HbA1c (venipuncture whole blood; immuno-assay certified by the National Glycohemoglobin Standardization Program and insensitive to hemoglobinopathies (Tina-quant HbA1c Gen. 2 Cobas Integra 400, Roche Diagnostics)) (NGSP, NGSP). Testing for DM was performed at enrolment in both controls and TB patients (45% screened after start of TB treatment

(median (IQR) of 2 days (1-4)), and repeated in TB patients after a median of five months of anti-TB treatment (67% screened under TB treatment).

DM is generally a chronic and permanent condition, however, for the sake of clarity, we refer to DM when repeated measurements are ≥7.0 mmol/l for FCG, ≥11.1 mmol/l for 2-hCG or ≥6.5% for HbA_{1c} and/or in the presence of history and treatment for DM according to the standard ADA and WHO criteria (2014, WHO, 2006, WHO, 2011a). Pre-diabetes (pre-DM) was defined according to WHO: 6.1≤FCG<7 mmol/l; 7.8≤2-hCG<11.1 mmol/l; 5.7≤HbA_{1c}<6.5% (39≤HbA_{1c}<48 mmol/mol) (WHO, 2006, WHO, 2011a). Hyperglycemia refers to patients with pre-DM or DM. Every abnormal glycemic (FCG ≥5.6 mmol/l; 2-hCG ≥7.8 mmol/l) or HbA1c value (HbA_{1c} ≥5.7% (39 mmol/mol))) was confirmed by repeat testing two to five days later. 2-hCG testing was omitted if FCG was ≥11.1 mmol/l. Participants diagnosed with DM were referred to the local DM clinic, but none was started on DM treatment.

Data analysis

Case-control study at enrolment

TB patients and controls with complete glucose status data (FCG, 2-hCG and HbA1c) at enrolment were included in the cross-sectional analysis (Figure 4-1). Characteristics of TB patients were compared to controls (+/-HIV stratification) and differences were tested with Wilcoxon-Mann-Whitney and chi-square tests. The independent association of pre-DM and DM with TB status was evaluated by logistic regression (+/-HIV stratification) for every DM screening test independently. Covariates included in the final models were age, sex, BMI, SES and HIV status (non-stratified analysis only). Lifestyle variables were not included in the models as they had no influence on the reported association. Additional sensitivity analysis excluded patients previously known and treated for DM.

Longitudinal course of hyperglycemia in TB patients

Longitudinal analysis of changes in blood glucose over follow-up of a median of five months of TB treatment was restricted to patients with complete DM screening data at enrolment and follow-up (Figure 4-1). Baseline characteristics of patients with and without follow-up were compared using Wilcoxon-Mann-Whitney and chi-squared tests. The longitudinal pattern of the association between TB and hyperglycemia was

assessed for each DM screening test separately a) with two cross-sectional comparisons (TB patients at enrolment versus controls and TB patients at follow-up versus controls; +/-HIV stratification), b) using a binary GEE-model (+/-HIV stratification) to assess the change in the prevalence of hyperglycemia from the time of enrolment to follow-up among TB patients. Potential confounding variables were included in the final models (age, sex, BMI, SES and HIV status (non-stratified analysis only)). Lifestyle variables were not included in the models as they had no influence on the reported association.

Hyperglycemia at enrolment and TB treatment outcome

Baseline characteristics of patients with and without adverse TB outcome were compared using Wilcoxon-Mann-Whitney and chi-squared tests. The association between adverse TB outcome and hyperglycemia was assessed for each DM screening test separately with logistic regression adjusted for potential confounding variables (age, sex, BMI, SES, HIV status, duration of TB symptoms before diagnosis and cavity on X-ray). Lifestyle variables were not included in the models as they had no influence on the reported association.

Statistical analyses were performed using Stata software (StataCorp, College Station, TX, USA, version 12).

Ethical considerations

All participants consented in writing to interview and health examination. The Ifakara Health Institute Institutional Review Board and the Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania, gave ethical clearance.

4.4 Results

Study sample at enrolment

A total of 539 TB patients and 496 controls were enrolled in the study. Nine TB patients and five controls were excluded because of missing FCG, 2-hCG or HbA1c data at enrolment (Figure 4-1). Characteristics of the study sample are described in Table 4-1. TB patients were more often HIV-infected (32 versus 14%), under-weight and

anemic. Only 10 TB patients and 2 controls, all HIV-negative, were already diagnosed and treated for DM at enrolment. DM prevalence at enrolment was significantly higher in TB patients (4.5%, 6.8% and 9.3%) compared to controls (1.2%, 3.1% and 2.2%) for FCG, 2-hCG and HbA1c ($p<0.01$). This was also true for pre-DM, irrespective of HIV status. Mean FCG, 2-hCG and HbA1c values were significantly higher in TB patients (mean values of 5.6mmol/L, 7.8mmol/L and 6.1%) compared to controls (4.8mmol/L, 6.7mmol/L and 5.6%).

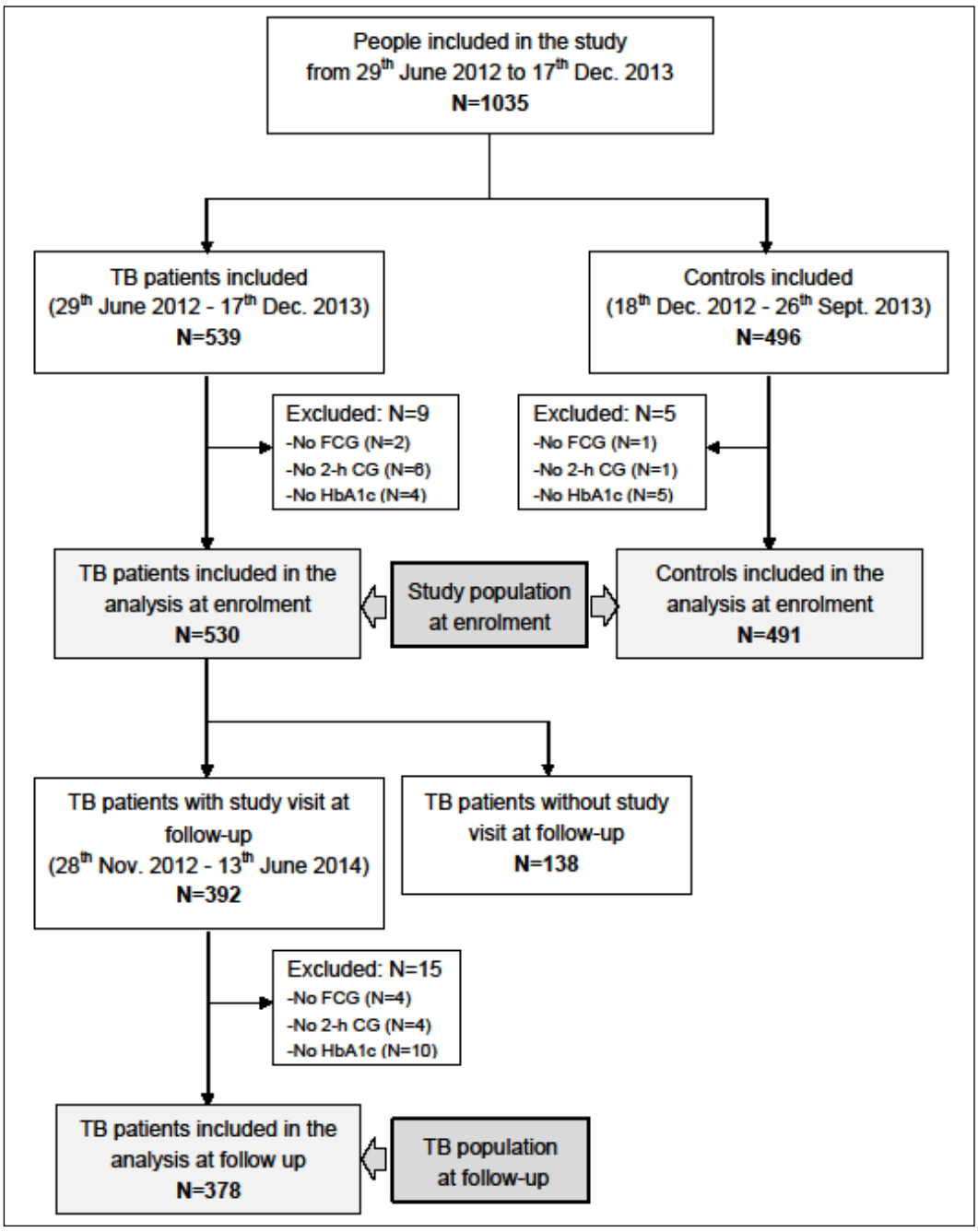

Figure 4-1. Flow chart of study participants.

Table 4-1. Characteristics of the case-control sample at enrolment, all subjects and stratified by HIV status.

	All			HIV-negative			HIV-infected		
	TB patients N=530	Controls N=491		TB patients N=355	Controls N=423		TB patients N=166	Controls N=66	
	N(%) or Mean(sd)		p	N(%) or Mean(sd)		p	N(%) or Mean(sd)		p
Age	35.9 (12)	36.7 (13)	0.75	35.0 (12)	36.0 (14)	0.41	37.0 (9)	41.0 (11)	0.06
Male sex	341 (64)	255 (52)	<0.001	262 (74)	233 (55)	<0.001	74 (45)	21 (32)	0.08
History of smoking	149 (28)	99 (20)	0.003	113 (32)	89 (21)	0.001	34 (21)	10 (15)	0.34
Alcohol misuse	62 (12)	27 (6)	<0.001	47 (13)	24 (6)	<0.001	14 (8)	3 (5)	0.31
Socioeconomic status			*<0.001*			*<0.001*			*<0.001*
Low	170 (32)	85 (18)	<0.001	110 (31)	80 (19)	<0.001	57 (35)	5 (8)	<0.001
Medium	253 (48)	243 (50)	0.3	165 (47)	196 (47)	1	83 (51)	46 (71)	0.008
High	103 (20)	158 (33)	<0.001	79 (22)	143 (34)	<0.001	23 (14)	14 (22)	0.17
Body Mass Index	19.6 (4)	25.4 (5)	<0.001	19.3 (4)	25.3 (6)	<0.001	20.2 (4)	25.9 (5)	<0.001
High blood pressure	44 (8)	74 (15)	0.001	33 (9)	66 (16)	0.008	10 (6)	8 (13)	0.11
HIV infection	166 (32)	66 (14)	<0.001						
Anemia	425 (81)	172 (35)	<0.001	271 (77)	142 (34)	<0.001	149 (91)	30 (45)	<0.001
Previously known DM	10 (1.9)	2 (0.4)	0.03	10 (3)	2 (0.5)	0.008	0 (0)	0 (0)	-
FCG			*<0.001*			*<0.001*			*0.02*
Normal	459 (86.6)	479 (97.6)	<0.001	316 (89.0)	414 (97.9)	<0.001	135 (81.3)	63 (95.5)	0.007
Pre-diabetes	47 (8.9)	6 (1.2)	<0.001	24 (6.8)	5 (1.2)	<0.001	23 (13.9)	1 (1.5)	0.004
Diabetes	24 (4.5)	6 (1.2)	0.002	15 (4.2)	4 (1.0)	0.004	8 (4.8)	2 (3)	0.73
2-hCG			*<0.001*			*<0.001*			*<0.001*
Normal	365 (68.9)	446 (90.8)	<0.001	257 (72.4)	387 (91.5)	<0.001	101 (60.8)	57 (86.4)	<0.001
Pre-diabetes	129 (24.3)	30 (6.1)	<0.001	75 (21.1)	26(6.2)	<0.001	53 (31.9)	4 (6.1)	<0.001
Diabetes	36 (6.8)	15 (3.1)	0.006	23(6.5)	10 (2.4)	0.007	12 (7.2)	5 (7.6)	1
HbA1c			*<0.001*			*<0.001*			*<0.001*
Normal	224 (42.3)	329 (67)	<0.001	158 (44.5)	283 (66.9)	<0.001	62 (37.4)	45 (68.2)	<0.001
Pre-diabetes	257 (48.5)	151 (30.8)	<0.001	164 (46.2)	135 (31.9)	<0.001	88 (53.1)	15 (22.7)	<0.001
Diabetes	49 (9.3)	11 (2.2)	<0.001	33 (9.3)	5 (1.2)	<0.001	16 (9.6)	6 (9.1)	1
TB symptoms >3months	97 (18)			69 (19)			25 (15)		
TB									
Smear positive	385 (73)			269 (76)			111 (67)		
Smear negative	111 (21)			64 (18)			44 (27)		
Extrapulmonary	34 (6)			22 (6)			11 (6.6)		
Cavity on X-ray	250 (50)			184 (55)			61 (38)		

Abbreviations and definitions: **FCG**: fasting capillary glucose: Normal: <6.1 mmol/L / Pre-diabetes: 6.1-6.9 mmol/L / Diabetes: ≥7 mmol/l | **2-hCG**: 2-hour capillary glucose: Normal: <7.8 mmol/L / Pre-diabetes: 7.8-11 mmol/L / Diabetes: ≥11.1 mmol/L | **HbA1c**: glycated haemoglobin: Normal:<5.7% / Pre-diabetes: 5.8-6.4% / Diabetes: ≥6.5%

Association between TB and DM at enrolment

Irrespective which DM test was used and whether diabetics previously diagnosed and treated were excluded or not, DM and pre-DM were independently associated with TB (Table 4-2). The OR for DM criterion based on HbA1c was substantially lower in HIV-infected participants (interaction p value=0.048). The prevalence of DM among HIV-infected patients was not different according to the presence or not of cART.

Table 4-2. Cross-sectional association of pre-diabetes and diabetes with tuberculosis at enrolment, all subjects and stratified by HIV status.

	All				HIV-negative				HIV-infected			
	N=530 Patients with tuberculosis (TB) N=491 Healthy controls (HC)				N=355 Patients with tuberculosis (TB) N=423 Healthy controls (HC)				N=166 Patients with tuberculosis (TB) N=66 Healthy controls (HC)			
	TB N (%)	HC N (%)	OR (95% CI) Unadjusted	OR (95% CI) Adjusted *	TB N (%)	HC N (%)	OR (95% CI) Unadjusted	OR (95% CI) Adjusted *	TB N (%)	HC N (%)	OR (95% CI) Unadjusted	OR (95% CI) Adjusted *
FCG												
Normal	459 (86.6)	479	*Ref.*	*Ref.*	316	414	*Ref.*	*Ref.*	135	63 (95.5)	*Ref.*	*Ref.*
Pre-diabetes	47 (8.9)	6 (1.2)	**8.2 (3.5-19.3)**	**8.8 (3.1-25.1)**	24 (6.8)	5 (1.2)	**6.3 (2.4-16.7)**	**7.2 (2.1-25.0)**	23 (13.9)	1 (1.5)	**10.7 (1.4-81.3)**	**15.5 (1.8-136)**
Diabetes	24 (4.5)	6 (1.2)	**4.2 (1.7-10.3)**	**10.6 (3.2-4.1)**	15 (4.2)	4 (1.0)	**4.9 (1.6-15.0)**	**8.8 (2.1-36.6)**	8 (4.8)	2 (3.0)	1.9 (0.4-9.1)	**17.1 (1.6-179.4)**
2-hCG												
Normal	365 (68.9)	446	*Ref.*	*Ref.*	257	387	*Ref.*	*Ref.*	101	57 (86.4)	*Ref.*	*Ref.*
Pre-diabetes	129 (24.3)	30 (6.1)	**5.3 (3.5-8.0)**	**8.2 (4.6-14.6)**	75 (21.1)	26 (6.2)	**4.3 (2.7-7.0)**	**7.6 (3.9-14.8)**	53 (31.9)	4 (6.1)	**7.5 (2.6-21.7)**	**11.1 (3.1-39.9)**
Diabetes	36 (6.8)	15 (3.1)	**2.9 (1.6-5.4)**	**3.7 (1.6-8.3)**	23 (6.5)	10 (2.4)	**3.5 (1.6-7.4)**	**3.8 (1.4-10.5)**	12 (7.2)	5 (7.6)	1.4 (0.5-4.0)	3.8 (1.0-15.3)
HbA1c												
Normal	224 (42.3)	329 (67)	*Ref.*	*Ref.*	158	283	*Ref.*	*Ref.*	62 (37.4)	45 (68.2)	*Ref.*	*Ref.*
Pre-diabetes	257 (48.5)	151	**2.5 (1.9-3.3)**	**3.6 (2.5-5.1)**	164	135	**2.2 (1.6-2.9)**	**3.1 (2.1-4.6)**	88 (53.1)	15 (22.7)	**4.3 (2.2-8.3)**	**7.9 (3.1-20.1)**
Diabetes	49 (9.3)	11 (2.2)	**6.5 (3.3-12.9)**	**10.7 (4.5-26)**	33 (9.3)	5 (1.2)	**11.8 (4.5-31)**	**19.3 (6.1-61)**	16 (9.6)	6 (9.1)	1.9 (0.7-5.3)	**4.7 (1.1-20.8)**

*Adjusted for age, sex, socioeconomic status, body mass index and HIV status (non-stratified models only)

Adjusted p value of the interaction between pre-diabetes / diabetes and HIV status: FCG: p= 0.66 / p=0.83 ; 2-hCG: p=0.81 / p=0.73; HbA1c: p=0.16 / p=0.048

Adjusted p value of the interaction between HbA1c based pre-diabetes / diabetes and anemia: in All: p=0.93 / p=0.12; in HIV-negative; p=0.54 / p=0.63; in HIV-infected: p=0.94 / p=0.17

Abbreviations and definitions: TB: tuberculosis; HC: healthy controls; OR: odds ratio / 95% CI: 95% confidence interval

FCG: fasting capillary glucose: Normal: <6.1 mmol/L / Pre-diabetes: 6.1-6.9 mmol/L / Diabetes: ≥7 mmol/l | **2-hCG**: 2-hour capillary glucose: Normal: <7.8 mmol/L / Pre-diabetes: 7.8-11 mmol/L / Diabetes: ≥11.1 mmol/L | **HbA1c**: glycated haemoglobin: Normal:<5.7% / Pre-diabetes: 5.8-6.4% / Diabetes: ≥6.5%

Statistical significance: in bold if p<0.05

Study sample at follow-up

Of the 530 TB patients, 392 (73%) presented for a follow-up visit; 15 TB patients were excluded from the analysis because of missing FCG, 2-hCG or HbA1c at follow-up (Figure 4-1). Characteristics and glycemic status of TB patients with and without follow-up were comparable, except for underweight and extrapulmonary TB being more common among subjects without follow-up (Table 4-3).

Table 4-3. Comparison of TB patients with and without follow-up study visit.

	TB patients N=530	TB with follow-up N=392 (74%)	TB without follow-up N=138 (26%)	
	N(%) or Mean(sd)			p
Age	35.9 (11.6)	36 (11)	35 (13)	0.27
Male sex	341 (64)	251 (64)	90 (65)	0.80
History of smoking	149 (28)	104 (27)	45 (33)	0.15
Alcohol misuse	62 (11.7)	42 (10.7)	20 (14.5)	0.24
Socioeconomic status				
Low	170 (32)	116 (30)	54 (40)	0.04
Medium	253 (48)	195 (50)	58 (43)	0.14
High	103 (20)	79 (20)	24 (18)	0.53
Body Mass Index				
Under-weight	223 (42)	153 (39)	70 (52)	0.02
Normal weight	257 (49)	202 (52)	55 (40)	0.02
Over-weight	46 (9)	35 (9)	11 (8)	0.86
High blood pressure	44 (8)	31 (8)	13 (10)	0.56
HIV infection	166 (32)	125 (32)	41 (31)	0.72
Anemia	425 (81)	315 (81)	110 (83)	0.66
Previously known DM	10 (2)	8 (2)	2 (2)	0.78
FCG				
Normal	459 (86.6)	342 (87.2)	117 (84.8)	0.47
Pre-diabetes	47 (8.9)	33 (8.4)	14 (10.1)	0.60
Diabetes	24 (4.5)	17 (4.3)	7 (5.1)	0.81
2-hCG				
Normal	365 (68.9)	272 (69.4)	93 (67.4)	0.83
Pre-diabetes	129 (24.3)	96 (24.5)	33 (23.9)	1
Diabetes	36 (6.8)	24 (6.1)	12 (8.7)	0.33
HbA1c				
Normal	224 (42.3)	168 (42.9)	56 (40.6)	0.69
Pre-diabetes	257 (48.5)	189 (48.2)	68 (49.3)	0.84
Diabetes	49 (9.3)	35 (8.9)	14 (10.1)	0.73
TB symptoms >3M	97 (18.4)	76 (19.5)	21 (15.4)	0.30
TB				
Smear positive	385 (72.6)	286 (73)	99 (71.7)	0.82
Smear negative	111 (20.9)	87 (22.2)	24 (17.4)	0.27
Extrapulmonary	34 (6.4)	19 (4.9)	15 (10.9)	0.02

Abbreviations and definitions: **FCG**: fasting capillary glucose: Normal: <6.1 mmol/L / Pre-diabetes: 6.1-6.9 mmol/L / Diabetes: ≥7 mmol/l | **2-hCG**: 2-hour capillary glucose: Normal: <7.8 mmol/L / Pre-diabetes: 7.8-11 mmol/L / Diabetes: ≥11.1 mmol/L | **HbA1c**: glycated haemoglobin: Normal:<5.7% / Pre-diabetes: 5.8-6.4% / Diabetes: ≥6.5%

Longitudinal evolution of glycemic status and its association with TB

The value of all three screening tests decreased over time (mean (SD) fall in FCG, 2-hCG and HbA1c: 0.3mmol/l (1.1), 1.0mmol/l (2.1) and 0.3% (0.5)).

After exclusion of previously-known DM, most TB patients with glucose levels consistent with DM at enrolment were not diabetic at follow-up (75%, 64% and 71%, using FCG, 2-hCG and HbA1c, respectively). Irrespective of HIV status, most TB patients diagnosed with pre-DM at enrolment had a normal glycemic status at follow-up (100%, 90% and 59% using FCG, 2-hCG and HbA1c, respectively) (Figure 4-2). There was a tendency for HbA1c based DM and pre-DM to more likely persist as hyperglycemia at follow-up. More than 99% of TB patients without DM at enrolment using any of the three screening methods did not have DM at follow-up.

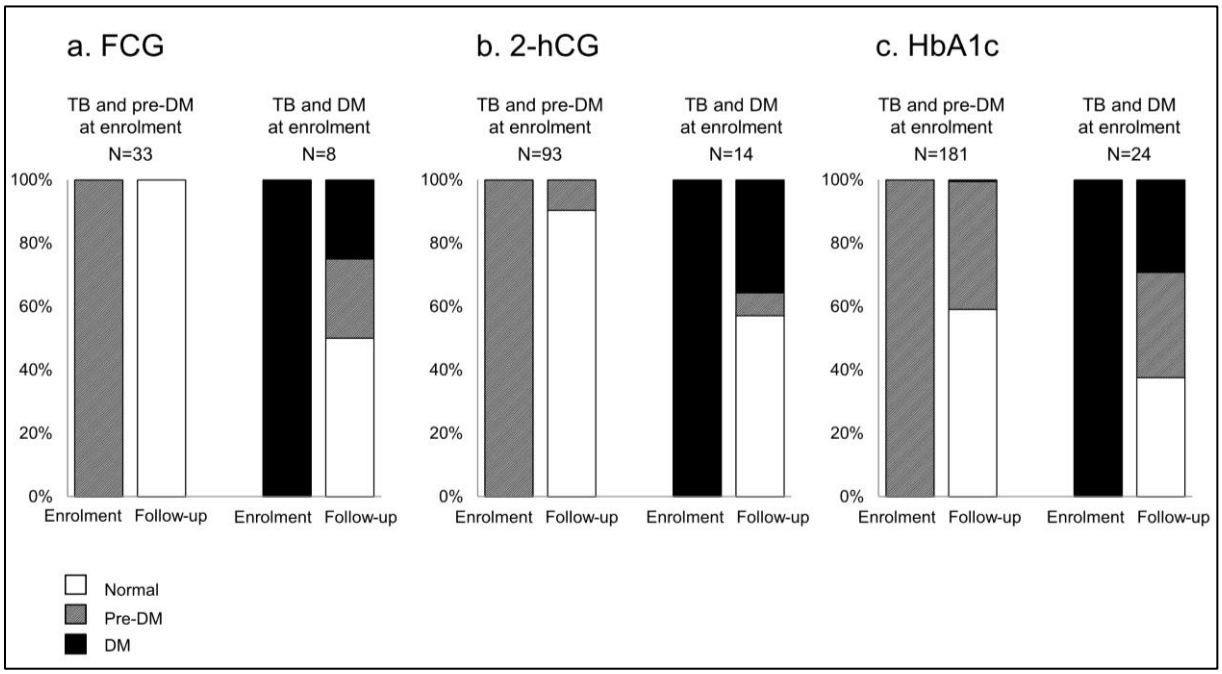

Figure 4-2. Longitudinal evolution of pre-diabetes (left 2 bars) and diabetes (right 2 bars) among tuberculosis patients from enrolment (left bar) to follow-up (right bar) using a.) Fasting capillary glucose (FCG), b.) 2-hours capillary glucose (2-hCG), c.) Glycated hemoglobin (HbA1c). Each screening test is compared to itself between enrolment and follow-up. Diabetics known and treated for the disease at baseline are excluded.

Prevalence of newly diagnosed DM at follow-up was similar in TB patients (0.8%, 1.4% and 2.2%) and controls (0.8%, 2.7% and 1.8%) for FCG, 2-hCG and HbA1c (p>0.1). Based on epidemiological data, these patients present probably type 2 DM (Hall et al., 2011, Swai et al., 1993). Irrespective of HIV status and of previous DM status, the adjusted ORs of the association between TB and hyperglycemia at enrolment consistently reverted to the null value (OR 1) during follow-up for all three DM

screening tests (Figure 4-3). The changes in TB-hyperglycemia association between enrolment and FUP were significant for all DM tests.

None of DM patients newly diagnosed at baseline was treated for DM. Hypoglycemic drugs were proposed only to two patients with FCG >10mmol/l, which is usual practice in Tanzania. One was lost to follow-up and the other did not start hypoglycemic treatment and still had high glycemia at follow-up.

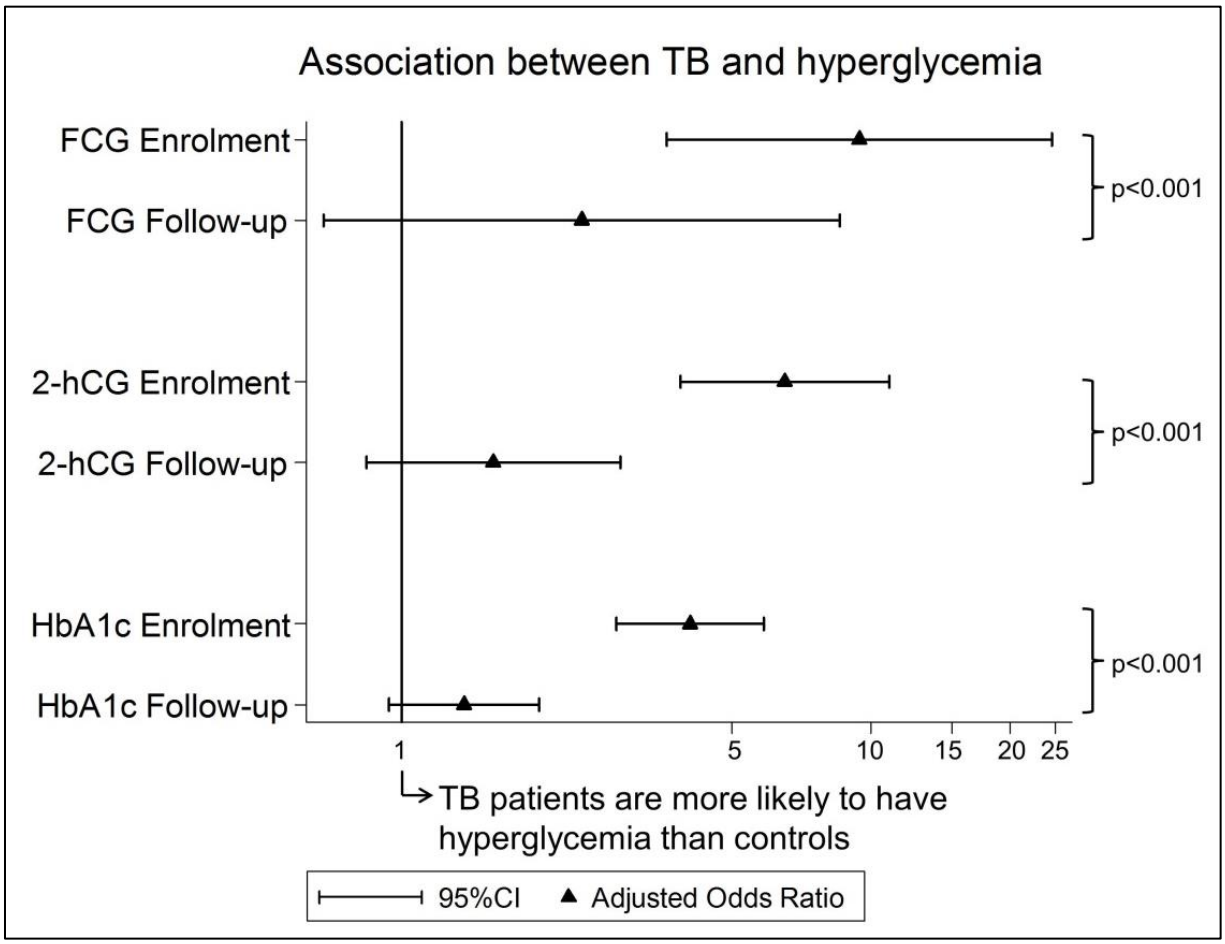

Figure 4-3. Longitudinal evolution of the association between TB and hyperglycemia, adjusted for age, sex, body mass index and socioeconomic status. Diabetics known and treated for the disease at baseline are excluded. Abbreviations: FCG: fasting capillary glucose; 2-hCG: 2-hours capillary glucose; HbA1c: glycated hemoglobin.

Hyperglycemia at enrolment and TB treatment outcome

Information on TB treatment outcome was available for patients with and without follow-up through NTLP (Figure 4-4). FCG and 2-hCG values in the range of DM or pre-DM at enrolment were significantly associated with adverse TB outcome (lost to follow-up, treatment failure or death) (Table 4-4). Hyperglycemia detected by FCG at enrolment was the only test significantly associated with an increased risk of failure or death (adjusted OR (95% CI): 3.32 (1.20-9.14)).

Table 4-4. Association of hyperglycemia with adverse tuberculosis outcome (lost to follow-up, treatment failure or death) and with failure or death

	All patients with tuberculosis N=530			
	Adverse outcome of tuberculosis		Failure or death	
	OR (95% CI) Unadjusted	OR (95% CI) Adjusted *	OR (95% CI) Unadjusted	OR (95% CI) Adjusted *
FCG Hyperglycemia	**2.29 (1.10-4.76)**	**2.46 (1.08-5.57)**	**3.33 (1.38-8.06)**	**3.32 (1.20-9.14)**
2-hCG Hyperglycemia	**2.29 (1.24-4.24)**	**2.26 (1.12-4.54)**	1.88 (0.83-4.24)	2.08 (0.84-5.18)
HbA1c Hyperglycemia	**0.50 (0.27-0.94)**	0.57 (0.28-1.14)	0.46 (0.20-1.04)	0.58 (0.23-1.45)

* Adjusted for age, sex, socioeconomic status, body mass index, HIV, duration of TB symptoms before diagnosis, cavity.

Abbreviations and definitions: Adverse outcome of tuberculosis: lost to follow-up, failure or death

FCG Hyperglycemia: fasting capillary glucose ≥6.1 mmol/L | 2-hCG Hyperglycemia: 2-hour capillary glucose ≥7.8 mmol/L | HbA1c Hyperglycemia: glycated haemoglobin ≥5.8-6.4% (40-46 mmol/mol)

Statistical significance: in bold if $p<0.05$

Diagnostic discordance of DM screening tests at enrolment

Using any of the three screening tests, 68 TB patients were diagnosed as DM at enrolment and 19 at follow-up. DM screening tests gave concordant results among 22% of TB patients at enrolment and 53% at follow-up.

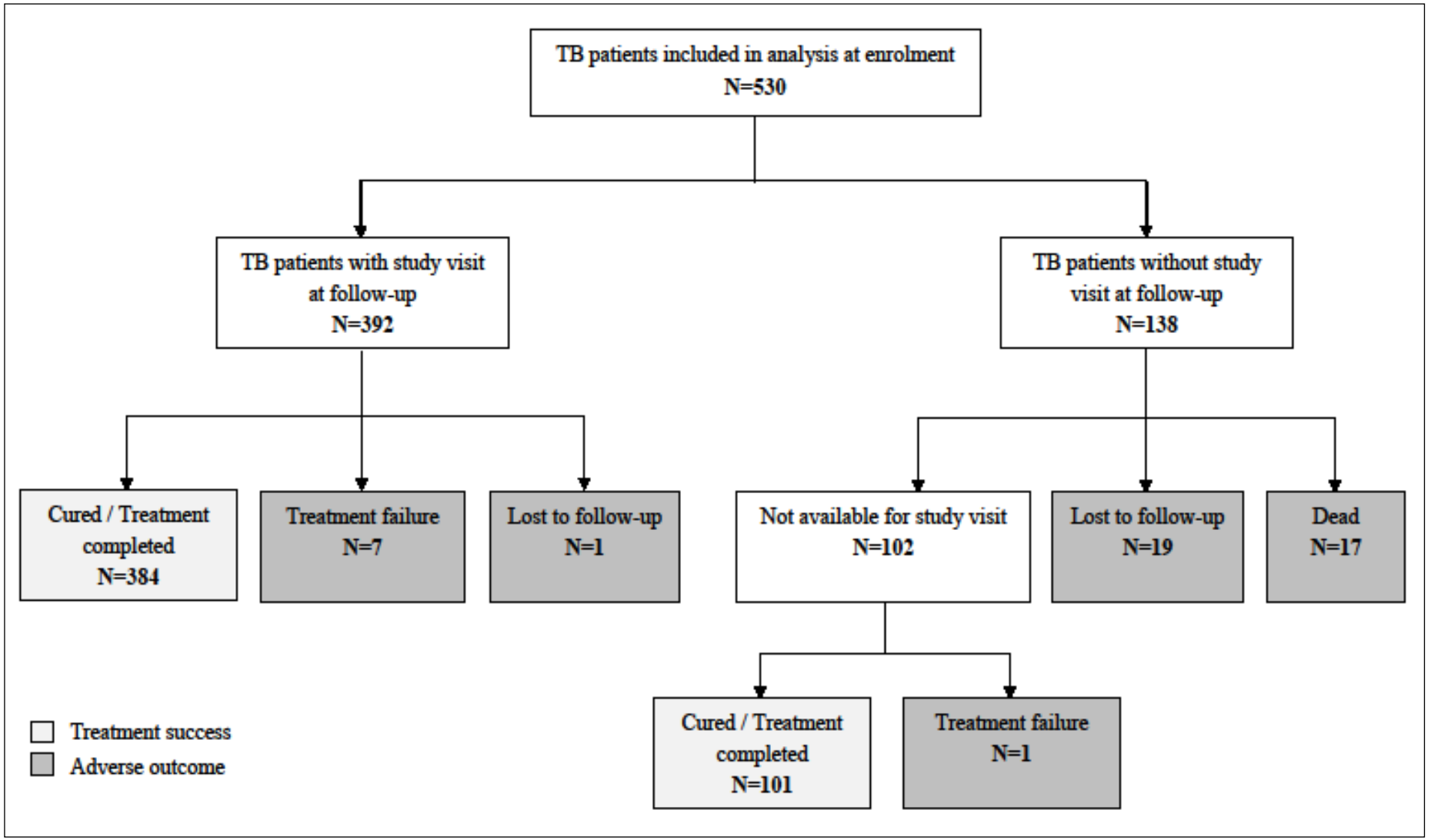

Figure 4-4. Flow chart of patients with tuberculosis and their outcome.

4.5 Discussion

In this sub-Saharan Africa urban setting, both DM and pre-DM at enrolment were more prevalent in TB patients than in controls irrespective of HIV status. But, in this environment with low DM prevalence, new hyperglycemia at the level of DM or pre-DM seem to be the consequence rather than the cause of TB and DM diagnosis must be confirmed after TB treatment. Testing for the presence of transient hyperglycemia with FCG detects TB patients at higher risk of adverse TB outcome and gives the opportunity to manage hyperglycemia, but the impact of such intervention on TB outcome needs to be confirmed.

TB and hyperglycemia at enrolment

The positive cross-sectional associations between TB and hyperglycemia, irrespective of the type of DM test, are in agreement with the literature (Jeon and Murray, 2008). Previous data from Africa suggests that the associations may be weaker in HIV-infected persons (Faurholt-Jepsen et al., 2011, Kibirige et al., 2013). In this study, this was only true for an HbA1c-based DM which is difficult to interpret due to a high prevalence of anemia particularly among HIV-infected patients. The complex interplay between HIV-related immunosuppression, the immunosuppressive effect of pre-existing DM, and the hyperglycemia subsequent to TB infection cannot be disentangled cross-sectionally.

TB and transient hyperglycemia

Among TB patients with glucose levels consistent with DM at enrolment, 58-80% (depending on the screening test) had newly-diagnosed DM (which is similar to the study conducted in Mwanza, 77%), but different from studies in India or China where >50% are known for DM before the onset of clinical TB (Viswanathan et al., 2012, Wang et al., 2013). Most patients with newly-diagnosed DM had a moderately increased glycemia.

Hyperglycemia was transient in the large majority of TB patients with newly diagnosed pre-DM and DM at the time of TB diagnosis. This transient hyperglycemia is well known in patients with sepsis (Dungan et al., 2009). The normalization of glycemic status during TB treatment has already been described in small study samples (N=20-50)

using oral glucose tolerance tests (Oluboyo and Erasmus, 1990, Singh et al., 1984). A larger study conducted in Iran also reported that one third of patients with high HbA1c at TB diagnosis returned to a normal concentration after three months of TB treatment (Tabarsi et al., 2014). The cause of this transient hyperglycemic status is likely multifactorial and might reflect inflammation induced by TB, the hyperglycemic effect of TB treatment and patient predisposition. Stress hyperglycemia results from a complex interplay between disturbed cytokine and hormone production leading to excessive hepatic glucose production and insulin resistance (Dungan et al., 2009). This high frequency of transient hyperglycemia in untreated TB patients raises the question of reverse causality between TB and DM and highlights the necessity to repeat DM screening later in the course of TB treatment. Furthermore, studies are needed to assess if TB patients with transient hyperglycemia are at increased risk to develop DM later (Chen et al., 2012).

Hyperglycemia testing for improved TB treatment

Hyperglycemia testing and control at TB diagnosis may have clinical utility in improving TB treatment outcome. A recent review showed that DM at TB diagnosis increases risk of failure and death during TB treatment and a large study conducted in Mwanza, Tanzania showed an increased risk of death among patients with TB and DM, particularly among HIV-negative patients (Baker et al., 2011, Faurholt-Jepsen et al., 2012). In our study, patients with fasting hyperglycemia at TB diagnosis had a higher risk of TB treatment failure or death while patients with elevated HbA1c were not at higher risk as previously described (Baker et al., 2011, Tabarsi et al., 2014). Short-term glycemic control with insulin has been linked to better outcome in septic patients, but only when using non intensive glycemic targets (Dungan et al., 2009). This evidence indirectly suggests that short-term management of glycemia might also improve TB outcome. A recent study pointed to metformin as a potentially useful adjunct antituberculosis therapy. In mice, it enhances *Mycobacterium tuberculosis*-specific host immunity and reduces inflammation. In TB-DM patients metformin, but not other DM drugs, improved TB outcome (Singhal et al., 2014). The effect of controlling transient hyperglycemia, particularly with metformin, during TB treatment was not yet studied in a randomized trial (Riza et al., 2014).

DM screening algorithm in TB patients

The concordance between the three DM tests used in this study is imperfect as previously described (2015, Kumpatla et al., 2013). A number of factors may be involved. The diagnostic cut-offs of the DM tests may need to be adapted in different populations as they were defined in non-African people. HbA1c appears to overestimate DM prevalence in iron-deficient populations (Hardikar et al., 2012). The fact that HbA1c, despite its longer half-life, does not capture long term DM better than other tests among TB patients, might be related to the long duration of the disease before TB diagnosis and to prevalent anemia in these patients (Hardikar et al., 2012, Roy, 2010). According to our study, FCG testing at the time of TB diagnosis is preferable to HbA1c testing, which, in contrast to FCG, failed to detect patients at risk of adverse TB outcome. Finally, glucose based tests are less expensive than HbA1c, which is a main consideration in low and middle income countries.

Our study has several strengths. We evaluated three recommended DM tests side-by-side and the HbA1c test was non sensitive to prevalent hemoglobinopathies (Mwakasungula et al., 2014). The study population was well defined and the patients categorized according to WHO recommendations. Its limitation was that TB diagnosis was based on NTLP guidelines and smear results rather than culture or GenXpert. However, the study was conducted under the real case scenario of TB treatment in this population. We did not screen for the presence of multidrug resistant *M. tuberculosis*, but their prevalence is still low (incidence of 1.1% in 2014) in Tanzania and have probably not affected the association between TB and DM (WHO, 2015). Fasting and 2-h glucose were assessed on capillary whole blood using a point-of-care test and not in venous blood. However, we used a plasma-calibrated glucometer which accuracy conformed to the International Standardization Organization guidelines (Essack et al., 2009). Our study was under-powered to investigate the DM course according to HIV status. TB patients and controls were not matched by SES and TB patients had a lower SES. However, SES variable was included in every multivariate analysis. Thirty percent of TB patients had no glycemic follow-up, but they are unlikely to have biased the observed results to a relevant degree as sensitivity analyses using inverse probability weighting and multiple imputation gave similar results than the one presented in the paper.

In conclusion, our study showed that mild hyperglycemia is very common in TB patients, but, in most of them, the glucose status is normalized with TB treatment. For the setting of sub-Saharan Africa, the clinical implications of this first study describing the longitudinal evolution of the three recommended DM tests during TB treatment are twofold. First, a new diagnosis of DM in TB patients must be confirmed after TB treatment. Larger studies using point-of-care DM tests that are insensitive to infection-induced hyperglycemia need to assess the role of DM as a risk factor for active TB in low DM prevalence-high TB burden settings like Sub-Saharan Africa. Second, FCG testing at enrolment best captures TB patients at risk of treatment failure and death. This may give the opportunity to manage hyperglycemia, but the effect of such interventions on TB outcome requires confirmation through randomized controlled trials including both HIV-infected and HIV-negative patients.

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Conflicts of interest

No conflict of interest to declare for all authors.

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5 Association between Tuberculosis, Diabetes Mellitus and Vitamin D in Tanzania: a Longitudinal Case Control Study

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5.1 Summary

Background

Vitamin D concentration is inversely associated with TB and DM. So, vitamin D could mediate the known increased risk of TB associated with DM. We aim to describe the associations between vitamin D, TB and DM.

Methods

Consecutive adults with TB and sex- and age-matched volunteers were included in a case-control study between February-December 2013 in Dar es Salaam, Tanzania. Glycemia and 25-hydroxyvitamin D (25(OH)D) were measured at enrolment and after TB treatment in cases. Association between 25(OH)D and TB was evaluated by linear and logistic regression adjusted for age, sex, body mass index, socioeconomic status, sunshine exposure and HIV.

Findings

Prevalence of 25(OH)D insufficiency (<75nmol/l) was not different between TB patients and controls (25.9 versus 31.0%; p=0.26). But the association between 25(OH)D insufficiency and TB was modified by hyperglycemia ($p_{interaction}$=0.01). 25(OH)D insufficiency was associated with TB risk in the presence of underlying hyperglycemia (OR(95%CI): 4.94(1.16-21.0)), but not in the case of normoglycemia (OR(95%CI): 0.68(0.39-1.17)) when compared to participants without hyperglycemia and 25(OH)D insufficiency.

Interpretation

25(OH)D insufficiency seams to increase TB risk only if associated with hyperglycemia. Diabetic patients living in high TB burden settings might benefit from preventive vitamin D supplementation.

5.2 Background

Diabetes mellitus (DM) triples the risk of tuberculosis (TB) and the global increase of DM in developing countries is expected to have a strong impact on TB incidence (Stevenson et al., 2007b, Wild et al., 2004, Jeon and Murray, 2008). Several studies have shown an association of low vitamin D with both, TB as well as DM (Martineau, 2012, Pilz et al., 2013, Xuan et al., 2013). Vitamin D is generated in the skin after sun exposure and, to a lower extent, trough the diet (Rosen, 2011). Vitamin D is usually measured as total blood level of 25 hydroxyvitamin D (25(OH)D) which is the major circulating form of vitamin D, for a large part protein-bound, but is less than one percent as potent as the free vitamin D, 1,25 dihydroxyvitamin D ($1,25(OH)_2D$), the most active form of vitamin D. Unfortunately, measurement of the free biological active vitamin D is not yet easily available and epidemiological studies must rely, for the time being, on total levels of 25(OH)D which can be affected by the level of binding proteins (Powe et al., 2013).

Vitamin D deficiency was first described as the cause of rickets and osteomalacia. However, the vitamin D receptor is expressed in most cells in the body and vitamin D seems to have an effect on a number of other health outcomes (Rosen, 2011). The association between type 2 DM and poor vitamin D status has been described in several cross-sectional and prospective studies and a metaanalysis of 21 prospective studies showed an inverse association between level of vitamin D and risk of type 2 DM (Song et al., 2013). Vitamin D affects pancreatic β-cell function, boosts the antimicrobial activity of human macrophages against *Mycobacterium tuberculosis*, and modulates adaptive response (Hawn et al., 2015). Building on these findings, it was suggested that vitamin D insufficiency could mediate some of the association between DM and TB (Handel and Ramagopalan, 2010). A cross-sectional Indian study confirmed the association between vitamin D deficiency, TB and DM while another study conducted in China did not show any association (Chaudhary et al., 2013, Zhan and Jiang, 2015).

It has been shown that vitamin D supplementation accelerates the resolution of inflammatory responses during TB treatment and may be used as a host-directed therapy (Coussens et al., 2012). However, the role of adjunctive vitamin D on TB

outcome remains uncertain as randomized clinical trials of vitamin D supplementation did not show a beneficial effect on TB outcome or mortality (Kota et al., 2011, Martineau et al., 2011b, Nursyam et al., 2006, Wejse et al., 2009, Daley et al., 2015).

Vitamin D supplementation might have a role in the prevention and treatment of TB in targeted subgroups of patients with low vitamin D levels, such as diabetic patients. In this study, we aimed to describe the association between vitamin D insufficiency and TB according to DM status in an African equatorial population.

5.3 Methods

Study design and setting

This case-control study with longitudinal follow-up of cases was part of a study on the association between TB and DM (Boillat-Blanco et al.). TB patients were recruited between February and December 2013 and followed up for a median time of 5.8 months (IQR 4.7-9.9) after the start of anti-TB treatment. Controls were recruited between March and September 2013 and did not have follow-up visits. Glucose and vitamin D were measured in blood collected at baseline from cases (88% before onset of TB treatment) and controls and in blood collected at follow-up from cases (54% still under TB treatment). The study was carried out in Kinondoni, the most populated District of Dar es Salaam. Patients were recruited in Mwananyamala Regional Hospital and connected health facilities (Sinza Hospital, Magomeni Health Centre, Tandale Dispensary). Dar es Salaam is located at a latitude of -6.81° and longitude of 39.28° which ensures a good ability of sunlight to synthesize vitamin D (Rosen, 2011).

Study participants

At enrolment, 280 TB patients and 358 controls had blood sampled for vitamin D and glucose measurement. Of the 280 TB patients, 205 (73%) presented for a follow-up visit after ≥5 months of TB treatment. The final study sample consisted of 167 TB cases with follow-up information and 358 controls, all of whom had complete information on fasting glucose, 25(OH)D and relevant covariates (Figure 5-1).

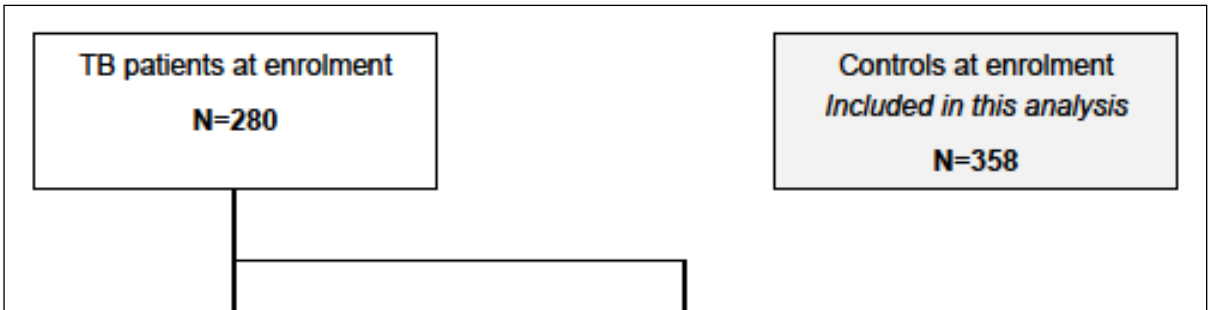

Figure 5-1. Flow chart of study participants.

Cases

Consecutive adult patients (age ≥ 18 years; living in Kinondoni District) presenting in the participating hospitals with new active TB diagnosed by the National TB and Leprosy Control Programme (NTLP) were screened for study inclusion.

TB diagnosis was based on sputum smear microscopy, chest X-ray (read by an experienced radiologist), clinical evidence of TB and decision by the clinician to treat with a full-course of anti-TB therapy (2013). TB patients were treated for 6 months with a standard regimen or longer if necessary (2013, WHO, 2010). Sputum microscopy was repeated after two-three months and again after five months of TB treatment by NTLP to evaluate the response to treatment in pulmonary smear-positive TB patients (2013, WHO, 2010). TB treatment outcome was defined according to WHO guidelines as treatment success (cure, treatment completed) or adverse outcome (failure, death, lost to follow-up) (WHO, 2010).

Controls

We used frequency matching on sex and age (10-year age groups) to select controls among adults accompanying patients, other than the one included in the study, to the outpatient departments and living in Kinondoni District. Exclusion criteria were a biological relationship to case, TB history, symptoms or signs of TB, other acute infection or major trauma within the last three months.

Study procedures

Demographic characteristics, health history (DM and HIV) and symptoms, socioeconomic status (SES; indicators of education, occupation, wealth using factor analysis), smoking (ever daily smoking), and alcohol misuse (≥three drinks per day or ≥six drinks per occasion) were obtained. Daily sunshine hours data were provided by the Tanzanian meteorological agency and month at enrolment was classified as high sunshine exposure if the monthly mean number of daily sunshine hours was ≥8 hours. Data were entered directly into an open data kit in a personal digital assistant with real-time error, range and consistency checks (Hartung et al., December 2010). Participants were screened for HIV infection according to the national algorithm (rapid immune-chromatographic test (Alere Determine™ HIV-1/2) confirmed by a second rapid test (Trinity Biotech Uni-gold™ Recombigen® HIV-1/2)).

Total 25 hydroxyvitamin D measurement

Serum was kept at -80°C within 6 hours of blood withdrawal and total 25(OH)D level was assessed by electrochemoluminescence immunoassay (Cobas® 8000, Roche Diagnostics) in Switzerland. Vitamin D deficiency was defined as a 25(OH)D <50nmol/l and insufficiency as vitamin D <75nmol/l as suggested by the Endocrine Society clinical practice guidelines (Holick et al., 2011, Rosen, 2011).

Hyperglycemia screening

Blood glucose testing was conducted after an overnight fast of ≥8 hours (fasting capillary glucose, FCG) and 2-hour glucose in an oral glucose tolerance test (2-hCG; standard 75-gram OGTT; GlucoPlus™, Diabcare, plasma-calibrated glucometer which accuracy conformed to the International Standardization Organization guidelines) (Essack et al., 2009). Abnormal glycemic result (FCG ≥5.6 mmol/l or 2-hCG ≥7.8 mmol/l) were confirmed by repeat testing two to five days later. 2-hCG testing was

omitted if FCG was ≥11.1 mmol/l. Hyperglycemia refers to patients with repeated FCG≥6.1 mmol/l and/or 2-hCG≥7.8 mmol/l according to WHO (WHO, 2006). Participants diagnosed with DM (FCG>7 mmol/l or 2-hCG>11 mmol/l) were referred to the local DM clinic, but none was started on DM treatment.

Data analysis

Characteristics of TB patients were compared to controls. Differences were tested with Wilcoxon-Mann-Whitney and chi-square tests. To identify factors associated with 25(OH)D insufficiency, study participants with 25(OH)D insufficiency were compared to those without insufficiency using Wilcoxon-Mann-Whitney and chi-square tests.

The independent association of 25(OH)D level and insufficiency at enrolment and follow-up with TB status was evaluated by linear and logistic regression, respectively. Covariates included in the final models were age, sex, body mass index (BMI), SES, sunshine exposure at enrolment and HIV status. To assess the combined effect of 25(OH)D insufficiency and hyperglycemia on TB risk, a 4-level categorical variable reflecting the combination of glycemic and 25(OH)D status was used as predictor variable in the logistic regression models. Interaction between 25(OH)D and hyperglycemia in linear regression models was assessed by adding each predictor as well as their interaction term to the model. Hyperglycemia status was defined based on glucose measured at follow-up as a proxy for pre-existing DM because hyperglycemia at enrolment is more likely to reflect stress hyperglycemia as a result of TB (Boillat-Blanco et al.).

Statistical analyses were performed using Stata software (StataCorp, College Station, TX, USA, version 12).

Ethical considerations

All participants gave written informed consent prior to interview and health examination. The Ifakara Health Institute Institutional Review Board and the Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania, gave ethical clearance.

Role of funding source

No involvement.

5.4 Results

Study sample

Characteristics of the study sample are described in Table 5-1. Compared to controls, TB patients were more often HIV-infected, had a lower BMI, higher 25(OH)D level and therefore, less often 25(OH)D insufficiency, particularly at follow-up. At enrolment, hyperglycemia was more common in TB patients than controls, but there was no longer a difference at follow-up after resolution of TB, probably because of stress hyperglycemia secondary to TB infection (Boillat-Blanco et al.). The level of 25(OH)D increased during TB treatment (mean increase 6.5 nmol/L (SD$\pm$21)) independently of whether TB treatment was still ongoing at the time of measurement. Correspondingly, the prevalence of vitamin D insufficiency decreased (24.6 versus 20.4%, $p<0.001$).

Glycemic and vitamin D status of TB patients with and without follow-up data were comparable, but males, patients with alcohol misuse, smoking and lower weight were more common among subjects without follow-up (data not shown).

Table 5-1. Characteristics of the case-control sample

	TB patients	Healthy Controls

	N=167	N=358	
	N(%) or Mean(sd)		p
Age	33.7 (10.7)	36.1 (13.0)	0.05
Male sex	95 (56.9)	191 (53.4)	0.45
History of smoking	25 (15.0)	90 (25.3)	0.008
Alcohol misuse	16 (9.6)	22 (6.2)	0.16
Socioeconomic status			
Low	41 (24.6)	71 (19.9)	0.25
Medium	82 (49.1)	185 (52.0)	0.64
High	44 (26.4)	100 (28.1)	0.75
High sunshine exposure	105 (62.9)	214 (59.8)	0.50
Body Mass Index (kg/m^2)	22.5 (4.2)	25.1 (5.1)	<0.001
HIV infection	51 (30.7)	51 (14.3)	<0.001
Previously known DM	5 (3.0)	2 (0.6)	0.02
VALUES AT ENROLMENT			
Hyperglycemia	38 (22.8)	37 (10.3)	<0.001
Vitamin D deficiency	3 (1.8)	21 (5.9)	0.04
Vitamin D insufficiency	43 (25.8)	111 (31.0)	0.22
Vitamin D level (nmol/l)	94.0 (26.9)	89.6 (26.9)	0.08
VALUES AT FOLLOW-UP			
Hyperglycemia	19 (11.4)	37 (10.3)	0.72
Vitamin D deficiency	7 (4.2)	21 (5.9)	0.43
Vitamin D insufficiency	34 (20.4)	111 (31.0)	0.01
Vitamin D level (nmol/l)	100.5 (29.8)	89.6 (26.9)	<0.001
TB CHARACTERISTICS			
TB symptoms >3M	21 (12.6)		
TB			
Smear positive	136 (81.4)		
Smear negative	27 (16.2)		
Extrapulmonary	4 (2.4)		
Cavity on X-ray	86 (52.8)		

Vitamin D and glycemia have been measured at follow-up only in TB patients.
Abbreviations and definitions: Alcohol misuse: ≥3 drinks per day or ≥6 drinks per occasion / Socioeconomic status: assessed with indicators of scholar education, occupation and wealth ownership using factor analysis / High sunshine exposure: monthly mean number of daily sunshine hours ≥8 hours
Hyperglycemia: fasting capillary glucose: ≥6.1 mmol/l and/or 2-hCG hyperglycemia:≥7.8 mmol/l
TB symptoms >3M: >3 month duration of tuberculosis symptoms before diagnosis

Correlates of vitamin D level and vitamin D insufficiency

In the control group vitamin D insufficiency was more common in women, but less common in HIV infected persons. The only other variable associated with vitamin D insufficiency was underlying DM (assessed as either a previous DM diagnosis or as hyperglycemia measured at follow-up), but only in TB patients and not in controls (Boillat-Blanco et al.). Vitamin D insufficiency was more frequent among TB patients previously known for DM (9.3% versus 0.8%; p=0.005) and those with hyperglycemia at follow-up (18.6% versus 8.9%; p=0.08) (Table 5-2). Indeed, 25(OH)D was lower among TB patients previously known for DM (mean 76.1 nmol/l (SD±29.3) versus 94.5

(±26.7); p=0.13) and those with hyperglycemia at follow-up (mean 81.5 nmol/l (±21.1) versus 95.6 (±27.2); p=0.03) (Table 5-3).

Table 5-2. Factors associated with vitamin D insufficiency among healthy controls and tuberculosis patients at enrolment.

	TB patients (N=167)			Healthy Controls (N=358)		
	Insufficient vitamin D N=43 (25.7%)	No insufficient vitamin D N=124 (74.3)		Insufficient vitamin D N=111 (31.0%)	No insufficient vitamin D N=247 (69.0%)	
	N(%) or Mean(sd)	N(%) or Mean(sd)	p	N(%) or Mean(sd)	N(%) or Mean(sd)	p
Age	35.1 (12.0)	33.3 (10.2)	0.53	35.5 (12.4)	36.4 (13.3)	0.79
Male sex	20 (46.5)	75 (60.5)	0.11	46 (41.4)	144 (58.8)	0.002
History of smoking	4 (9.3)	21 (16.9)	0.23	25 (22.5)	65 (26.8)	0.40
Alcohol misuse	0 (0)	7 (5.7)	0.11	3 (2.7)	19 (7.7)	0.07
Socioeconomic status						
Low	11 (25.6)	30 (24.2)	0.84	17 (15.3)	54 (22.2)	0.2
Medium	22 (51.2)	60 (48.4)	0.86	57 (51.4)	127 (52.3)	1
High	10 (23.3)	34 (27.4)	0.84	37 (33.3)	62 (25.5)	0.13
High sunshine exposure	29 (67.4)	76 (61.3)	0.47	64 (57.7)	150 (60.7)	0.58
Body mass index (kg/m^2)	19.8 (3.1)	20.4 (3.9)	0.54	25.8 (5.5)	24.9 (4.8)	0.11
HIV infection	12 (27.9)	39 (31.7)	0.64	6 (5.4)	45 (18.4)	0.001
Previously known for DM	4 (9.3)	1 (0.8)	0.005	0 (0)	2 (0.8)	0.34
Enrol. Hyperglycemia	9 (20.9)	29 (23.4)	0.74	11 (9.9)	26 (10.5)	0.86
Follow-up Hyperglycemia	8 (18.6)	11 (8.9)	0.08			
TB symptoms >3M	8 (18.6)	13 (10.5)	0.17			
TB						
Smear positive	36 (83.7)	100 (80.7)	0.82			
Smear negative	6 (14.0)	21 (16.9)	1			
Extrapulmonary	1 (2.3)	3 (2.4)	1			
Cavity on X-ray	20 (48.8)	66 (54.1)	0.56			

Abbreviations and definitions: Alcohol misuse: ≥3 drinks per day or ≥6 drinks per occasion / Socioeconomic status: assessed with indicators of scholar education, occupation and wealth ownership using factor analysis / High sunshine exposure: monthly mean number of daily sunshine hours ≥8 hours / DM: diabetes
Hyperglycemia: fasting capillary glucose: ≥6.1 mmol/l and/or 2-hCG hyperglycemia:≥7.8 mmol/l
TB symptoms >3M: >3 month duration of tuberculosis symptoms before diagnosis

Table 5-3. Factors associated with vitamin D level among TB patients and healthy controls.

	TB patients N=167			Healthy Controls N=358		
	N(%)	25(OH) D (nmol/L)	P	N(%)	25(OH) D (nmol/L)	p
Age (years)			0.47			0.42
<45	156 (93.4)	94.4 (26.9)		321 (89.7)	90.0 (27.4)	
≥45	11 (6.6)	88.3 (27.4)		37 (10.3)	86.2 (22.6)	
Sex			0.002			<0.001
Female	72 (43.1)	86.6 (24.2)		167 (46.6)	84.3 (25.9)	
Male	95 (56.9)	99.6 (27.6)		191 (53.4)	94.3 (27.0)	
Smoking			0.16			0.41
No	145 (86.8)	92.9 (26.9)		271 (75.7)	88.9 (27.8)	
Yes	22 (13.2)	101.5 (26.1)		85 (23.7)	91.7 (24.3)	
Alcohol			0.31			0.007
No misuse	160 (95.8)	93.5 (27.0)		336 (93.9)	88.6 (26.7)	
Misuse	7 (4.2)	104.1 (24.2)		22 (6.1)	104.6 (26.9)	
Socioeconomic status			0.59			1
Low	41 (24.6)	90.3 (25.2)		71 (19.9)	89.4 (25.7)	
Medium	82 (49.1)	94.7 (28.9)		185 (52.0)	89.7 (25.7)	
High	44 (26.3)	96.0 (24.8)		100 (28.1)	89.5 (30.4)	
Sunshine exposure			0.86			0.85
<8 hours/day	62 (37.1)	94.5 (25.8)		144 (40.2)	90.5 (27.7)	
≥8 hours/day	105 (62.9)	93.7 (27.6)		214 (59.8)	89.0 (26.5)	
BMI (kg/m^2)			0.79			0.006
<18.5	52 (31.1)	95.2 (28.8)		14 (4.0)	75.4 (25.4)	
18.5-24.9	94 (56.3)	92.8 (26.7)		189 (53.4)	93.3 (28.0)	
≥25	21 (12.6)	96.4 (23.6)		151 (42.6)	85.9 (24.8)	
HIV infection			0.74			0.005
No	115 (69.3)	93.6 (26.7)		306 (85.7)	88.0 (26.7)	
Yes	51 (30.7)	95.1 (27.9)		51 (14.3)	99.5 (26.5)	
Known for DM			0.13			0.87
No	162 (97.0)	94.5 (26.7)		356 (99.4)	89.6 (27.0)	
Yes	5 (3.0)	76.1 (29.3)		2 (0.6)	86.5 (2.5)	
Enrol. Hyperglycemia			0.90			0.83
No	129 (77.2)	94.1 (28.1)		344 (96.1)	89.5 (27.1)	
Yes	38 (22.8)	93.5 (22.8)		14 (3.9)	91.1 (24.7)	
FUP Hyperglycemia			0.03			
No	148 (88.6)	95.6 (27.2)				
Yes	19 (11.4)	81.5 (21.1)				
TB symptoms duration			0.23			
<3 months	146 (87.4)	94.9 (26.6)				
>3 months	21 (12.6)	87.4 (28.9)				
TB			0.16			
Smear positive	136 (81.4)	92.2 (25.6)				
Smear negative	27 (16.2)	103.1 (32.7)				
Extrapulmonary	4 (2.4)	94.4 (20.5)				
Cavity on X-ray			0.78			
No	77 (47.2)	93.6 (87.9)				
Yes	86 (52.8)	94.8 (28.7)				

Abbreviations and definitions: Alcohol misuse: ≥3 drinks per day or ≥6 drinks per occasion / High sunshine exposure: monthly mean number of daily sunshine hours ≥8 hours / DM: diabetes / Enrol.: enrolment / FUP: follow-up / Hyperglycemia: fasting capillary glucose: ≥6.1 mmol/l and/or 2-hCG hyperglycemia:≥7.8 mmol/l

TB symptoms >3M: >3 month duration of tuberculosis symptoms before diagnosis

Association between TB and 25(OH)D level in all subjects and according to hyperglycemia at follow-up

In all subjects combined, 25(OH)D levels at baseline and follow-up were slightly higher in TB patients compared to controls (baseline: mean 94.0 nmol/l (SD 26.0) vs. 89.6 (26.9); follow-up: 100.5 (29.8) vs. 89.6 (26.9). However the association between 25(OH)D level and particularly vitamin D insufficiency with TB depended on the presence of hyperglycemia at follow-up as a proxy for DM. In the absence of DM, vitamin D levels were higher in TB patients than in controls, irrespective of the time point of vitamin D measurement. In the presence of DM, though, circulating 25(OH)D concentrations were not higher in TB patients. In fact, when assessing the joint associations of vitamin D insufficiency and hyperglycemia status with TB status, we observed the risk for TB to be highest in participants with a combination of vitamin D insufficiency and hyperglycemia. This associations was stronger for vitamin D insufficiency at baseline than at follow-up (OR 4.94 (95% CI 1.16-21.0 vs. 3.68 (0.77-17.70)). In contrast, vitamin D insufficiency at follow-up in the absence of hyperglycemia was protective against TB (OR 0.56 (0.31-0.99)) (Table 5-4).

Table 5-4. Joint association of vitamin D and hyperglycemia at follow-up with tuberculosis

	TB patients N=167	Healthy Controls N=358	OR (95% CI)	OR (95% CI)	
	N (%) or mean(SD)		Unadjusted	Adjusted ▪	p-interaction
VITAMIN D AT ENROLMENT					
Vitamin D (nmol/l)					
Normal glycemia	95.6 (27.2)	89.7 (26.7)	**1.01 (1.00-1.02)**	1.01 (1.00-1.02)	
Hyperglycemia	81.5 (21.1)	88.8 (29.3)	0.99 (0.97-1.01)	0.98 (0.94-1.04)	0.07
Vitamin D Insufficiency					
No Insufficiency and Normal glycemia	113 (67.7)	221 (61.7)	Ref.	Ref.	
No Insufficiency and Hyperglycemia	11 (6.6)	26 (7.3)	0.83 (0.39-1.74)	0.80 (0.31-2.07)	
Insufficiency and Normal glycemia	35 (21.0)	100 (27.9)	0.68 (0.44-1.07)	0.68 (0.39-1.17)	
Insufficiency and Hyperglycemia	8 (4.8)	11 (3.1)	1.42 (0.56-3.64)	**4.94 (1.16-21.0)**	**0.01**
VITAMIN D AT FOLLOW-UP					
Vitamin D (nmol/l)					
Normal glycemia	102.0 (29.9)	89.7 (26.7)	**1.02 (1.01-1.02)**	**1.01 (1.01-1.02)**	
Hyperglycemia	88.7 (27.4)	88.8 (29.3)	1.00 (0.98-1.02)	1.01 (0.96-1.05)	0.18
Vitamin D Insufficiency					
No Insufficiency and Normal glycemia	121 (72.5)	221 (61.7)	Ref.	Ref.	
No Insufficiency and Hyperglycemia	12 (7.2)	26 (7.3)	0.84 (0.41-1.73)	0.98 (0.41-2.37)	
Insufficiency and Normal glycemia	27 (16.2)	100 (27.9)	**0.49 (0.31-0.80)**	**0.56 (0.31-0.99)**	
Insufficiency and Hyperglycemia	7 (4.2)	11 (3.1)	1.16 (0.44-3.08)	3.68 (0.77-17.70)	**0.04**

▪ Adjusted for age, sex, body mass index, socioeconomic status, sunshine exposure and HIV status
p-interaction: adjusted p value of the interaction between hyperglycemia at follow-up and vitamin D
Statistical significance: in bold if p<0.05

Vitamin D status and TB treatment outcome

Information on TB treatment outcome was available for patients with and without study follow-up through NTLP. Vitamin D level at baseline was not associated with adverse TB outcomes (lost to follow-up, treatment failure or death), but power for this analyses was limited as the number of adverse TB outcomes was small (5%; data not shown).

5.5 Discussion

In this sub-Saharan Africa equatorial setting, we provide novel evidence for the association between low 25(OH)D and TB to depend on underlying hyperglycemia and DM, irrespective of HIV status. In normoglycemic participants, 25(OH)D levels were higher in TB cases compared to controls. Only in the presence of DM, we observed lower levels of 25(OH)D and higher prevalence of vitamin D insufficiency in TB cases compared to controls. Furthermore, DM was a determinant of vitamin D insufficiency among TB patients. The longitudinal blood sampling in TB cases allowed to have a better estimation of vitamin D insufficiency and hyperglycemia in TB cases after treatment and therefore, after the time point of acute inflammatory reactions related to active TB.

The associations between vitamin D insufficiency and DM, vitamin D insufficiency and TB as well as TB and DM have been demonstrated in many studies. These data point to vitamin D as a potential mediator in the link between DM and TB which is in line with our results (Handel and Ramagopalan, 2010). Our data are concordant with an Indian study showing that the prevalence of vitamin D insufficiency is higher among TB patients with DM compared to those without DM (Chaudhary et al., 2013). The absence of link between DM and 25(OH)D level among healthy controls may be explained by the low number of controls with previously known DM. Our results from a longitudinal study underline the limitations of studying the combined impact of circulating glucose and 25(OH)D on TB in a cross-sectional manner. At the time of diagnosis of active TB, the inflammation status influences both hyperglycemia and likely also 25(OH)D. Cross-sectional studies may miss the importance of DM in the association between TB and vitamin D when only considering the results of baseline glycemic screening at the time of active TB that most likely represent stress hyperglycemia (Boillat-Blanco et al.). Consistent with two other studies, 25(OH)D level increased during TB treatment,

despite the described lowering effect of isoniazid and rifampicin (Brodie et al., 1982, Friis et al., 2013, Koo et al., 2012, Sloan et al., 2015). The improved vitamin D status during TB treatment may be related to increased exercise, sun exposure and food consumption during recovery. However, 25(OH)D results have to be interpreted carefully particularly during active TB. Indeed, as previously discussed, 25(OH)D is mainly protein-bound and does not represent the free active form of vitamin D. During inflammation, the concentration of VDBP which is an acute-phase reactant is probably affected and therefore, the level of active or "free" serum $1,25(OH)_2D$ is modified. So, the measured total 25(OH)D might not be a suitable marker of vitamin D status during infection. Studies showed inconsistent results regarding changes in VDBP concentration during inflammation. Lower levels of VDBP were observed in critically ill children whereas an increase of VDBP was shown after allergen-induction of endobronchial inflammation (Bratke et al., 2014, Madden et al., 2015).

The importance of vitamin D for active TB development was demonstrated in a prospective study conducted among HIV-infected adults in Tanzania where a significant association between vitamin D deficiency and incident pulmonary TB was identified (Sudfeld et al., 2013). In keeping with this study, most case control studies reported a lower 25(OH)D in TB patients compared to the control group (Martineau et al., 2011a, Nnoaham and Clarke, 2008). However, consistent with a large study conducted in Mwanza, Tanzania, we observed a higher 25(OH)D and lower prevalence of vitamin D insufficiency among TB patients compared to healthy controls, particularly after treatment of TB (Friis et al., 2013). Contrasting with most studies, the prevalence of vitamin D deficiency was low and mean 25(OH)D was in the normal range in the Tanzanian studies which may have restricted the impact of low 25(OH)D on TB to diabetic patients who had lower 25(OH)D levels.

There is a high variability in vitamin D deficiency between studies conducted in Africa, ranging from 4.3% in Tanzania to 62.7% in South Africa (Martineau et al., 2011a). Highly different vitamin D levels between countries are likely explained by the distance of the studies sites to the Equator and perhaps also to differences in food composition, prevalence of comorbidities such as DM, laboratory techniques, as wells as possibly genetic polymorphisms (Powe et al., 2013).

In keeping with a recent study from Malawi, lower vitamin D was not associated with adverse TB treatment outcome in our study (Sloan et al., 2015) which contrasts with other studies conducted in setting where vitamin D levels at baseline were lower. However, this is in line with results of randomized control studies of adjunctive vitamin D that failed to detect an improvement of TB outcome (Daley et al., 2015, Davies and Martineau, 2015, Martineau et al., 2011b).

Our study has several strengths. First, the longitudinal design allows assessing the association between TB, DM and vitamin D after resolution of the inflammation status which influences vitamin D level and glycemic status. The study population was well defined and the patients categorized according to WHO recommendations. Its main limitation is the measurement of total 25(OH)D and not bioavailable “free” serum 25(OH)D which represents the active form of vitamin D. VDBP binds 85-90% of 25(OH)D and makes it unavailable to act on target cells (Powe et al., 2013). This might have been even more important during the course of an infection which modifies the levels of protein. The measurement of “free” 25(OH)D is only available in few laboratories, it is not routinely used and thresholds to define vitamin D sufficiency have been based on total serum 25(OH)D levels. TB diagnosis was based on NTLP guidelines and smear results rather than culture or GenXpert. However, the study was conducted under the real case scenario of TB treatment in this population. Fasting and 2-h glucose were assessed on capillary whole blood using a point-of-care test and not in venous blood. However, we used a plasma-calibrated glucometer which accuracy conformed to the International Standardization Organization guidelines (Essack et al., 2009). Our study was under-powered to investigate vitamin D levels among hyperglycemic patients.

In this equatorial population with a low prevalence of vitamin D deficiency, vitamin D insufficiency seems to increase the risk of TB only if associated with hyperglycemia suggestive of underlying DM and may even be inversely associated with active TB otherwise. Diabetic patients might be an appropriate target for vitamin D supplementation to improve TB outcome or prevent active TB, but the effect of such interventions requires confirmation through randomized controlled trials.

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Conflicts of interest

No conflict of interest to declare for all authors.

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6 Poor glycemic control in latent and active tuberculosis is inversely correlated with BCG-specific CD4 T cell immunity in Tanzanian adults

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6.1 Abstract

Background

Diabetes mellitus (DM) constitutes a risk factor for development of active tuberculosis (TB). Hyperglycaemia affects negatively innate immune functions. The recent increase of type 2 DM in developing countries with high TB endemicity poses novel challenges to these health systems. The impact of DM on TB specific adaptive immune responses remains poorly addressed.

Methods

Latent and active TB patients with or without DM were recruited in Dar es Salaam, Tanzania. Peripheral blood mononuclear cells were restimulated in vitro with ESAT-6 and CFP10 peptide pools or live *M. bovis* BCG. We analysed by polychromatic flow cytometry the intracellular cytokine expression of Th1, Th2, Th9 and Th17 in different T cell subsets. Cell culture supernatants harvested after antigen stimulation were analysed by Luminex.

Results

Our results show a lower frequency of INF-γ $CD4^+$ T cells and a lower proportion of $CD4^+$ T cells producing both TNF-α and IFN-γ in patients with active TB and DM after live *M. bovis* BCG but not after peptide pools stimulation. An inverse correlation between INF-γ or TNF-α $CD4^+$ T cell frequencies and poor glycaemic control after live *M. bovis* BCG stimulation was observed.

Conclusions

DM negatively affects IFN-γ and TNF-α producing $CD4^+$ T cell response in active TB after live BCG but not *Mtb* peptide re-stimulation indicating that antigen processing and/or presentation is primarily affected.

6.2 Introduction

Diabetes mellitus (DM) increases the risk of active tuberculosis (TB) development by about threefold (Jeon and Murray, 2008). The recent and rapid increase of type 2 DM prevalence in countries suffering already from high TB burden is expected to negatively impact TB control efforts (Corbett et al., 2003, Stevenson et al., 2007b). The observed interaction between active TB and DM suggests that host defence mechanisms for control of *Mycobacterium tuberculosis* (*Mtb*) infection are impaired. Hyperglycaemia affects innate immune functions of neutrophil granulocytes, macrophages and Natural Killer cells (Delamaire et al., 1997, Gan, 2013, Schuetz et al., 2011). T cell-mediated immune responses are clearly critical in the outcome of an *Mtb* infection (Jasenosky et al., 2015) as demonstrated by the marked susceptibility and reactivation of TB in HIV-co-infected persons (Sester et al., 2010). Defects in pro-inflammatory T Helper 1 (Th1) cellular polarization and cytokine production, particularly IFN-γ, are well-defined risk factors for mycobacteriosis (Bustamante et al., 2014). CD4 T^+ cell poly-functionality, i.e. the ability to produce simultaneously different combinations of IFN-γ, TNF-α and IL-2 correlates with improved effector and proliferative functions (Jasenosky et al., 2015). These responses can be substantially influenced by treatment and disease status and could therefore play a role in determining outcome of natural infection.

The immunological consequences of DM co-morbidity on TB specific adaptive immunity has been evaluated in different human studies but a defect in protective immunity has not yet been identified (Martinez and Kornfeld, 2014). A mouse model study provided interesting data showing a delayed but unimpaired cellular immune response to *Mtb* among mice with DM (Vallerskog et al., 2010). The conclusions of several human studies gave conflicting results. A higher production of Th1 cytokines following PPD stimulation among diabetic patients with active TB compared to non-diabetic patients with active TB has been reported but not reproduced in an Indonesian setting (Kumar et al., 2013b, Restrepo et al., 2008, Stalenhoef et al., 2008).

To our knowledge, the immunological features underlying TB and DM comorbidity have not been analysed in sub-Saharan Africa and the hypothesis of delayed adaptive immune response has not been tested in humans. We performed a broad phenotypic and functional characterisation of T cells in subjects with active or latent *Mtb* infection

stratified by their diabetic status following stimulation with either live *M. bovis* BCG or *Mtb*-specific peptide pools that respectively requires or not antigen processing by antigen presenting cells to recall antigen-specific T cell responses in vitro.

6.3 Methods

Study design and setting

This prospective case-control study was part of a study on the association between TB and DM (Boillat-Blanco et al.). Patients with active TB were recruited between April and December 2013 and controls between May and October 2013. Patients were recruited in Kinondoni District in Dar es Salaam (Mwananyamala Regional Hospital, Sinza Hospital, Magomeni Health Centre and Tandale Dispensary). Controls were recruited among adults accompanying patients, other than the one included in the study, to the outpatient departments of the same hospitals and living in Kinondoni District.

Study participants

Cases

Active TB patients (≥ 18 years) with new smear positive pulmonary TB were screened for study inclusion. Patients were eligible if they were not on TB treatment and sputum Xpert MTB/RIF (Cepheid, Sunnyvale, USA) assay was positive, HIV screening (Alere Determine™ HIV-1/2 and Trinity Biotech Uni-gold™ Recombigen® HIV-1/2) was negative. Exclusion criteria were pregnancy (positive urinary pregnancy screening test) and first week of lactation, any kind of immunosuppression during the last 6 months and severe anaemia (<5g/dL).

Controls

Healthy volunteering adults and diabetic patients attending the diabetes clinic in Mwananyamala hospital were screened for inclusion. Eligibility criteria for healthy controls were: HIV negative, no past TB history, absence of symptoms or signs of

active TB or other acute infection or major trauma within the last three months. Exclusion criteria were identical to TB cases.

Diabetes status

Blood glucose testing was conducted before the start of TB treatment after an overnight fast of ≥8 hours (fasting capillary glucose-FCG; GlucoPlus^{TM}, Diabcare); two-hours capillary glucose (2-hCG; standard 75-gram OGTT); glycated hemoglobin HbA1c (venipuncture whole blood; immuno-assay certified by the National Glycohemoglobin Standardization Program and insensitive to hemoglobinopathies (Tina-quant HbA1c Gen. 2 Cobas Integra 400, Roche Diagnostics)) (ADA, 2014, Essack et al., 2009, NGSP). DM was defined as repeated measurements ≥7.0 mmol/l for FCG, ≥11.1 mmol/l for 2-hCG and ≥6.5% for HbA_{1c} and/or in the presence of history and treatment for DM according to the standard ADA and WHO criteria (2014, WHO, 2006, WHO, 2011a). Normal glycaemia was defined as FCG<6.1 mmol/l and 2-hCG<7.8 mmol/l. The capillary glucose values were confirmed by plasma testing (Cobas Integra 400 plus). DM status was confirmed after 5 months of TB treatment to avoid including patient with transient stress hyperglycemia (Boillat-Blanco et al.). Patients with pre-DM were excluded.

Latent TB

Healthy controls were classified as latently infected by *Mycobacterium tuberculosis* (*Mtb*) based on positive *Mtb*-specific Interferon-Gamma enzyme-linked immunospot assay (ELISPOT) or positive flow cytometry result (frequency of IFN-γ, TNF-α or IL-2 $CD4^+$ T cells after *Mtb*-specific stimulation >0.03% and >2 times higher than negative control) or positive Luminex results (defined as IFN-γ, TNF-α or IL-2 after *Mtb*-specific stimulation >5 times higher than negative control). Immunologically irrelevant, healthy control samples that did not show evidence of latent TB infection were excluded from the analysis.

Study procedures

Data and sample collection

Demographic characteristics, health history and symptoms, socioeconomic status (indicators of education, occupation, and wealth using factor analysis) were obtained. Complete blood count was performed (Sysmex XS-800i automated haematology analyzer). Data were entered directly into an open data kit in a personal digital assistant with real-time error, range and consistency checks (Hartung et al., December 2010). Before TB treatment initiation, Peripheral Blood Mononuclear cells (PBMC) were isolated within five hours of phlebotomy and cryopreserved in temperature monitored liquid nitrogen tanks.

Immunological assays

Immunological assays were performed after thawing and 5-6 hours resting of cryopreserved PBMCs. For flow cytometry assays, PBMC ($1x10^6$) were cultivated overnight in the presence of Brefeldin A (BD GolgiPlug, Becton Dickinson) either untouched (negative control), or stimulated with Staphylococcal enterotoxin B (SEB; Sigma; 200 ng/ml^{-1}; positive control), or live *M. bovis* bacilli Calmette-Guérin vaccine (20µl BCG Vaccine solution, Staten Serum Institute) or *Mtb* specific peptide pools covering the 10-kDa culture filtrate antigen (CFP10) and the 6-kDa early secretory antigen target (ESAT6) protein sequences as described previously (Harari et al., 2011).

IFN-γ ELISPOT assays

Rested PBMCs were stimulated for 16 hours with Mtb-specific peptide pools or SEB (positive control). Only cell samples with >80% viability were analysed. Assays with <50 SFU for negative control and >500 SFU per 10^6 cells after SEB stimulation were considered as valid. ELISPOT result was defined positive when Mtb-specific stimulation led to ≥55 SFU per 10^6 cells and fourfold higher than negative control.

Intracellular cytokine staining and flow cytometry analyses

Rested PBMCs were incubated in 100 µl PBS containing 1 µl Aqua solution (LIVE/DEAD kit, Invitrogen), and then stained with antibodies specific for CD3 (APC-H7, SK7, BD Biosciences), CD4 (PE-CF594, RPA-T4, BD Biosciences), CD8 (eFluor625NC, RPA-T8, eBioscience) and CD45RA (BV711, 92430, Biolegend) before fixation, and permeabilization with BD Cytofix/Cytoperm kit (BD Biosciences). PBMCs

were then stained with antibodies directed against IFN-γ (AF700, B27, BD Biosciences), TNF-α (PECy7, Mab11, BD Biosciences), IL-2 (PerCpCy5.5, MQ1-17H12, Biolegend), IL-10 (BV 421, JES3-9D7, Biolegend), IL-17 (FITC, BL168, Biolegend), IL-4 (PE, 3010.211, BD Bioscienecs), IL-5 (PE, TRFK5, Biolegend), IL-13 (PE, JES10-5A2, Biolegend) and IL-9 (Alexa647, MH9A4, BD Biosciences). Cells were fixed, acquired on a BD LSRII apparatus equipped with 405, 488, 532 and 633 nm lasers and data analysed with FlowJo version 8.8.6 (Tree Star Inc.) and SPICE version 5.1 (Roederer et al., 2011). Gating strategy is shown on Figure 6-1. Only samples with a cytokine response to SEB at least twice higher than unstimulated control were considered valid. A positive flow cytometry response was considered when the frequency of cytokine-specific cells after live *M. bovis* BCG vaccine or *Mtb*-specific peptide pools stimulation was above 0.03% and twice higher than the unstimulated specimen for any of the investigated cytokine.

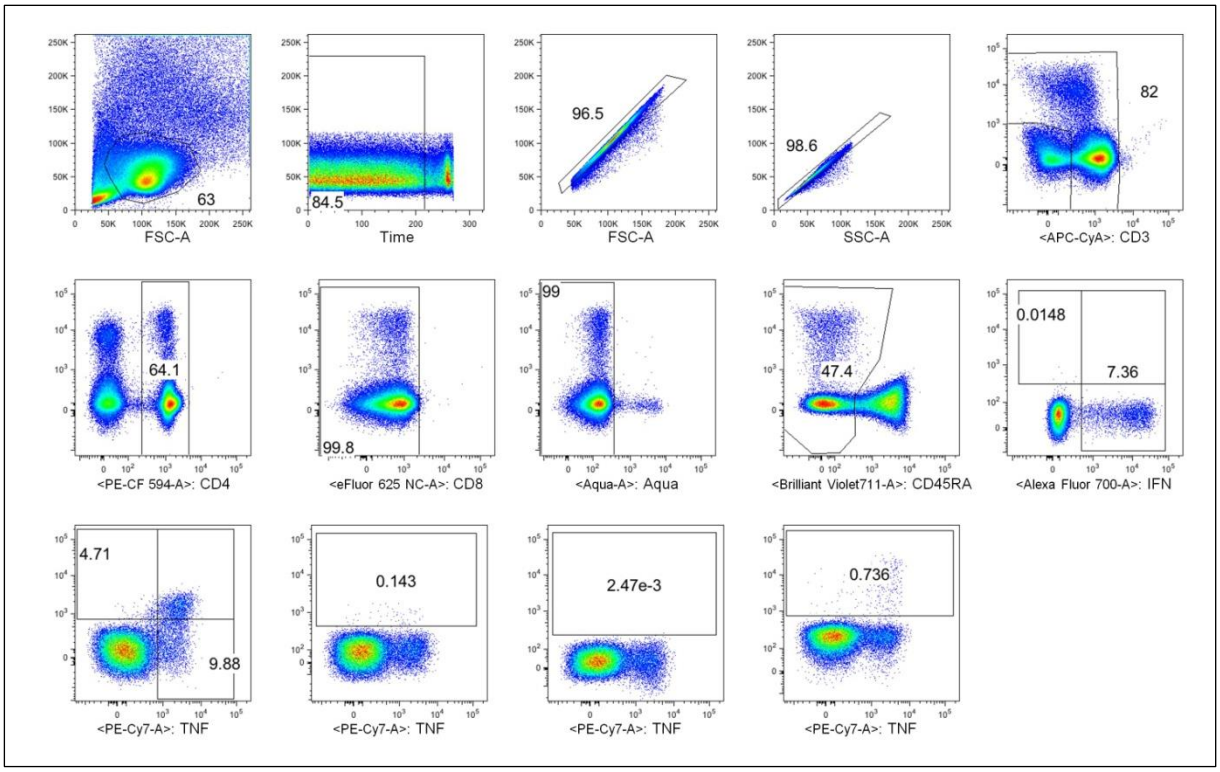

Figure 6-1. Gating strategy for the flow cytometry analysis of CD4⁺ T cell cytokine responses

Cytokine quantification with Luminex assay

After overnight stimulation as described above but in the absence of BrefeldinA, cell culture supernatants were harvested and stored at -20°C before analysis with ProcartaPlex Human Cytokine & Chemokine Panel (Affymetrix ebioscience) and a Bio-Plex 200 analyzer (Biorad). Data were processed using Hmisc, nCal, rjags, gWidgetstcltk R packages. Luminex response was defined as a cytokine or chemokine concentration after *Mtb*-specific peptide pools stimulation fivefold higher than the unstimulated specimen.

Data analysis

Frequencies of CD4⁺ T cells producing cytokines as well as quantitative cytokine responses were adjusted by subtracting the background level (negative control).

Frequency of responders based on flow cytometry of the tested cytokines was compared between the 4 study groups with Fischer's exact test. Only cytokines with a flow cytometry responder's frequency ≥50% in at least one study group were kept in subsequent analyses.

Frequencies of $CD4^+$ T cells producing cytokines after live *M. bovis* BCG vaccine and *Mtb*-specific peptide pools stimulation were compared between the different study groups by Wilcoxon-Mann-Whitney test using GraphPad Prism 6.

SPICE analysis of $CD4^+$ T cells frequencies was compared between the different study groups by unpaired two-tailed Student's *t* test.

Frequencies of $CD4^+$ T cells producing cytokines were analysed according to fasting glycaemia by linear regression after adjustment for age and sex. HbA1c results were not considered as they appeared to overestimate DM prevalence in this TB population with a high prevalence of anemia and hemoglobinopathies (Boillat-Blanco et al.).

Frequency of responders per cytokine and cytokine concentration level after live *M. bovis* BCG vaccine and *Mtb*-specific peptide pools stimulation were compared between the study groups by Wilcoxon-Mann-Whitney test. Statistical analyses were performed using Stata software (StataCorp, College Station, TX, USA, version 12).

Ethical considerations

All participants consented in writing to interview and health examination. The Ifakara Health Institute Institutional Review Board and the Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania, gave ethical clearance.

6.4 Results

Study sample

In total, 26 latent and 28 active TB cases with and without DM were enrolled into this immunological study with their anthropometric and glycemic status summarized in Table 6-1. Participants suffering from DM were significantly older than the one with normal glycemia while gender and body mass index did not differ between the study groups. Figure 6-2 depicts the distribution of participants with latent TB, active TB or no TB in the two groups according to their glycaemic status (DM versus normal plasma glucose). Among patients with active TB and DM co-morbidity, 5/8 received already DM treatment prior to TB diagnosis (4/8 were treated with metformin and 1/8 with

insulin). All volunteers with latent TB and DM comorbidity were under DM treatment at the time of blood collection (11/12 received metformin and 1/12 received insulin). Clearly, glycaemia was uncontrolled with values in the DM range, most likely due to poor treatment adherence.

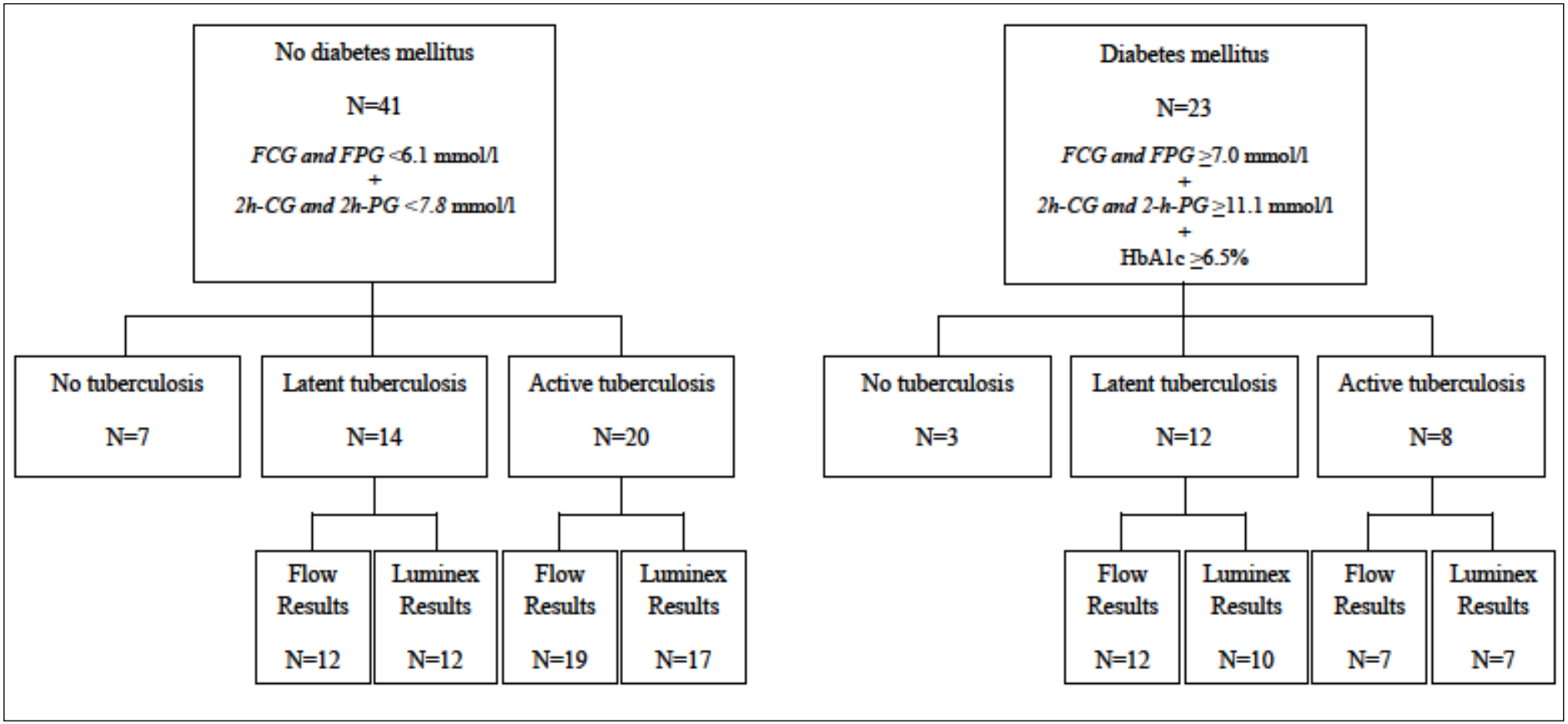

Figure 6-2. Flow chart of study participants and immunological tests.

Table 6-1. Study population.

	Latent *Mtb* N=14	Latent *Mtb* DM N=12		Active TB N=20	Active TB DM N=8	
	N (%) / median (IQR)		p	N (%) / median (IQR)		p
Age	35 (10)	45 (27)	<0.001	28 (11)	57 (7)	<0.001
Male sex	10 (71)	5 (42)	0.13	17 (85)	7 (88)	0.86
Low socio-economic status	3 (23)	2 (17)	0.69	7 (37)	3 (43)	0.78
Body mass index (kg/m^2)	23.4 (5.5)	26.3 (8.8)	0.96	18.4 (4.3)	18.7 (3.6)	0.57
Under treatment for diabetes	-	12 (100)	-	-	5 (63)	-
TB symptom duration>1month	-	-	-	15 (79)	6 (86)	0.70
Fasting capillary gluc. (mmol/L)	4.8 (0.7)	10.1 (4.6)	<0.001	5.0 (0.5)	18.3 (12)	<0.001
Fasting plasma gluc. (mmol/L)	5.1 (1.1)	16.4 (4.6)	<0.001	5.1 (0.6)	20.7 (6.2)	<0.001
Glycated Hemoglobin (%)	5.4 (0.6)	11.2 (3.4)	<0.001	5.8 (0.7)	13.6 (4.5)	<0.001

Abbreviations and definitions:
Latent *Mtb*: *Mycobacterium tuberculosis* latent infection defined as positive *Mtb*-specific Interferon-Gamma enzyme-linked immunospot assay or positive *Mtb*-specific flow cytometry response or positive *Mtb*-specific cytokine release assay / TB: tuberculosis / DM: diabetes mellitus
P value calculated with two-sided Wilcoxon-Mann-Whitney or chi-square tests when appropriate.

Mtb peptide and BCG specific responses

We determined the intracellular expression of the cytokines IFNγ, IL-2, TNFα, IL-4, IL-9, IL-10 and IL-17 by CD4$^+$ T cells using multi-parameter flow cytometry following stimulation of PBMCs with *Mtb*-specific peptide pools or live BCG. The percentage of volunteers responding to *Mtb*-specific peptide pools or live BCG did not vary significantly amongst study groups for any of these cytokines (Table 6-2). Participants with latent *Mtb* showed a trend towards lower frequency of *Mtb*-specific TNF-α CD4$^+$ T cell responses compared to patients with active TB (p=0.08). After live BCG stimulation, patients with active TB and DM had a lower frequency of IFN-γ CD4$^+$ T cell responses compared to participants with latent *Mtb* infection (p=0.07). Positive staining for cytokines IL-4, IL-9, IL-10 or IL-17 was low and was not evaluated further (Table 6-2).

Table 6-2. Percentage of responding volunteers per cytokine and per study group.

	Mtb-specific peptide pools stimulation					Live *M. bovis* BCG stimulation				
	All N=50	Latent *Mtb* N=12	Latent *Mtb* DM N=12	Active TB N=19	Active TB DM N=7	All N=50	Latent *Mtb* N=12	Latent *Mtb* DM N=12	Active TB N=19	Active TB DM N=7
Any	88[1]	92	75	89	100	92	92	100	89	86
Th1	84	75	75	89	100	88	92	92	84	86
IFN-γ	70	67	75	74	57	62	75	75	58	29**¥**
TNF-α	80	58**§**	75	89	100	82	83	92	79	71
IL-2	60	50	67	53	86	36	58	33	26	29
IL-4	8	8	8	11	0	2	0	0	5	0
IL-9	4	8	0	5	0	4	0	0	11	0
IL-10	6	17	0	0	14	12	0	17	21	0
IL-17	0	0	0	0	0	2	0	0	5	0

Abbreviations and definitions:
Mtb-specific stimulation: *Mycobacterium tuberculosis* antigens (CFP-10 and ESAT-6) / Latent *Mtb*: defined as positive *Mtb*-specific Interferon-Gamma enzyme-linked immunospot assay or positive *Mtb*-specific flow cytometry response or positive *Mtb*-specific cytokine release assay / TB: tuberculosis / DM: diabetes mellitus / Th1: IFN-γ, TNF-α or IL-2.
§: P value=0.08 compared to active TB group under same stimulation condition; **¥:** P value=0.07 compared to latent TB group under same stimulation condition (Fischer's exact test).

Frequencies of CD4⁺ T cells producing Th1 cytokines

<Following live BCG stimulation, we observed lower absolute frequency of CD4⁺ T cells producing IFN-γ (p=0.04; Figure 6-3a) and a similar trend in CD4⁺ T cells producing TNF-α among patients with active TB and DM compared to normo-glycaemic patients with active TB. In contrast, after *Mtb*-specific peptide pools stimulation, no difference in cytokine producing CD4⁺ T cells frequencies was observed between study groups (Figure 6-3b). As positive control documenting comparable PBMC viability polyclonal SEB stimulation was performed. No difference in frequencies of IFN-γ or TNF-α producing CD4⁺ T cells was measured (Figure 6-3c).

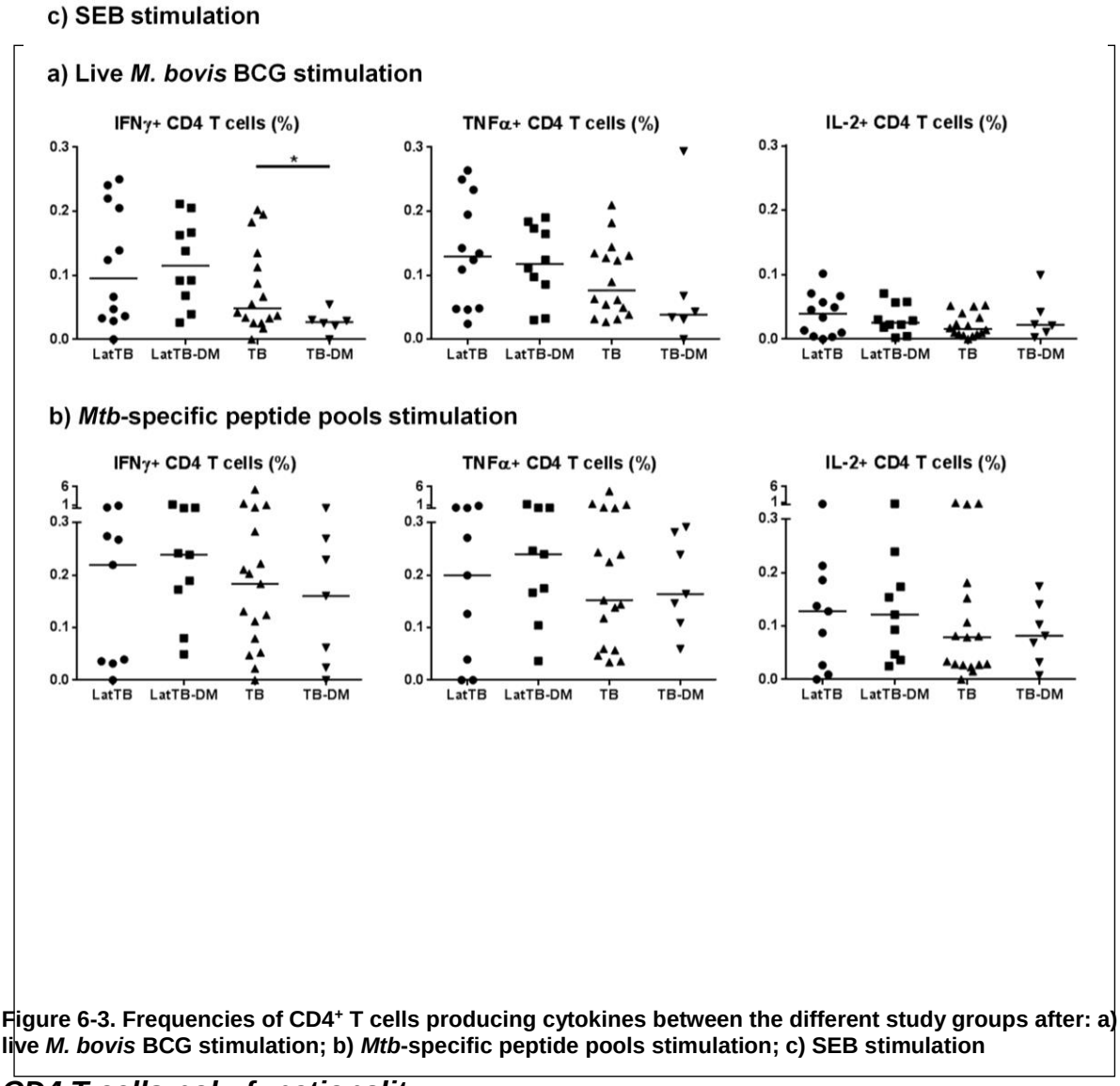

Figure 6-3. Frequencies of CD4⁺ T cells producing cytokines between the different study groups after: a) live *M. bovis* BCG stimulation; b) *Mtb*-specific peptide pools stimulation; c) SEB stimulation

CD4 T cells poly-functionality

We next investigated the representation of the CD4+ T cell subset producing different combinations of cytokines. After live *M. bovis* BCG stimulation, we observed a lower proportion of $CD4^+$ T cells producing both TNF-α and IFN-γ in the group of patients with active TB and DM compared to normoglyacemic participants with latent *Mtb* infection (p=0.06, Figure 6-4a). There was a lower proportion of BCG-specific $CD4^+$ T cells producing IFN-γ only in the group of participants with latent *Mtb* infection and DM compared to normo-glycaemic patients with active TB (p=0.08; Figure 6-4a). The proportion of *Mtb*-specific $CD4^+$ T cells double positive for TNF-α and IFN-γ was lower among participants with latent *Mtb* infection compared with all other study groups (Figure 6-4b).

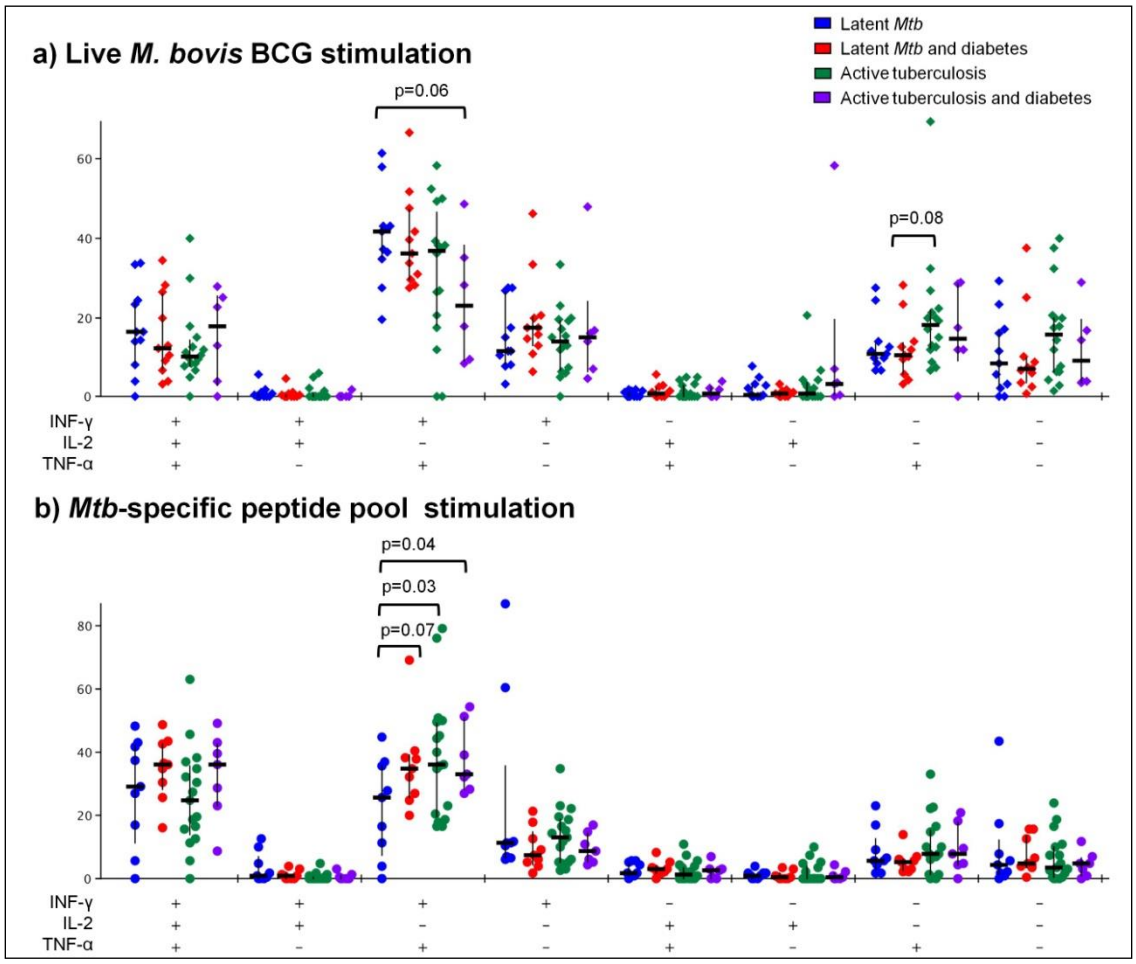

Figure 6-4. Representation of mono/polyfunctional $CD4^+$ T cell subset responses between the different study groups after a) live *M. bovis* BCG stimulation; b) *Mtb*-specific peptide pools stimulation. Bars represent the median, lines the interquartile range.

Frequencies of $CD4^+$ T cells producing cytokines according to glycaemia

We next sought to investigate whether the intensity of the cytokines responses detected by flow cytometry would correlate with DM disease severity irrespectively of TB disease status. An increase in glycaemia was found negatively correlated with INF-γ and TNF-α CD4⁺ T cells frequency under live *M. bovis* BCG stimulating condition (p=0.02 and 0.03 respectively) while there was no difference under *Mtb*-specific peptide pools or SEB control stimulation (Figure 6-5).

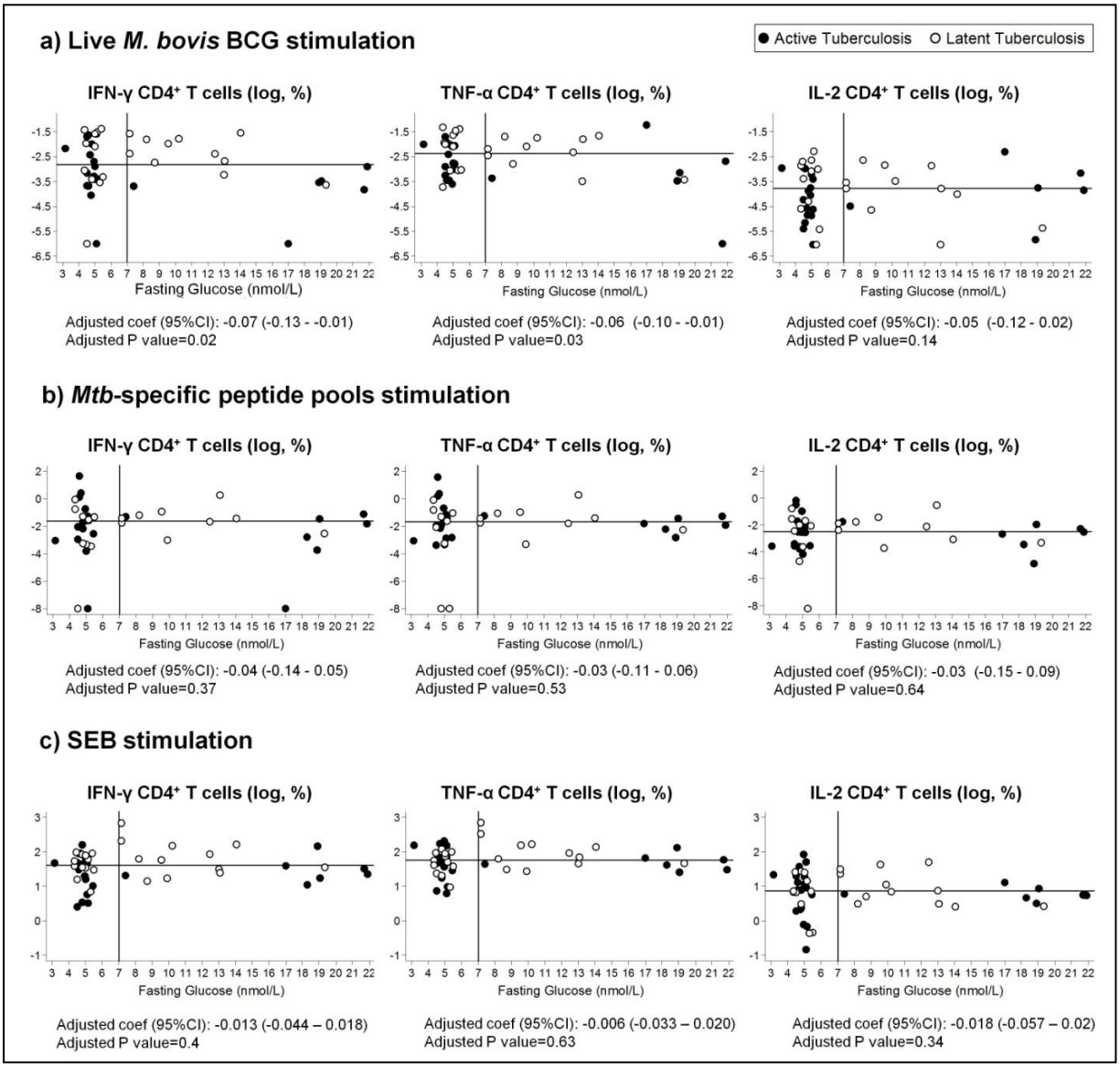

Figure 6-5. Dot-plot graphs of the frequencies of CD4⁺ T cells producing cytokines against fasting capillary glucose levels after: a) live *M. bovis* BCG stimulation; b) *Mtb*-specific peptide pools stimulation; c) SEB stimulation. Linear regression was adjusted for age and sex. The horizontal line represents the median value of the logarithmic frequency of CD4⁺ T cells producing cytokines and the vertical line represents the fasting glucose level cut-off used for diabetes mellitus disease classification

Cytokine and chemokine release assay response per study groups

Finally, we measured the secretion of cytokines and chemokines in cell culture supernatant after stimulation with *Mtb*-specific peptide pools (Table 6-3) or live *M. bovis* BCG (Table 6-4). Overall, *Mtb*-specific stimulation did not elicit substantial cytokine and chemokine production. Inflammatory mediators detected in BCG stimulated samples for more than 50% of patients in at least one arm and for which the production could statistically discriminate between two arms is summarized in Table 6-5). We observed a significantly lower frequency of responders for IL-9 and IL-22 among participants with latent *Mtb* infection and DM compared to those with a normal glycaemic status ($p=0.005$ and $p=0.03$). The production of IL-6 and MIP-1α was significantly higher among participants with latent *Mtb* infection and DM when compared to those with a normal glycaemic status ($p=0.03$ and $p=0.007$). In return, the level of IL-21 was lower among in participants with latent *Mtb* infection and DM compared to those with a normal glycaemic status ($p=0.05$). Among patients with active TB and DM and compared to those with a normal glycaemic status, the IL-18 frequency of responders was found significantly reduced ($p=0.02$).

Table 6-3. Frequency of responders based on Luminex data analysis per cytokine and per study group, and cytokine level in all participants per study group after *Mtb*-specific peptide pool stimulation.

	All N=46	Latent *Mtb* N=12		Latent *Mtb* DM N=10		Active TB N=17		Active TB DM N=7	
	%	%	Median (IQR)	%	Median (IQR)	%	Median (IQR)	%	Median (IQR)
IFN-γ	83	75	23(85)	70	24(603)	94	10(36)	86	55(346)
TNF-α	28	33	1.2(4.9)	40	0.8(4.5)	29	0(1.3)	10	0.6(9.1)
IL-2	46	50	11(21)	30	9.8(66)	53	2.5(9.3)	43	7.7(81)
IL-4	30	17	2(14)	30	2.4(15)	41	0(9.1)	29	14(23)
IL-5	13	8	0(0)	20	0(0)	12	0(0)	14	0(0)
IL-13	46	58	0.9(4.5)	60	3.5(5.4)	29	0(2.1)	43	0(22)
IL-9	20	25	0(24)	0	0(5.8)	24	0(5.8)	29	2.5(17)
IL-10	9	0	0(1.4)	10	0(1)	12	0(0)	14	0(1.6)
IL-17A	15	25	0(1.2)	10	0(1.3)	6	0(0)	29	0(3)
IL-6	20	17	15(83)	30	180(362)	12	24(59)	29	20(668)
IL-7	4	8	0(0)	10	0(0)	0	0(0)	0	0(0)
IL-8	0	0	209(673)	0	472(605)	0	1892(3357)	0	258(384)
IL-12p70	2	0	0(0)	10	0(0)	0	0(0)	0	0(0)
IL-15	20	25	0(2.3)	20	0(3.6)	18	0(1.7)	14	0(2.6)
IL-18	15	17	0(0)	30	0(14)	6	0(0)	14	0(0)
IL-21	15	17	0(13)	10	1.8(7.1)	12	0(2)	29	4.6(11)
IL-22	15	17	37(100)	20	25(131)	12	0(66)	14	12(167)
IL-23	100	100	0(5.2)	100	0(7)	100	0(7)	7	3.2(8.5)
IL-27	13	25	43(66)	10	0(45)	12	0(0)	0	0(0)
IL-31	9	8	0(48)	0	0(27)	12	0(29)	14	4.5(37)
IP-10	85	83	185(1398)	70	425(823)	88	183(669)	100	164(1318)
MCP-1	4	8	498(1147)	0	146(795)	6	447(690)	0	173(1191)
MIP-1 α	39	50	196(587)	40	402(290)	29	58(451)	43	125(281)
MIP-1 β	15	17	448(1536)	10	974(1612)	18	354(1382)	14	498(1363)
RANTES	4	0	1.3(37)	10	3.6(34)	0	16(54)	14	0(4.3)
SDF-1 α	4	8	123(378)	0	167(320)	6	359(790)	0	109(199)
TNF-β	9	8	0(8.4)	0	9.2(31)	**0**	0(21)	**43**	0(12)
INF-α	28	50	0.2(0.8)	30	0.4(0.6)	18	0(0)	14	0(0.8)
Eotaxin	33	50	0.6(0.8)	20	0.4(1)	29	0(0.5)	29	0(1)
GM-CSF	28	42	4.2(19)	20	0(22)	**12**	**0(0)**	**57**	**8.7(42)**
IL-1RA	17	25	300(1757)	20	150(1126)	18	371(933)	0	24(861)
IL-1α	7	0	0.2(0.8)	0	0.4(0.6)	18	0(0)	0	0(0.8)
IL-1β	41	42	1.2(4)	50	8(20)	35	1.6(4.1)	43	0.9(12)
GROα	11	8	77(152)	0	229(262)	18	87(216)	14	47(175)

P value<0.05 in bold. P values calculated with Wilcoxon-Mann-Whitney.

Table 6-4. Frequency of responders based on Luminex data analysis per cytokine and per study group, and cytokine level in all participants per study group after stimulation with live *M. bovis* BCG.

	All N=46	Latent *Mtb* N=12		Latent *Mtb* DM N=10		Active TB N=17		Active TB DM N=7	
	%	%	Median (IQR)	%	Median (IQR)	%	Median (IQR)	%	Median (IQR)
IFN-γ	89	83	576(1908)	100	792(2277)	94	209(279)	71	84(7057)
TNF-α	96	92	214(658)	90	200(193)	100	317(226)	100	244(699)
IL-2	76	67	28(34)	60	45(35)	88	37(20)	86	34(48)
IL-4	85	75	57(61)	80	79(58)	94	74(32)	86	74(51)
IL-5	78	83	6.5(11)	60	9.4(16)	88	4.5(11)	71	5.7(11)
IL-13	93	92	5.4(23)	100	9.5(12)	100	3.9(9)	71	5.4(60)
IL-9	30	**58**	18(38)	**0**	0(35)	29	0(11)	29	0(37)
IL-10	91	92	39(85)	80	39(36)	100	71(78)	86	33(186)
IL-17A	41	58	1.1(3.1)	30	1.2(5)	29	0(2.1)	57	1.6(10)
IL-6	72	67	**2665(1528)**	60	**6802(5655)**	76	3617(1565)	86	3672(3732)
IL-7	15	17	0(0)	10	0(0)	12	0(0)	29	0(0.4)
IL-8	0	0	159(895)	0	664(1620)	0	1469(1688)	0	367(7715)
IL-12p70	41	58	7.6(24)	50	2.1(21)	29	0(5.1)	29	0(8.6)
IL-15	48	67	7.9(7.6)	40	3.6(8)	35	0(1.7)	57	3.6(12)
IL-18	85	75	13(23)	90	17.3(15)	**100**	12(8.6)	**57**	10(35)
IL-21	30	50	**14(11)**	10	**2.5(9.6)**	24	1.9(8.9)	43	8.6(18)
IL-22	50	**75**	249(649)	**29**	183.5(316)	47	106(106)	57	165(766)
IL-23	15	8	22(96)	9	20.2(8.7)	29	39(73)	14	42(80)
IL-27	24	42	**150(215)**	10	**13.1(76)**	24	11(30)	14	18(215)
IL-31	15	17	41(87)	10	8(55)	18	4.4(43)	14	32(106)
IP-10	28	50	10(49)	10	19.6(46)	29	15(29)	14	10(24)
MCP-1	2	0	**0(194)**	0	**0(0)**	6	42(558)	0	0(0)
MIP-1 α	43	50	**1069(779)**	30	**1684(205)**	35	1747(715)	43	1781(1497)
MIP-1 β	43	50	8730(14356)	30	13384(23089)	35	16154(26790)	43	37571(185385)
RANTES	7	0	35(120)	0	62(80)	18	100(411)	0	19(251)
SDF-1 α	9	8	205(656)	0	456(953)	18	**889(787)**	0	**105(458)**
TNF-β	9	8	0(34)	10	0(7.6)	6	0(12)	14	0(1.6)
INF-α	30	58	0.7(0.9)	30	0(0.3)	18	0(0)	14	0(0.8)
Eotaxin	41	58	2(1.4)	20	1.2(1.3)	47	1.3(0.9)	29	0.7(1.7)
GM-CSF	98	92	453(654)	100	432(208)	100	559(571)	100	532(1231)
IL-1RA	28	33	1021(2117)	10	604(1664)	35	**1780(2539)**	29	**401(629)**
IL-1α	33	25	0.7(0.9)	20	0(0.3)	47	0(0)	29	0(0.8)
IL-1β	93	92	751(1186)	90	1275(1198)	94	1028(1144)	100	800(1366)
GROα	30	25	249(494)	20	560(344)	24	434(230)	71	450(4519)

P value<0.05 in bold. P values calculated with two-sided Wilcoxon-Mann-Whitney

Table 6-5. Frequency of responders based on Luminex data analysis per cytokine and per study group, and cytokine level in all participants per study group after stimulation with live *M. bovis* BCG vaccine.

	All N=46	Latent *Mtb* N=12		Latent *Mtb* DM N=10		Active TB N=17		Active TB DM N=7	
	%	%	Median (IQR)	%	Median (IQR)	%	Median (IQR)	%	Median (IQR)
IL-9	30	**58**	18(38)	**0**	0(35)	29	0(11)	29	0(37)
IL-6	72	67	**2665(1528)**	60	**6802(5655)**	76	3617(1565)	86	3672(3732)
IL-18	85	75	13(23)	90	17.3(15)	**100**	12(8.6)	**57**	10(35)
IL-21	30	50	**14(11)**	10	**2.5(9.6)**	24	1.9(8.9)	43	8.6(18)
IL-22	50	**75**	249(649)	**29**	183.5(316)	47	106(106)	57	165(766)
MIP-1 α	43	50	**1069(779)**	30	**1684(205)**	35	1747(715)	43	1781(1497)

P value<0.05 in bold. P values calculated with two-sided Wilcoxon-Mann-Whitney

6.5 Discussion

Under live *M. bovis* BCG stimulation, we observed a lower frequency of responders and reduced frequencies of IFN-γ $CD4^+$ T cells as well as lower proportion of $CD4^+$ T cells producing both TNF-α and IFN-γ in patients with active TB and DM compared to normo-glycaemic TB patients.

This study presented here has the strengths that our study population is well characterized and that DM diagnosis is based on three currently recommended screening tests that were repeated after TB treatment to avoid inclusion of patients with stress hyperglycaemia (2014). Patients with with pre-DM were excluded from the immunological study. PBMC collection was performed prior initiation of TB treatment since it is well known that cellular immune responses change rapidly under TB treatment (Jasenosky et al., 2015). We have studied PBMC instead of whole blood to circumvent potential bias linked to the extent of hyperglycaemia present in a whole blood assay set up. Importantly, we compared PBMC stimulation using whole live BCG side by side with peptide pools stimulation assuming that live BCG bacteria require steps of antigen processing and presentation for T cell stimulation while peptides can readily be loaded onto MHC class II molecules and presented to $CD4^+$ T cells. Finally, we have used one of the most exhaustive Luminex cytokine detection panels available to compare the production of immune mediators in the different study groups. The study limitation mainly resides in the small sample size. Despite systematic screening

of TB patients for DM comorbidity, the recruitment of patients with active TB and DM has been greatly impeded by the exclusion of individuals with HIV co-infection. Also, only volunteers showing hyperglycaemia in all three screening tests used were included which allowed to select DM patients with persistent hyperglycaemia at the end of TB treatment rather than transient stress-induced hyperglycaemia (Boillat-Blanco et al.).

To our knowledge, only one study previously examined by using multiparameter flow cytometry the influence of DM on $CD4^+$ T cells responses from patients with active TB after stimulation with various *Mtb* antigens including PPD, ESAT-6 and CFP-10. Contrasting to our observations, higher frequencies of $CD4^+$ T cells expressing single or combinations of IFN-γ, IL-2 and TNF-α was found in active TB patients with DM compared to non-diabetic TB patients, suggesting a sustained, strong adaptive immune response in the presence of DM co-morbidity (Kumar et al., 2013b). However, Kumar et al. reported higher frequencies of $CD4^+$ T cell producing cytokines at baseline than under antigen specific stimulation while our gating strategy used the signals detected among negative controls to solely detect T cell signals specifically induced by the stimuli after minimal background subtraction. Another study used ELISA to assess PBMC cytokine responses after stimulation with a battery of mycobacterial antigens and whole-mycobacteria and did not detect significantly different responder frequencies due to DM among patients with active TB (Al-Attiyah and Mustafa, 2009). These results suggested that adaptive immunity is not affected by DM or that TB specific $CD4^+$ T cells may develop excessively. Different factors related to the host and pathogen genetics and to the selection of the study participants make comparison of the published results difficult. The study of Kumar *et al.* was conducted in India and DM diagnosis was based on glycated haemoglobin or random blood glucose measured at TB diagnosis while Al-Attiyah *et al.* included TB patients in Kuwait and defined DM using two blood glucose measurements (Al-Attiyah and Mustafa, 2009, Kumar et al., 2013b). In our study, DM stratification was the most stringent using repeated fasting glucose, an oral glucose tolerance test and glycated haemoglobin that were further confirmed to be high after 5 months of TB treatment to avoid inclusion of patients with transient stress-induced hyperglycemia rather than DM (Boillat-Blanco et al.). In addition, pre-DM patients were excluded. Furthermore, most our diabetic participants

were previously known and treated for DM and did not take their treatment the day before study inclusion while this information is not available in other studies. For instance, Metformin, which is frequently used for DM treatment, facilitates phagosome-lysosome fusion and enhances *Mtb*-specific immune response. This could improve antigen presentation and bias the interpretation of assay result (Singhal et al., 2014).

We report an extensive multiplex cytokine/chemokine Luminex based detection, highlighting notably or the first time a lower frequency of responders of IL-9 and IL-22 producing cells among diabetic participants with latent TB compared to normo-glycaemic volunteers. We also observed a lower response frequency of IL-18 producing cells and a trend towards lower IFN-γ producing cells among patients with TB and DM compared to normo-glycaemic active TB patients.

The lower response frequency of IL-9 in patients with latent TB and DM is in line with previous results showing that IL-9 was significantly reduced in patients with DM compared to normo-glycaemic patients (Vasanthakumar et al., 2015). The higher IL-6 release in patients with latent TB and DM following BCG stimulation also supports the generally elevated levels of circulating IL-6 described in type 2 DM and in patients with TB and high HbA1c level or in TB patients with DM and severe hyperglycemia (Kumar et al., 2013a, Restrepo et al., 2008, Stalenhoef et al., 2008). IL-22 has an important function in regulating metabolism including hyperglycaemia and insulin resistance (Wang et al., 2014, Wilson et al., 2010). Consistent with some of the findings of Kumar et al, we found a lower frequency of IL-22 responders among patients with latent TB and DM which is produced by macrophages and stimulating T cells to release INF-γ (Kumar et al., 2013a, Maartens and Wilkinson, 2007, Okamura et al., 1995). IL-18 has a decisive role in *Mtb* protective immunity (Schneider et al., 2010). The observed lower IL-18 response among patdiabetic patients with active TB and DM has not been previously documented.

The lower frequency of IFNγ or IFNγ/TNFα $CD4^+$ T cells among diabetic patients with active TB that is observed solely when PBMCs were stimulated with live *M. bovis* BCG but not with peptide pools, points to an antigen processing defect in this population. Our interpretation of a delayed T cell recall in live *M. bovis* BCG stimulated samples is in line with human experimental studies showing a defective function of antigen-

presenting cells among diabetics and with the diminished interaction of *Mtb* with monocytes among patients with TB and DM (Delamaire et al., 1997, Gomez et al., 2013, Jansen et al., 1995). Our results also go along with experimental data in mice showing that although the adaptive immune response against *Mtb* among diabetic mice is conserved, a delayed priming of $CD4^+$ T cells to produce IFN-γ was observed and further sustained by the late migration of antigen presenting cells to the lung (Martens et al., 2007, Vallerskog et al., 2010).

Of note, we observed a significant negative correlation between increasing levels of hyperglycaemia and the ability to measure antigen-specific $CD4^+$ T cells following live *M. bovis* BCG stimulation in both, latent and active TB cases. This observation highlights the negative impact of high blood glucose levels on antibacterial immunity and has not been assessed in this way before.

In conclusion, an impaired function of antigen presenting cells supported by other reports strongly reconcile the different $CD4^+$ T cell recall responses observed between BCG and *Mtb*-specific peptide pools stimulation. These findings suggest that DM may not quantitatively affect T-cell mediated immunity in Tanzanian TB patients, but would rather delay its priming by affecting antigen processing and presentation. This undoubtedly warrants further investigation notably in the context of Metformin adjunct therapy during TB treatment that may specifically compensate for this defect and explain the benefits observed when Metformin was administered together with regular TB treatment regimen (Singhal et al., 2014).

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Conflicts of interest

No conflict of interest to declare for all authors.

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7 Discussion

The main purpose of this PhD thesis was to analyze the epidemiological and immunological backgrounds underlying the association between DM and TB in Tanzania, a setting with high HIV prevalence.

The main findings of these studies are the following. Irrespective of HIV status, hyperglycemia was very common in TB patients, but, in most of them, the glucose status was normalized with TB treatment. Fasting hyperglycemia screening at TB diagnosis captured patients at risk of adverse TB outcome. In this equatorial African setting, vitamin D insufficiency seemed to increase the risk of TB only if associated with hyperglycemia measured after TB treatment. The immunological analyses suggested that DM does not quantitatively affect *Mtb*-specific T-cell mediated immunity in TB patients based on in vitro stimulation using ESAT-6 and CFP-10 peptide pools. However, poor glycemic control correlated with reduced in vitro IFN-γ secretion after live *M. bovis* BCG stimulation most likely reflecting impaired antigen processing or presentation.

In this section we will discuss questions that have arisen throughout the project on comorbidity between communicable and non-communicable diseases. We will present how our studies contributed to generation of new knowledge on (i) the real burden of DM / low vitamin D and TB (7.1), (ii) the optimal methods of DM screening during TB (7.2), and (iii) the management of comorbid patients with DM and TB (7.3)

7.1 Assessment of tuberculosis risk related to hyperglycemia and vitamin D: Advantage of a longitudinal study design

Most studies assessed the link between TB and DM with a cross-sectional design and reported an increased risk of TB in the presence of DM (Jeon and Murray, 2008, Stevenson et al., 2007a). It is highly plausible that DM predisposes to TB as hyperglycemia leads to an impaired innate immune response which is the cornerstone of TB resistance, but TB also induces hyperglycemia through inflammation-driven insulin resistance and neoglucogenesis. Similarly, many cross-sectional studies reported that low vitamin D is a risk factor for TB which is compatible with the biological

activity of vitamin D as a booster of the innate antimicrobial activity. However, the inflammation induced by TB can possibly alter vitamin D metabolism and affect the concentration of the free active vitamin D by modifying the concentration of vitamin D-binding proteins that are acute phase reactants (Bratke et al., 2014, Madden et al., 2015).

Therefore, cross-sectional study designs are limited by the reverse causality between TB and DM / vitamin D and quantification of the increased risk of TB attributable to DM based on the available evidence is questionable. The dynamic nature of these diseases calls for different study designs to understand the real burden of the association between TB and DM / vitamin D as well as the long term effect of each disease on the other.

Ideally, a longitudinal population-based study with blood collected for DM screening / vitamin D measurement before the onset of active TB would provide the best evidence. However, such studies would need a very large sample size as the incidence of active TB is rather low. Registry-based studies could be an interesting alternative providing a large sample size, but mostly lack of access to prospective blood to allow DM and Vitamin D screening. So, given the challenges associated with an appropriate cohort or population-based study with prospectively sampled blood, the second best way to evaluate the real dual burden of TB and DM / vitamin D is to conduct a longitudinal study with glycemic and vitamin D measurements after treatment and resolution of active TB as we did in our study. By measuring these parameters after TB has been treated, we assumed that they were the best representation of the situation before the onset of TB. We used post-treatment blood levels as surrogate markers of the baseline situation.

We will discuss below how our longitudinal study has advanced understanding of the correlation between hyperglycemia / low vitamin D and the risk to develop active TB.

7.1.1 Hyperglycemia and risk of active TB and impact on TB outcome

We observed a high frequency of transient hyperglycemia during active TB which highlighted the necessity to repeat DM screening later in the course of TB treatment to confirm DM and understand better the potential impact of DM on the burden of TB. As a result of the normalization of glycemic status during TB treatment, the observed

association between TB and DM at baseline disappeared after TB treatment. So, in cross-sectional studies, the impact of DM on active TB may be overestimated due to capture of stress hyperglycemia. However, detection of stress hyperglycemia was useful in predicting adverse TB outcome.

Most studies evaluating the link between TB and DM have been conducted in settings with low HIV prevalence and only few studies have been conducted in the African region where HIV is the leading cause of TB. Our results suggest that DM may not be a major contributor to TB risk in this African setting. A study conducted in Mwanza, Tanzania showed that DM was a risk factor for TB only in HIV-negative patients.

HIV is contributing to both, high TB and high DM prevalence as these diseases were both more common in HIV-infected individuals. While the contribution of HIV to these diseases independently is well known and was confirmed in our study, we did not observe a synergistic effect of HIV and DM on the risk of TB or the course of TB.

7.1.2 Low vitamin D and risk to develop active TB

We observed that vitamin D was lower among TB patients with underlying DM (assessed as either a previous DM diagnosis or as hyperglycemia measured at follow-up) when compared to TB patients with a normal glycemia at follow-up. Vitamin D level was not affected by baseline hyperglycemia. Furthermore, we identified an increased risk of TB linked to vitamin D insufficiency only in the presence of hyperglycemia at follow-up.

Considering the above, it is easy to understand how cross-sectional studies may miss the importance of DM in the association between TB and vitamin D when only considering the results of baseline glycemic screening at the time of active TB and its diagnosis.

7.1.3 Importance of longitudinal glycemic evaluation in immunological studies

We observed a lower frequency of IFN-γ or IFN-γ/TNF-α $CD4^+$ T cells among diabetic patients with active TB after stimulation with live *M. bovis* BCG whereas in another study using the same methodology, higher frequencies of IFN-γ, IL-2 and TNF-α $CD4^+$ T cells were found in active TB patients with DM compared to non-diabetic TB patients. The definition of DM was very different between studies. Indeed, we included only

patients with previously known DM or persisting DM at the end of TB treatment while the other studies categorized their DM patients based on HbA1c and/or glycemia measurement done at TB diagnosis. They probably included many TB patients with stress hyperglycemia rather than DM. The impact of stress hyperglycemia on TB-specific immune response might be completely different than the impact of preexisting DM. Indeed, stress hyperglycemia results in a new glucose balance that might be beneficial for not insulin dependent tissues, like central and peripheral nervous system, bone marrow, white and red blood cells for which glucose movements is dependent on this concentration gradient. Stress hyperglycemia provides a source of fuel for the immune system and the brain at a time of stress (Marik and Bellomo, 2013). In contrast, in the presence of chronic hyperglycemia, the innate responses have impaired performance with decreased mobilization, chemotaxis, phagocytic activity and cytokine expression (Schuetz et al., 2011). So, the complete DM assessment done in our immunological study allowed to have comparable DM patients within the TB group as well as between the TB and control groups which was a major limitation of previous studies.

Indeed, the recognition of stress hyperglycemia versus DM is of primary importance to identify the immunological mechanisms underlying the link between TB and DM.

7.2 Methods of diabetes screening

According to WHO and the American Diabetes Association (ADA), DM definition is based on three different biomarkers, fasting plasma glucose (FPG), 2-h plasma glucose (2-hPG) in an oral glucose tolerance test and, more recently, HbA1c. As shown in a recent review of data from several world regions, different biomarkers and definitions for DM provide different estimates of DM prevalence (2015).

Each screening test has advantages and disadvantages (Figure 7-1). FPG is a cheap test, widely available and easy to perform. But, the test has to be done in the morning after an 8-hour overnight fast which can be challenging in sick patients. 2-hPG is a more sensitive test. However, it is not convenient to perform and this particularly in very sick people. HbA1c has several advantages over the other tests. Most assays have been standardized by the National Glycohemoglobin Standardization Program

which has improved precision and accuracy of the tests. It is more convenient for the patient as fasting is not required and it can be done at any time during the day. It has less day-to-day perturbations during acute illnesses due to its long half-life. So, we assumed that it would better capture preexisting DM during stress and illness. However, HbA1c is an expensive test which is not affordable and available in many low- and medium-income countries. Point of care HbA1c assays have not been validated for DM diagnostic. Furthermore, it has a lower sensitivity and Hba1c-based definition will not identify a substantial proportion of DM people (2015). HbA1c level may vary with patients ethnicity and HbA1c cut-off for DM diagnosis has not been validated in some part of the world. It might also be difficult to interpret HbA1c result in the presence of anemia and hemoglobinopathies.

Figure 7-1. DM screening tests done during the study a. fasting capillary glucose; b. 2-hour glucose measurement after an oral glucose tolerance test; c. glycated hemoglobin in venous blood.

Most studies assessing the link between TB and DM used one or two different DM screening tests and few evaluated Hba1c, while we used the three screening tests in

parallel which gives a unique opportunity to compare their performance during active TB.

We will discuss below how using the three recommended DM biomarkers has advanced understanding of the optimal DM screening algorithm among TB patients.

7.2.1 Non-overlap of the three screening tests

We observed a very low concordance between tests, primarily due to a high number of Hba1c-only based DM diagnoses among TB patients (Figure 7-2).

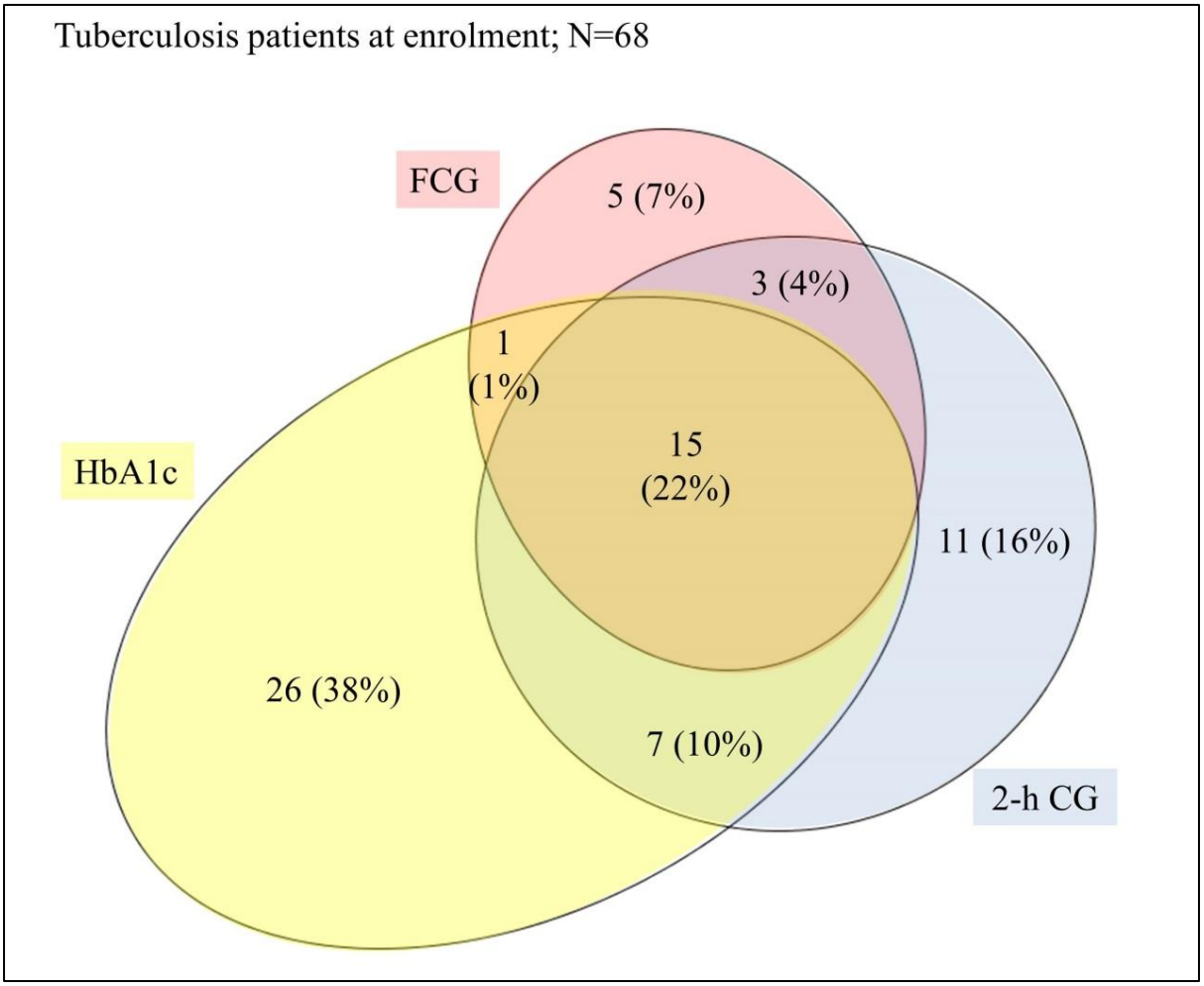

Figure 7-2. Concordance between DM screening tests for DM diagnosis among TB patients at TB diagnostic in the epidemiological study described in Chapter 4.

7.2.2 Performance of glycated hemoglobin

Surprisingly, HbA1c did not perform better than other screening tests in capturing preexisting DM. The use of Hba1c for DM diagnosis was never validated in East Africa, a setting with a high prevalence of hemoglobinopathies and infectious diseases. Patients with active TB have a high prevalence of anemia which probably affects the reliability of the assay. Moreover, TB has unique characteristics as most of the patients have been sick for several months before diagnosis and the belief that preexisting underlying DM can be identified by Hba1c which is not affected by acute disease is wrong. This was also shown in a study conducted in Iran and confirmed by our findings (Tabarsi et al., 2014).

HbA1c performed less well than fasting glucose to identify TB patients who will have an adverse outcome during TB treatment. Therefore, fasting glucose seems to be the optimal DM screening test to identify stress hyperglcycemia and potential associated adverse TB outcome.

7.2.3 Repeated testing

Most studies on the association between TB and DM based their DM diagnosis on a single unrepeated screening test value. We confirmed every elevated result by repeated testing as recommended by ADA (ADA, 2014) and observed a high intra-individual variability particularly for FCG and 2-hCG (Figure 7-3). Part of this variability can probably be explained by an inappropriate fasting state.

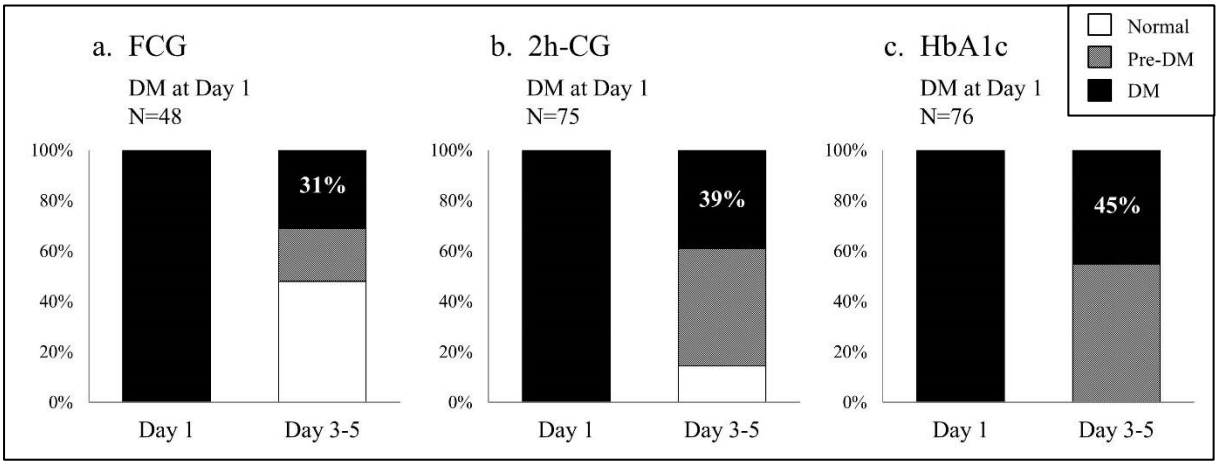

Figure 7-3. Intra-individual variability of repeated DM screening tests among TB patients at TB diagnostic in the epidemiological study described in Chapter 4.

These results highlight the need for repeated testing particularly during an acute disease making fasting difficult. They show the potential overdiagnosis of hyperglycemia in the absence of diagnosis confirmation.

7.2.4 Impact for the immunological study

During our immunological study, the TB group with DM consisted of patients having the three confirmed DM screening tests in the DM range. This allowed selecting patients with underlying DM as it was confirmed during follow-up. We discussed above the importance of DM definition as the expected *Mtb*-specific immune response is probably highly different in the presence of chronic underlying DM and stress hyperglycemia.

7.3 DM management in TB patients

We observed that fasting hyperglycemia was associated with poor TB outcome. Therefore, management of DM during TB treatment might be beneficial. However, all studies examining the effect of hyperglycemia management have been conducted in intensive care units. In this setting, short-term glycemic control with insulin has been linked to better outcome in septic patients, but only when targeting non intensive glycemic control and therefore avoiding the occurrence of hypoglycemia (Dungan et al., 2009). This evidence indirectly suggests that short-term management of glycemia might also improve TB outcome. However, the optimal glucose cutoff for treatment introduction and the appropriate drug to be used in the outpatient setting have to be defined. Important considerations about the management of hyperglycemia among TB patients are that we are dealing with outpatients having limited glucose monitoring and having an unpredictable nutritional intake which hamper the use of insulin. In this setting, metformin, the most prescribed drug for type 2 DM worldwide, might be a good choice. It has the advantage to reduce blood glucose without inducing hypoglycemia. Furthermore, metformin is currently being investigated for novel applications as host-directed therapy for TB (Pryor and Cabreiro, 2015). Our immunological study suggests that DM delays the priming of T-cell mediated immunity by affecting antigen processing and presentation, a defect that may be specifically compensated by metformin.

The optimal glycemic cut-off to decide on hypoglycemic treatment and the benefit of such treatments is controversial. The long term DM risk among patients with transient hyperglycemia is unknown. However, detection of hyperglycemia is an opportunity to raise awareness of lifestyle influence on DM risk.

8 Recommendations

We will now discuss the potential impact of our results on a change in medical practice and how NCDs programs can learn from communicable diseases in low- and middle-income countries.

Recommendations for policy and care

- Integration of care between TB and DM programs
- Screen TB patients for DM with fasting glycemia at TB diagnosis
- Treat hyperglycemia among TB cases to improve TB outcome
- Confirm DM at the end of TB treatment to decide on long-term DM treatment
- Screen DM patients for TB

8.1 Integration of care between TB and DM programs

The new post-2015 Global TB Strategy aims to end the global TB epidemic by 2035. To reach this target, there is a need to accelerate the reduction in TB incidence. Given the accepted link between TB and DM (increased TB risk and adverse TB outcome) and the expected sharp rise in DM during the next decade, it is important to include DM in strategic plans to control TB. TB programs could also be used to raise awareness of the importance of healthy diet and physical activity in case of hyperglycemia. So, there is a need to develop interactions, synergies and integration of care between TB and NCDs. These synergies and care integrations can be done at low cost through educational tools. These measures are most pressing in low- and middle-income countries, precisely those which are least prepared to confront this huge-scale DM pandemic. The link between TB and DM and the implementation of the collaborative framework for care and control have the potential to stimulate and strengthen the scale-up of NCD care and prevention programs, which may help in reducing not only the global burden of DM but also the global burden of TB.

- Integration of care between TB and DM programs should start without delay and address NCDs prevention and treatment in TB clinics as well as education about signs and symptoms of TB in diabetic clinics. This could help to reduce the burden of both communicable and non-communicable diseases.

8.2 DM screening algorithm among TB patients

Fasting hyperglycemia is very common in TB patients and is associated with an adverse outcome, but, in most of them, the glucose status is normalized with TB treatment. In most countries, there is a high yield from DM screening among TB patients at TB diagnosis (Harries et al., 2015). The yield is particularly high in older TB patients (>40 years) living in an urban setting and programs could chose to target higher risk patients according to their local epidemiology. DM screening at TB diagnosis gives the opportunity first, to identify patients at higher risk of adverse TB outcome and second, to manage hyperglycemia in these patients. However, DM needs confirmation later during TB treatment because of the high prevalence of transient hyperglycemia and the poor performance of HbA1c to detect preexisting DM.

- Systematic fasting glucose screening of patients at the start of anti-TB treatment should be implemented.
- In case of hyperglycemia, it should be treated to improve TB outcome.
- Because of the high rate of transient hyperglycemia, DM should be confirmed at the end of TB treatment.
- If DM diagnosis is confirmed, long-term DM treatment should be introduced and counselling about lifestyle modifications should be done.

8.3 TB screening in DM clinics

Systematic TB screening among diabetic people should be considered only in high TB burden countries because of very high number of diabetic patients needed to screen to detect one TB case in other settings. A recent Danish longitudinal study showed that the risk of TB drastically decrease within the first two years after diagnosis of DM (Kamper-Jorgensen et al., 2015). This raises some concern in parallel to our own observations, namely that at least some of the DM/TB association may be the result of

transient hyperglycemia rather than underlying DM. Therefore, at least in the African setting that we focused on, screening all DM patients for TB with expensive tests is not justified. But a simple and inexpensive TB screening method is to implement an education programme for care givers and patients to understand the increased TB risk and recognize early TB symptoms.

- Systematic TB symptoms screening of DM patients should be done and DM patients should be able to recognize early signs and symptoms of TB.

9 Future research

Recommendations for future research

- Large cohorts of DM patients to follow-up for the incidence of TB
- Cohorts of TB patients to follow-up for the long-term risk of DM after transient hyperglycemia
- Randomized control trials to evaluate the optimal treatment of hyperglycemia during TB
- Randomized control trials to evaluate the benefit of vitamin D supplementation to prevent TB among DM patients
- Immunological studies including TB patients with DM and with transient hyperglycemia
- Studies on the role of DM in other infections

9.1 Large DM and TB cohorts

The interaction between TB and DM is a dynamic process leading to difficult interpretation of cross-sectional data on the causal association between the two diseases. The dynamic nature of these diseases calls for appropriate longitudinal study design such as cohorts. Such studies will help to understand the relevance of dual or triple disease burden on the global burden of diseases. The integration of such data in the prediction models of the Global Burden of Disease rather than assessing the contribution of single diseases will improve the accuracy and reliability of the models.

Cohort studies can also address the long term risk of DM among people having experienced transient stress hyperglycemia. Indeed, by analogy with gestational DM, the presence of transient hyperglycemia might identify people at higher risk of developing type 2 DM.

9.2 Randomized control trials to evaluate the optimal treatment of hyperglycemia during TB

There is an urgent need to bring evidence to the management of comorbid patients. Intervention studies addressing the optimal glycemic cut-off for hypoglycemic treatment start as well as the choice of the appropriate treatment should be performed.

The clinical benefits of adjunctive therapy with metformin together with regular TB treatment regimen were observed in two retrospective cohorts (Singh et al., 1984). The use of metformin adjunct therapy undoubtedly warrants further investigation. The therapeutic potential of metformin treatment extends far beyond its use as an anti-hyperglycemic drug. It may be useful as a host-directed therapy for TB. Host-directed therapies include drugs that target the host and not the pathogen. They modulate the protective innate and adaptive immunity, reduce excess inflammation, repair or prevent tissue damage or enhance the effectiveness of TB drug therapy by modulating host factors (Hawn et al., 2015, Zumla and Maeurer, 2015). A key virulence mechanism of *Mtb* is its capacity to halt phagosome maturation in macrophages enabling an increase in lung bacterial load until adaptive immunity has been primed. Innate immune cells such as macrophages and dendritic cells play key roles in sensing infection, orchestrating adaptive immune priming and expressing enhanced antimicrobial functions once activated or targeted for cytolysis by antigen-specific T cells. Given the diversity of diabetic complications and the numerous pathways involved, the immune response to *Mtb* infection is probably affected at different levels (Martinez and Kornfeld, 2014). Data from mice suggested an impaired function of antigen presenting cells to initial infection with a resulting delay in the adaptive immune effector response as a key mechanism of susceptibility (Vallerskog et al., 2010). This is consistent with our data which suggest that DM may not quantitatively affect T-cell mediated immunity in TB patients, but would rather delay its priming by affecting antigen processing and presentation. Metformin which enhances macrophage autophagy by promoting phagolysosome fusion and increasing mitochondrial ROS production may specifically compensate for the observed defect in antigen presenting cells (Singhal et al., 2014).

9.3 Randomized control trials to evaluate vitamin D on TB outcome and for TB prevention in DM patients

Vitamin D is another candidate for host-directed therapy. It boosts the antimicrobial activity of human macrophages against *Mtb*. Vitamin D stimulates the vitamin D receptor to induce cathelicidin expression which has both immunoregulatory and direct antimicrobial activity (Hawn et al., 2015).

Randomized control trials using vitamin D as adjunct anti-TB therapy did not provide detectable improvement in clinical outcome or mortality among patients with TB. Patients with DM who have a lower vitamin D level might be an appropriate target for a personalized approach for vitamin D supplementation to improve TB outcome.

On the other hand, vitamin D supplementation is maybe more effective in prevention than treatment (Davies and Martineau, 2015). There is an ongoing trial testing the effectiveness of vitamin D in preventing pulmonary TB among HIV infected individual in Tanzania. Trials of vitamin D to prevent active disease in other high risk groups like people with DM are lacking and would provide interesting results.

9.4 Immunological studies including TB patients with DM and with transient hyperglycemia

For a better understanding of the immunological characteristics of patients with TB and DM, it will be important in future studies to differentiate patients with DM from those with transient hyperglycemia who will potentially not be affected in the same way.

9.5 Studies on the role of DM in other infections

The rising burden of DM in low- and middle-income countries might have a negative impact on other infections. The role of stress hyperglycemia and DM should be studied in other infections. Thanks to the expertise acquired during this PhD, I could bring my knowhow into a study on DM and dengue. Interestingly, we observed that DM was associated with an increased risk for a severe clinical presentation of dengue (Annex 1).

10 **Conclusions**

In this sub-Saharan African setting, HIV seems to be a stronger contributor to TB than DM when looking at the impact on the risk of active TB. However, screening for hyperglycemia at TB diagnosis is still relevant, particularly in HIV positive patients, to identify patients at risk of adverse TB outcome who would benefit from appropriate management of hyperglycemia. As glycemic status is normalized with TB treatment in most patients, DM needs confirmation after TB treatment. The present work highlighted the dynamics of glycemic status during TB and the usefulness to screen patients at different time points during their disease.

This work also provides interesting information on the association between low level of vitamin D, TB and DM. Low level of vitamin D seams to increase the risk of TB only if associated with hyperglycemia. These data move towards a potential use of vitamin D supplementation in a targeted diabetic population to prevent the development of active TB.

Furthermore, the findings of the immunological work give new insights in the mechanisms underlying the association between TB and DM. These data indicate that DM affects live *M. bovis* BCG-specific $CD4^+$ T cell responses through negative interaction with antigen processing or presentation events. This undoubtedly warrants further investigation notably in the context of Metformin adjunct therapy which may specifically compensate for this defect.

This observational work was the first step in a better understanding of the interaction between TB and DM. It is now time to move to the improvement of the management of these comorbid patients, to integration between TB and DM programs, and to the setup of intervention studies.

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12 Annex 1 - Is diabetes a risk factor for a severe clinical presentation of dengue? - Review and meta-analysis

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Author Summary

Both, dengue and diabetes have reached epidemic dimensions and pose a joint threat to a large proportion of populations in low- and middle-income countries. Dengue is no longer a disease primarily affecting children. Therefore the influence of non-communicable diseases such as diabetes, which are increasingly prevalent in adults, on the clinical presentation of a dengue episode becomes a public health priority. We conducted a systematic literature review to assess the available evidence on the effect of diabetes mellitus (DM) on the clinical presentation of dengue. The meta-analysis of published evidence combined with supporting biological evidence point to an increased risk for potentially life threatening symptoms of dengue among patients with diabetes. The current evidence is limited by statistical power and other study limitations and does not allow conclusions about a causal effect of diabetes. Yet, based on the currently available evidence, diabetes patients with fever and living in a dengue endemic region should seek confirmation of dengue infection as early as possible. Diabetes should be considered in the triage of patients for close observation and early intervention, which are challenges, particularly during dengue outbreaks. Timeliness of intervention is the most important factor averting serious complications and death in patients with acute dengue.

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Abstract

Background

The mean age of acute dengue has undergone a shift towards older ages. This fact points towards the relevance of assessing the influence of age-related comorbidities, such as diabetes on the clinical presentation of dengue episodes. Identification of factors associated with a severe presentation is of high relevance, because timely treatment is the most important intervention to avert complications and death. This review summarizes and evaluates the published evidence on the association between diabetes and the risk of a severe clinical presentation of dengue.

Methodology/Findings

A systematic literature review was conducted using the MEDLINE database to access any relevant association between dengue and diabetes. Five case-control studies (4 hospital-based, 1 population-based) compared the prevalence of diabetes (self-reported or abstracted from medical records) of persons with dengue (acute or past; controls) and patients with severe clinical manifestations. All except one study were conducted before 2009 and all studies collected information towards WHO 1997 classification system. The reported odds ratios were formally summarized by random-effects meta-analyses. A diagnosis of diabetes was associated with an increased risk for a severe clinical presentation of dengue (OR 1.75; 95% CI: 1.08-2.84, p =0.022).

Conclusions/Significance

Large prospective studies that systematically and objectively obtain relevant signs and symptoms of dengue fever episodes as well as of hyperglycemia in the past, and at the time of dengue diagnosis, are needed to properly address the effect of diabetes on the clinical presentation of an acute dengue fever episode. The currently available epidemiological evidence is very limited and only suggestive. The increasing global prevalence of both, dengue and diabetes, justify further studies. At this point, confirmation of dengue infection as early as possible in diabetes patients with fever if living in dengue endemic regions seems justified. The presence of this co-morbidity may warrant closer observation for glycemic

control and adapted fluid management to diminish the risk for a severe clinical presentation of dengue.

Introduction

With low- and middle-income countries (LMIC) experiencing a growing chronic non-communicable disease (NCD) burden and a continuously high communicable disease (CD) incidence rate, understanding the co-morbidity between the two disease groups is necessary to properly assess, monitor, evaluate, and control their prevalence (Bygbjerg, 2012, Remais et al., 2013). Cardiovascular diseases and their risk factors including diabetes mellitus (DM) are major contributors to the growing NCD burden (Murray et al., 2012). WHO projects that DM will be the 7th leading cause of death in 2030. Today there are 347 million people worldwide who have DM (Danaei et al., 2011), around 90% of them type 2 DM (WHO, 2014b). More than 80% of DM deaths occur in LMIC(WHO, 2014b). In high income countries DM has long been known for its association with increased susceptibility to infections such as tuberculosis (Martinez and Kornfeld, 2014). Although these associations have been attributed in part to DM associated alterations in innate immunity, related evidence is inconsistent and underlying mechanisms remain poorly understood. They are likely to vary by type of infection (Knapp, 2013). Yet, only few studies investigated the complex associations of diabetes with neglected tropical diseases (NTDs).

Dengue, one of 17 diseases assigned NTD status by WHO, is next to malaria the most important arthropo-borne (ARBO) tropical infection caused by the dengue virus. It is transmitted by several mosquito species within the genus *Aedes*, principally *A. aegypti* (Gubler, 1998). The number of Dengue virus infections has increased 30fold over the last decades. Today it is a major public health problem in tropical and subtropical regions (Who, 2014a). The absence of adequate public health awareness, surveillance and control, population growth, globalization and urbanization contributed to this increase. An estimated 2.5 billion people are at risk of infection in over 100 endemic countries(CDC, 2012). WHO estimates that currently between 50 and 100 million dengue infections occur annually. An estimated 500’000 dengue patients with potentially life threatening symptoms require hospitalization each year and about 2.5% of those affected die (WHO, 2014c). Dengue ranges from asymptomatic or self-limiting non-severe dengue (with or without warning symptoms) to severe dengue, characterized variously by

severe plasma leakage, severe bleeding or severe organ involvement (WHO, 2009). The studies reviewed in this paper were all except one conducted before 2009 and therefore routine clinical data was collected according to the WHO 1997 guidelines. These guidelines group symptomatic dengue virus infections into three clinical categories: undifferentiated fever, dengue fever (DF), and dengue hemorrhagic fever (DHF). DHF is further subclassified into four severity grades, with grades III and IV being defined as dengue shock syndrome (DSS) (WHO, 1997). The clinical presentation of a dengue infection is difficult to predict, although the presence of warning signs occurring within 3 to 7 days after the first symptoms warrant strict observation and medical intervention. Intervention, including intravenous rehydration as the therapy of choice, can reduce case fatality in severe dengue to less than 1 % (Who, 2012). Dengue has long been viewed as a pediatric disease, but the average age of dengue cases has been rising and there is a suggestion for adults to be at increased risk for dying from dengue. The increase of tourism in tropical regions also contributed to the increase in adult dengue cases (Tantawichien, 2012) .

There are five dengue virus (DENV) serotypes (DENV-1, DENV-2, DENV-3, DENV-4 and DENV-5), however, serotype 5 seems not to have a sustained transmission cycle in humans (Normile, 2013). Infection confers immunity to the infecting serotype which is life-long, but not to the remaining three. Subsequent infections with a different dengue virus serotype increase the risk of severe complications (Ooi et al., 2006). Other than that, factors increasing the risk of severe clinical manifestations remain poorly characterized (Cunha et al., 1999, Kouri et al., 1989, Malik et al., 2012). Present evidence suggests that beyond viral factors age, gender, social status, genetic background, sickle cell anemia, uremia, bronchial asthma, allergies, hypertension, chronic renal failure and also DM might adversely influence the clinical presentation of an infection (Cunha et al., 1999, Huy et al., 2013, Khor et al., 2011, Kouri et al., 1989, Silva et al., 2010, Thomas et al., 2010).

In acknowledging the shift of dengue to older ages and the steep increase in the prevalence of DM, the objective of this review is to access the current clinical and

epidemiological evidence for this NCD to contribute to a higher risk of a severe clinical presentation in dengue fever patients.

Methods

Search strategy

We conducted a systematic literature review using MEDLINE database to access any relevant publication describing an association between dengue and diabetes up to February 28 2014. The search terms used were “("dengue"[MeSH Terms] OR "dengue"[All Fields]) AND ("diabetes mellitus"[MeSH Terms] OR ("diabetes"[All Fields] AND "mellitus"[All Fields]) OR "diabetes mellitus"[All Fields] OR "diabetes"[All Fields]”.

Inclusion criteria

We included articles in all languages; articles which reported on epidemiology, clinical signs, and laboratory parameters for dengue-infected patients, or on severity assessment. There was no restriction in publication dates, place of study, study design or age of research participants. As a validity assessment, the PRISMA criteria were used (2009, David Moher, 2009).

For meta-analyses, we included studies that compared the prevalence of DM between persons affected by different dengue stages (case-control studies), reporting estimates of association and their 95% confidence intervals, or enough information to derive this.

Data extraction

We extracted the year during which dengue cases were diagnosed (year during which the study was conducted), year of the publication, country of the study, study design, study definitions of dengue infection and diabetes, and confounder adjustments. We extracted data on the sample size of enrolled persons and number of cases and controls and on the estimates (unadjusted and adjusted models) of the association (and their 95% confidence intervals) between diabetes and severe dengue. The transition of the patients data from clinical or hospital records to this review was based on published non-individual and non-identifying

data. Data were extracted from the published papers independently by two reviewers and disagreements were resolved by discussion.

Case Definitions

Dengue

The studies included in this review mostly included dengue diagnosed before 2009 and applied the WHO 1997 dengue classification criteria (WHO, 1997). A 2009 classification was proposed by the WHO/TDR group and in 2011 the WHO/SEARO group also suggested modifications (Who, 2012). We list the classification system applied in the respective studies in Table 12-1, which summarizes the available evidence. Control status and case status are predominantly defined as DF and as DHF or DSS respectively. Cases of DHF/DSS in the studies reviewed were hospitalized cases or deaths confirmed serologically, clinically with hemorrhagic manifestations or radiographically with a certain extent of plasma leakage. According to WHO 1997 classification, DHF is clinically subdivided into grades I-IV and all 4 grades could have some degree of at least subclinical plasma leakage. Grade I is the presence of fever with positive tourniquet test, grade II presents with spontaneous bleeding into the skin and elsewhere, grade III shows clinical signs of shock or circulatory failure, and grade IV presents with severe shock with undetectable blood pressure and pulse. Grade III and IV have also been labeled as “dengue shock syndrome” (DSS). The WHO 2009 classification differs from the 1997 classification scheme in that dengue cases are classified by the levels of severity with or without warning signs. Several clinical features specifically listed in the WHO 2009 classification were not directly obtained in the reviewed studies, e.g. clinical features such as lethargy, restlessness, and severe organ impairment or failure. We refer to DHF/DSS as “severe clinical presentation of dengue”.

In the reviewed publications, laboratory diagnosis methods to confirm dengue virus infection as the cause of disease varied across studies and involved detection of virus, viral nucleic acid, antigens or antibodies, or a combination of these techniques (WHO, 2009). The detection of virus, nucleic acid or antigen in the blood is confirmatory for an acute dengue infection. Antibody response to infection

differs by the host immune status. In the setting of a primary infection, IgM are the first antibodies to appear, but it takes 10 days for them to be detectable in 99% of patients. During a secondary dengue infection, anti-dengue IgG are detectable at high levels already in the acute phase, whereas IgM levels remain significantly lower than in primary dengue infection. A four-fold or greater increase in IgG antibody levels in paired sera (sample collected during the acute stage of illness and sample collected in the convalescent stage) indicates acute dengue (WHO, 2009). Identification of virus/viral RNA/viral antigen and antibody response are ideally combined for the confirmation of acute dengue. Yet, as specimen collection and processing is easier for serology, the laboratory methods applied in the studies mostly conducted in LMIC varied broadly. We list the laboratory confirmation methods applied in the respective studies in Table 12-1, together with the available evidence.

Diabetes Mellitus

In studies included in this review, the diagnosis of DM was mostly ascertained by self-reported diagnosis in the context of in-person interviews or DM diagnosis listed in clinical records. The DM diagnosis was further verified by requesting the prescription and/or medication package during the in-person interview in the only population-based case-control study (Figueiredo et al., 2010). An oral glucose tolerance test was performed in dengue patients in the case series published by Hasanat study (Hasanat et al., 2010).

Meta-analysis

We used random-effects models for meta-analyses of the association between DM and a severe clinical presentation of dengue (Lau et al., 1997). They take into consideration the variation between the true effects estimated by included studies unlike fixed effect models which assume a common true effect across studies. We used odds ratios as measure of association across all studies. We used the estimates reported by the authors as “primary model” and the I^2 metric and Tau^2 to describe the between study heterogeneity and variance respectively.

We conducted sensitivity analyses by using a fixed effect model and excluding a study, which was based on non-matched random controls and that only reported

unadjusted estimates (Karunakaran et al., 2014). We performed analyses with Stata version 12 (Stata Corporation, Texas) and considered $p<0.05$ as statistically significant.

Results

Our literature search resulted in 32 hits (Fig. 12-1). After excluding duplicates and non-relevant articles based on a full text analysis (single case reports, outbreak investigation reports, no specific factors studied, narrative overview of dengue infections in a specific country or globally), ten articles were retained. Among them, five studies were case-control studies (Karunakaran et al., 2014, Mahmood et al., 2013, Pang et al., 2012, Min-Sheng Lee, 2006) with one study being population-based (Figueiredo et al., 2010). The case-control studies compared the prevalence of DM in persons with acute or past non severe dengue (controls) to that in persons with an acute severe clinical presentation of dengue. Five additional articles (case series) characterized DM in mostly dengue patients with severe clinical manifestations, including fatal cases (Hasanat et al., 2010, Lahiri et al., 2008, Lye et al., 2010, Sam et al., 2013, Wieten et al., 2012). These studies lack a comparison group with acute or past non severe of dengue, and are therefore not included in the meta-analysis.

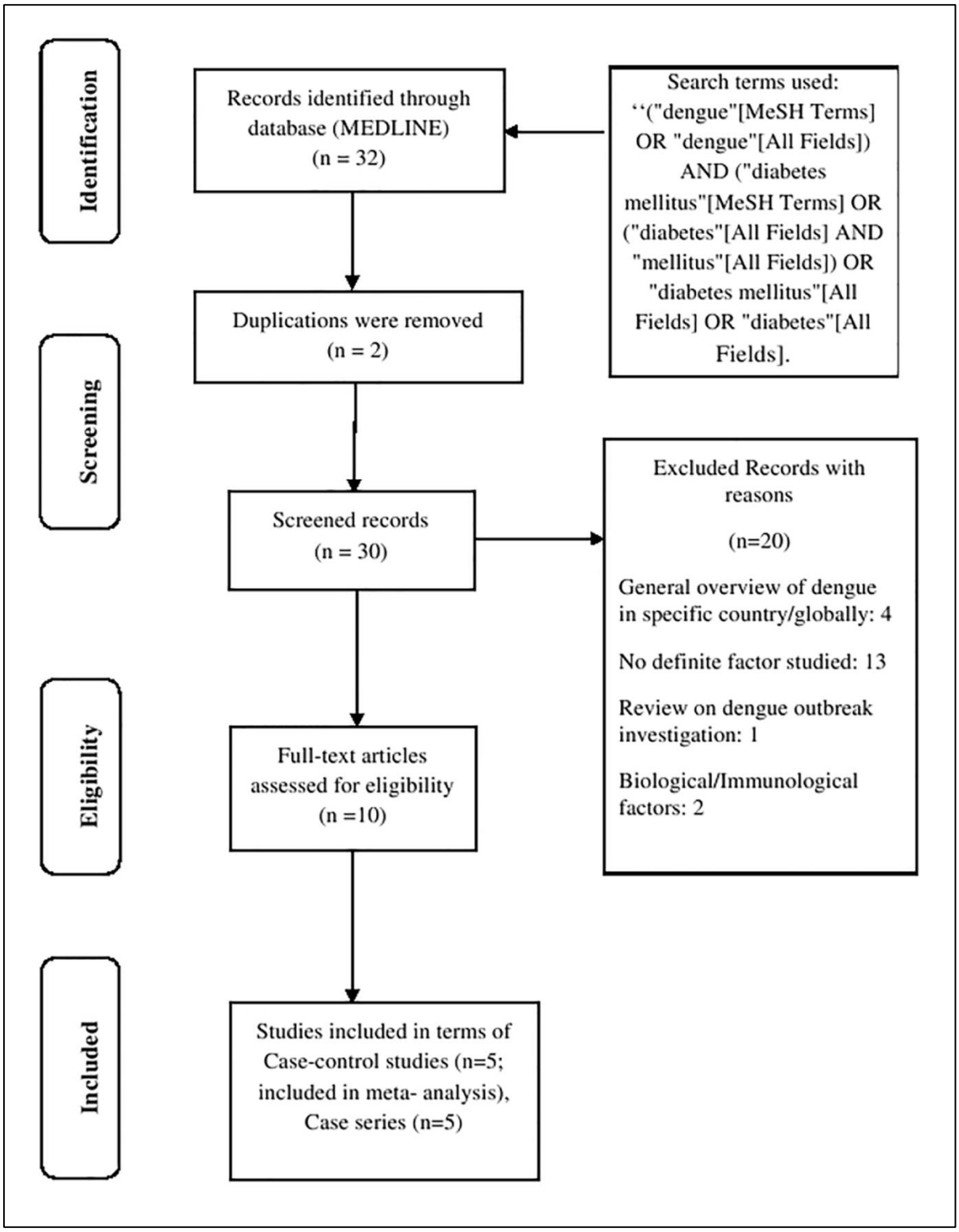


Figure 12-1. PRISMA Flow diagram of diabetes and dengue

Epidemiological evidence from case-control studies on the association between DM and a severe clinical presentation of dengue

Epidemiological studies included in the meta-analysis compared the prevalence of DM and other co-morbidities between patients suffering from acute dengue with a severe presentation and controls with acute or past dengue without severe clinical manifestations (Table 12-1). In the absence of controls without evidence for a dengue infection history, studies thereby compared the prevalence of DM in dengue patients with different degrees for severity in clinical presentation, rather than the risk of being infected with dengue virus.

The only population-based case-control study (Figueiredo et al., 2010) identified was conducted in two Brazilian cities and included both, children and adults. DHF cases were ascertained through the national surveillance system. The surveillance records were reviewed by two physicians. DHF was defined according to criteria used by Brazilian Healthy System, which were very close to WHO 1997 criteria. Controls were selected from the same neighborhood as cases. In addition, they were tested positive for anti-dengue IgG and matched to cases by age, sex and a report of a past dengue-like fever in the same year as the DHF diagnosis of cases. Additional information from both, cases and controls was obtained through in-person interviews. DM was based on a self-reported physician-diagnosis. Interviewers also asked for medication intake and verified it by seeing the prescription of packaging. DM was statistically significantly associated with DHF independent of age, sex skin color, income and educational level (aOR 2.75, 95% CI 1.12-6.73). The association of self-reported diabetes with DHF was stronger in diabetic patients being treated, especially if treated with insulin or more than 1 drug (aOR 3.36; 95% CI 0.72-15.61). In addition, white ethnicity (aOR 4.70, 95% CI 2.17-10.2), high income (aOR 6.84, 95% CI 4.09-11.43), high educational level (aOR 4.67, 95% CI 4.09-11.43) and a self-report of allergy treated with steroids (aOR 2.94, 95% CI 1.01-8.54) were also associated with a more severe clinical presentation of dengue.

A hospital-based study including persons aged 15 to 65 in Pakistan (Mahmood et al., 2013) compared hospitalized acute DHF patients (cases) to dengue IgG positive patients, but hospitalized for unrelated conditions (controls). The study was

conducted in two major tertiary care hospitals. A DHF case was defined as diagnosis by an experienced clinician applying WHO criteria, although the version applied was not specified. The categorization into persons with and without DHF points to the use of WHO 1997 criteria. Information was obtained through structured record review and in-person interview. Age- and sex-matched DHF cases were slightly more likely to report a DM diagnosis than control patients. In both groups, the reported prevalence of DM was exceptionally high, 41.8% in controls and 43.2% in cases. The association of DM with DHF adjusted for age, sex and duration of illness was statistically non-significant (aOR 1.26, 95% CI 0.78-2.03, p= 0.34).

In Singapore, a hospital-based study was conducted in the nation's largest clinic. It included all admitted patients with acute dengue without age restriction (Pang et al., 2012). Information on case and control status as well as comorbidities was exclusively derived from abstracting medical charts. Cases were defined as DHF patients, and controls were DF patients. Probable dengue patients had a positive acute dengue serology. Confirmed dengue patients had positive dengue polymerase chain reaction assays. Clinical diagnosis for DHF was based on WHO 1997 criteria. The data was analyzed separately for the two epidemic periods of 2006 (predominantly DENV-1) and 2007/2008 (predominantly DENV-2; larger sample size). No association between DM status and DHF was found in the 2006 dengue outbreak. DM was independently associated with DHF in the 2007/8 epidemic (aOR 1.78, 95% CI 1.06-2.97). The association was stronger if the diabetic patients additionally had hypertension (aOR 2.16, 95% CI 1.18-3.96) or asthma (aOR 4.38, 95% CI 0.80-23.85). Mean hospitalization days were longer for DM (4.99±3.34days) as compared to non-DM patients (4.04±1.62 days, p =0.001). Additional factors associated with DHF were Chinese ethnicity (compared to Malay or Indian ethnicity) (aOR 1.90, 95% CI 1.01-3.56 in 2006 epidemic periods and aOR 1.67, 95% CI 1.24-2.24 in 2007/2008 epidemic periods) as well as middle age in 2007/2008 (aOR 1.41, 95% CI 1.09-1.81 in 30-39 years and aOR 1.34, 95%CI 1.09-1.81 in 40-49 years of age group).

In 2002 in Taiwan, all patients with confirmed acute DF treated at the Kaoshiung Medical University Hospital during a large outbreak occurring in the southern

Taiwan were categorized into groups of DF and DHF/DSS by strictly adhering to clinical WHO 1997 criteria and laboratory confirmation under the auspices of the Taiwanese Centre of Disease Control (Min-Sheng Lee, 2006). Clinical information such as signs and symptoms and the results of blood investigation were abstracted from medical records. Cases were mostly adults, only 4.5% were below age 15 years. The prevalence of DM was 16.8% in DHF/DSS cases compared to 7.6% in DF patients (controls). The adjusted OR reported was 1.86 (95%CI 1.04-3.37), albeit covariates were not reported. In addition to the independent association of DHF/DSS with DM, statistically significant associations with hypertension and renal insufficiency, uremia, past history of dengue infection as well as male gender and older age were also found.

A hospital-based study in Southern India (Karunakaran et al., 2014) obtained information on patients admitted to the largest multi-specialty hospital in South Kerala for acute dengue between 2005 and 2008. The case group consisted of 10 in-hospital deaths of patients admitted with a clinical diagnosis of probable dengue, which was confirmed by either RT-PCR or IgM antibody tests, and review of clinical symptoms through medical record review. Forty non-matched controls were randomly selected among patients with a confirmed acute dengue, but recovering from the illness. The classification of dengue among the controls was not specified. Information on co-morbidities and other factors was abstracted from medical records. The prevalence of DM in controls was 2.5% compared to 40% in cases. DM was a strong predictor of mortality in the bivariate analysis (OR 26.0, 95% CI 2.47-273.67, p =0.004). In the same study, hypertension was also a strong predictor of mortality (OR 44.3, 95% CI 6.2-315.5, p= 0.000). Mortality was much higher in patients over 40 years (OR 9.3, 95% CI 1.9-44.4, p= 0.002). No adjusted odds ratios, which would facilitate the interpretation of independency in the reported associations, were reported.

Table 12-1. Summary of case-control studies and case series.

First author and publication date	Country & Year of study	Study design	Characteristics of Study population	Results(Odds ratios (OR) and 95% confidence intervals)
Epidemiological case-control studies				
Figueiredo et al.(2010)	Brazil, 2002-2003 (Salvador), 2003-2005 (Fortaleza)	Population-based case-control study	**Cases:**170 acute DHF	Predominantly DENV-3,Association DM – acute DHF vs. asymptomatic (IgG) (+)ve controls Adjusted OR (age, sex,income, neighborhood, skin colour, education)
			-registered in the national surveillance systems	DM yes vs. no.: aOR 2.75 (1.12-6.73)
			-residents of 2 cities	DM according to the number of medications: no medication vs. no DM :aOR 1.83 (0.18-18.67)
			-no age restriction	1 medicine vs. no DM: aOR 2.72 (0.86-8.60)
			-diagnostic criteria:	Insulin/ >1 medicine vs. no DM:aOR 3.36 (0.72-15.61)
			surveillance record review by 2 physicians;	DM prevalence: controls:2.6%,cases:5.3%
			Brazilian Health Service (very similar to WHO 1997) criteria;	
			fever & positive serology for anti-dengue IgM and/or viral isolation and characterization;	
			at least two signs or symptoms of dengue fever (headache or retroorbital pain, myalgia, arthralgia, prostration, exanthema);	
			all of the following signs: hemorrhagic manifestations, hemoconcentration with an increased haematocrit level; thrombocytopenia;	
			no consideration of ascites or pleural effusion (rarely recorded)	
			Controls: 1175	

			-neighborhood controls	
			-sero-positive (anti-dengue IgG)	
			-self-report of dengue like illness in same year as matched case	
			-no history of DHF	
			-matched for age and sex (within 5 years)	
			Information on dengue history, confounders, effect modifiers & comorbidities: in-person interviews with cases and controls	
			DM: self-report of physician diagnosis and verification of prescription /packaging of medication	
Mahmood et al (2013)	Pakistan (2011)	Hospital-based case-control study	**Cases: 132** acute DHF	Association DM – acute DHF vs. asymptomatic IgG positive controls: Adjusted OR (sex, age, duration of illness)
			-admitted to two major tertiary care hospitals of Lahore	DM yes vs. no: aOR 1.26 (0.78-2.03), p=0.34
			-age 15-65	DM according to its duration:
			-diagnostic criteria:	5-10 vs. <5 yrs aOR 2.76(0.77-9.84),p=0.11
			diagnosed as DHF by a trained clinician;	>10 vs. <5 yrs:aOR 1.86 (0.55-6.26),p=0.31
			WHO criteria (version not specified)	DM prevalence: controls: 42% ,cases:43%
			Controls: 249 patients without acute dengue	
			-random sample of patients from same health facilities admitted for reasons other than dengue;	
			-positive for anti-dengue IgG;	
			-matched for age and sex (within 5 years);	

			-Information on dengue history, confounders, effect modifiers & co-moribities: in-person interviews with cases and controls; checklist for clinical record review	
			DM: self-report or clinical record	
Pang et al (2010)	Singapore (2006-2008)	Hospital-based case-control study	2006 epidemic: Cases:149 acute DHF, Controls:326 acute DF	2006 epidemic: predominantly DENV-1
			2007/2008 epidemic: Cases:590 acute DHF, Controls:1141 acute DF	27.6% of patients PCR (+)ve
			-admitted to the largest hospital of Singapore for dengue	72.4% of patients sero-positive & PCR(-)ve
			-adult	Association DM- acute DHF vs. acuteDF: Adjusted OR (age, ethnicity)
			-diagnostic criteria:	DM yes vs. no: aOR 0.34 (0.06-1.89)
			probable DF: positive acute dengue serology (Dengue Duo IgM & IgG Rapid Strip Test) and clinical criteria of DF by WHO1997;	DM prevalence: controls: 2.2% ,cases: 1.3%
			confirmed DF: positive dengue PCR and clinical criteria of DF by WHO 1997	2007/8 epidemic: predominantly DENV-2
			-**DHF**: presence of all four criteria of fever, hemorrhagic manifestiations, thrombocytopenia, plasma leakage	32.6% of patients PCR positive
			Information on dengue history, confounders, effect modifiers & comoribities: clinical record review	67.4% of patients sero-positive & PCR (-)ve
			DM: clinical records	Association DM- acute DHF vs. acute DF: aOR (age, ethnicity, gender, hypertension)
				DM yes vs. no: aOR 1.78 (1.06-2.97)
				DM with hypertension: aOR 2.16 (1.18-3.96)
				DM with hyperlipidemia:aOR 1.62 (0.90-2.92)
				DM with asthma: aOR 4.38 (0.80-23.85)
				DM prevalence: controls:3.5%, cases:6.4%

Lee et al. (2006)	Taiwan (2002)	Hospital-based case-control study	**Cases:**232 acute DHF (12 DSS)	Association DM- acute DHF/DSS vs. acute DF: Adjusted OR(factors not reported)
			Controls:412 acute DF	DM yes vs. no: aOR 1.86 (1.04-3.37)
			-all confirmed acute DF treated at Kaohshiung Medical University Hospital in 2002	DM prevalence: cases:16.8%, controls:7.6%
			-no age restriction	Plasma leakage prevalence: Pleural effusion: 51% of DHF cases
			-diagnostic criteria for confirmed acute **DF:**	Ascites: 31% of DHF cases
			WHO 1997 criteria, meeting any of:	
			positive dengue virus by PCR;	
			4-fold increase of dengue virus-specific IgM or IgG in paired serum samples;	
			positive for dengue virus-specific IgM or IgG in a single serum sample;	
			Thrombocytopenia;	
			Evidence for hemorrhage and plasma leakage;	
			additional diagnostic criteria DSS:	
			hypotension, narrow pulse pressure, clinical signs of shock	
			Information on dengue history, confounders, effect modifiers & comoribities: clinical record review	
			DM: clinical records	
Karunakaran et al (2014)	India (2005-2008)	Hospital-based case-control study	**Cases**: 10 acute dengue patients, fatal	Association DM - mortality among confirmed acute dengue patients
			Controls: 40 acute dengue patients, non-fatal	DM yes vs. No: Crude OR 26.0 (2.5-273.7)
			-confirmed dengue patients admitted to in South Kerala hospital between 2005-2008	DM prevalence: controls:2.5%, cases:40%
			diagnostic criteria:	

			-confirmation by PCR or IgM antibody	
			Information on dengue history, symptoms, confounders, effect modifiers & comorbities: semi-structured interview ;assessment of warning signs according to WHO 2009 case definition	
			DM mellitus: self-report	
Case series				
Lye et al. (2009)	Singapore (2004)	Hospital-based	**Cases:** 1971 acute DF, DHF, and DSS cases	DM prevalence: Age <60:2% ,Age ≥ 60: 17%
			-all patients admitted to the Tan Tock Seng Hospital in Singapore in 2004	
			- fulfilling WHO 1997 criteria for acute dengue	
			- positive dengue diagnostic tests:	
			- probable dengue:(+)ve acute dengue serology (Dengue Duo IgM & IgG Rapid Strip Test)	
			- confirmed dengue:	
			- positive PCR	
			- no age restriction	
			Information on dengue history, symptoms, confounders, effect modifiers & comoribities: clinical records	
			DM: clinical records	
Wieten et al. (2012)	Netherlands (2006-2011)	Tropical and travel-medicine based case series	**Cases:** 132 acute dengue cases	DM prevalence: 8%
			-dengue patients serologically tested at Amsterdam Medical Center between 2006-2011	
			-diagnostic criteria:	
			serological confirmation:positive anti dengue IgM or at least fourfold increase in dengue specific IgG if possible based on one sample from the initial phase and one from the convalescent phase or else according to WHO 2009 criteria for a single sample;	
			clinical picture of probable dengue (WHO 1997 and 2009 criteria) or	

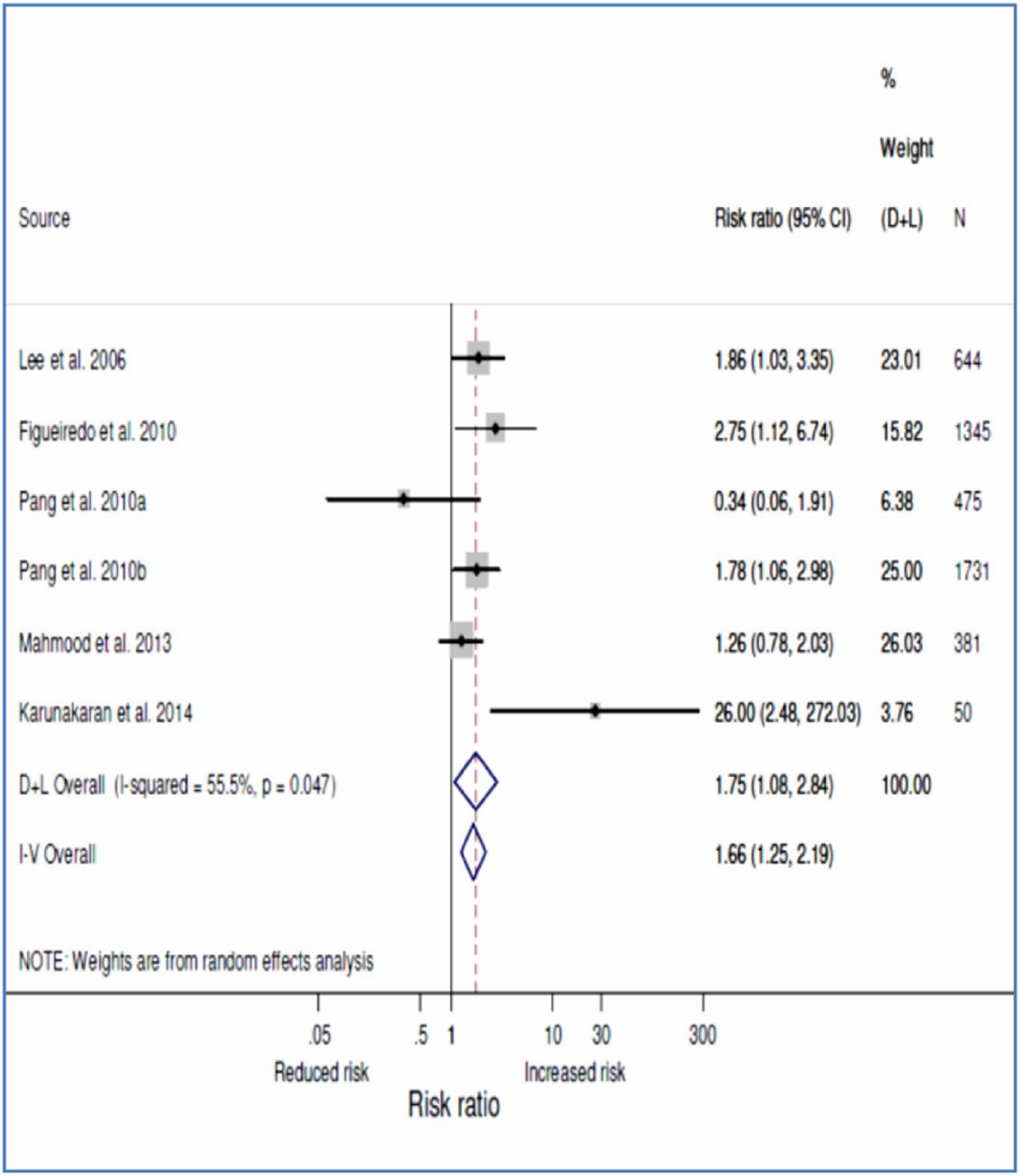
			DHF (WHO 1997) or dengue with warning signs (WHO 2009);	
			Information on dengue history, symptoms, confounders, effect modifiers & comoribities: clinical records	
			DM: clinical records	
Sam et al (2013)	Malaysia (2006-2007)	Hospital-based	**Cases**: 10 acute DF/DHF/DSS patients, fatal	DM prevalence: 30% (pre-existing)
			-fatal cases at the University Malaya Medical Center 2006-2007	Plasma leakage prevalence: 78% of all cases
			-diagnostic criteria:	(67% among diabetic patients)
			laboratory confirmation: acute phase dengue-specific IgM and IgG; RT-PCR;	
			disease severity classification WHO 1997 ;	
			age range: 11-59;	
			Information on dengue history, symptoms, confounders, effect modifiers & comoribities: clinical records	
			DM: clinical records	
Lahiri et al (2008)	Singapore (2004-2005)	Hospital-based	**Cases**: 9 acute dengue cases, fatal	DM prevalence: 78%
			-fatal cases of a total of 1235 admissions with acute dengue in 2004-2005	Plasma leakage prevalence: 28% of DM patients
			-all 9 patients had positive laboratory test for dengue (7 by IgM, 3 by PCR)	
			-all 9 patients had evidence for capillary leakage and hemorrhagic manifestations	
			-7 of 9 patients strictly met DHF WHO 1997 criteria	
			- age range: 37-71	
			Information on dengue history, symptoms, confounders, effect modifiers & comoribities:	
			clinical records	

			DM: clinical records	
Hasanat et al.(2010)	Bangladesh (2009)	Hospital-based (prospective)	**Cases**: 133 acute dengue cases	Status on 1st OGTT (n=133): Normal:25% , Glucose intolerant:54% ,DM:21%
			-patients admitted to Samoritha Hospital in Dakha for dengue	Status on 2nd OGTT (n=40):
			- laboratory confirmation by anti-dengue antibody test 1 week of onset of illness	Normal:83%
			-further diagnostic criteria not provided	Glucose intolerant:17%
			Information on dengue history, symptoms, confounders, effect modifiers & comorbities: interviews	Repeatedly normal:25%,
			DM: Oral glucose tolerance testing in consenting cases: 1st test between day 3 and 10 of admission and 2nd test before discharge	Repeatedly abnormal:18%
				Reverting to normal:55%

Meta-analysis of epidemiological evidence from case-control studies

We included the results of five above mentioned studies in a meta-analysis. One of these studies reported two separate estimates from two independent cross-sectional assessments; hence, we considered them as separate studies. The meta-analysis showed that the presence of a severe clinical presentation of dengue was positively associated with the presence of DM.

A diagnosis of DM was associated with an increased risk for severe clinical manifestations of dengue by 75% (95% CI: 1.08-2.84, p =0.022) compared to non-DM patients (Fig. 12-2). This OR remained robust across sensitivity analyses involving fixed effect analysis. We observed some heterogeneity across the studies, consistent with the broadly differing study settings. The small number of studies included in the meta-analysis did not provide statistical power for formal statistical assessment of heterogeneity (Fig. 12-2).



Where I-square is the variation in effect estimates attributable to heterogeneity, D+L is the pooled random effect estimate of all studies and I-V is the pooled fixed effect estimates of all studies.

Figure 12-2. Meta-analysis of case-control studies on the association between diabetes mellitus and severe clinical presentation of dengue.

Clinical case series on characteristics of DHF patients

We additionally identified five case series reporting dengue-related hospitalizations and the prevalence of DM in these cases. They are listed in Table 12-1 as reference of the prevalence of DM in dengue patients with a severe clinical presentation. The studies have been conducted mostly in Asian dengue endemic regions such as Malaysia (Sam et al., 2013); Bangladesh (Hasanat et al., 2010); Singapore (Lye et al., 2010, Lahiri et al., 2008). In the absence of a control group, these studies are of limited value for better understanding of the role of DM on the clinical presentation of dengue. In several instances the case definition was restricted to just being dengue seropositive. This does not allow differentiating between DM influencing the clinical manifestations of dengue infection versus dengue infection influencing the clinical presentation of DM. Of interest, in that respect is the study by Hasanat et al (Hasanat et al., 2010). Hospitalized DF patients underwent oral glucose tolerance testing (OGTT) between 3 and 10 days after the start of illness. A subset of these patients agreed to a second OGTT before discharge. The authors demonstrated a high rate of glucose intolerance in the early phase of disease, which returned though to normal in 55% of the patients.

Discussion

The few published studies specifically addressing the role of DM as a risk factor for a severe clinical presentation of dengue provide suggestive evidence for an adverse effect. This result merits further investigation in the context of study designs that overcome the weaknesses of currently available publications as addressed below.

Assigning causality to the modifying effect of diabetes on the clinical presentation of dengue is premature. First, the case-control studies conducted to date and summarized above are mostly retrospective in nature. The clinical and laboratory diagnostic criteria applied in different studies vary broadly, as does the definition for the control group. Second, the WHO 1997 dengue classification used in the studies reviewed, has itself a number of limitations. For example, the development of this classification was based on disease patterns of children in Thailand, potentially limiting its generalizability to other geographical regions and to older age groups. Furthermore, clinical assessments such as the Tourniquet test do not

differentiate between DF, DHF and other febrile illness (Deen JL, 2006, Rigau-Perez JG, 2006). It also fails to detect severe dengue manifestations in many patients (Balmaseda A, 2005). Third, information on DM was either self-reported or record-based and did not systematically allow differentiating between DM diagnosed before versus concurrent with the dengue episode. Given the high degree of under diagnosis of DM, especially in LMIC, misclassification of DM status in the studies is likely substantial. The problem of under diagnosis is not easy to overcome, as DM measured at the time of acute dengue is not necessarily reflecting the underlying DM status, but rather stress-related hyperglycemia, which disappears after recovery from dengue (Hasanat et al., 2010). Longitudinal studies of dengue patients are needed that allow studying both, the longitudinal course of hyperglycemia and the evolvement of clinical presentation of the dengue infection. In addition, whether better control of DM in early stages of dengue will prevent severe forms of dengue needs to be assessed in the context of intervention studies. The basis should be dengue surveillance programs which registers all dengue episodes and which includes routine collection of blood specimens for HbA1c and other hyperglycemia parameters and of information regarding diagnosis and treatment of DM. A fifth limitation of studies conducted to date is the fact that most of them were hospital-based case-control studies with a high potential for selection bias. But the results from the only population-based case-control study also point to DM increasing the risk for a severe clinical presentation of dengue (Figueiredo et al., 2010). Finally, study limitations also include the fact that the modifying effect of DM on different dengue serotypes could not be differentiated; that the age range of study subjects was not always reported; and that many studies included children and adolescents in whom DM is rare.The observed heterogeneity between the results of the individual studies is a limitation in the meta-analysis and reflects differences in the diagnostic and classification criteria for dengue and DM, differences in the controls selected, as well as differences in study design such as sample size, population and study setting or confounders considered in the analysis. Our systematic and broad search strategy, (not limiting to publication dates, place of study, study design or age of research participants) and the novelty of this topic are the strengths of our review and should stimulate further research into the topic, also in the light of supporting results from experimental studies.

Biological evidence exists to support the hypothesis for a high DHF: DF ratio in diabetes. One example for a line of reasoning is that the cytokine overload related to a Th1 to Th2 shift and a severe manifestation of dengue superimposed by the cytokine overload of a diabetic state may be particularly detrimental to the endothelium and for subsequent vascular leakage (Sierra et al., 2010, Daniel Limonta, 2008, Chaturvedi et al., 2000). The third space fluid shift such as pleural/pericardial effusion or ascites is an important clinical manifestation in dengue with severe clinical symptoms, which is a consequence of endothelial dysfunction and results in hemoconcentration, hypotension and shock (Lahiri et al., 2008). DM shares with a severe clinical presentation of dengue alterations in the innate immune response, a pro-inflammatory state and endothelial dysfunction. Lee and colleagues infected mononuclear cells from diabetic (n=33) and healthy individuals (n=29) with dengue virus. Cells from diabetics produced higher levels of IL-4 and of both, IL-4 and IL-10 as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) on the first and third day post-infection, respectively. No differences in viral load were found (Lee et al., 2013). In a post-mortem study of DHF/DSS related deaths, the intestinal serosa of 1 DM case with ascites, but not of non-DM cases showed apoptosis of microvascular endothelial cells (Daniel Limonta, 2008). Microvascular endothelial cells cultured with sera from acute dengue infection patients containing high TNF-α levels exhibited activation and apoptosis, a pathophysiological alteration likely related to vascular leakage (Sierra et al., 2010).

Currently available epidemiological studies on the DM/DHF, DSS association do not provide sufficient clinical details to specifically address this “cytokine overload” hypothesis. For example, the Brazilian study explicitly stated that it did not look into ascites or pleural effusion, as these were very rarely recorded (Figueiredo et al., 2010). The other studies did not mention plasma leakage such as pleural effusion or ascites except for the Taiwan study, where 51% of 164 DHF cases showed evidence of pleural effusion in X- ray and 31% of DHF cases had ascites in ultrasound (Min-Sheng Lee, 2006). Future studies thus need to specifically address the issue of plasma leakage in the overlap between severe dengue and DM. Clinical reporting of the presence of pleural effusion and ascites as signs of severe plasma leakage is essential, as hemoconcentration and shock symptoms in the

absence of plasma leakage can also arise from poorly controlled DM. The direct hypovolemic effect of hyperglycemia and the associated elevated hematocrit may even lead to misclassification of diabetics as DHF in the absence of objective evidence of plasma leakage. While recognition of hemoconcentration irrespecitive of its reason is of primary clinical importance for the provision of appropriate fluid therapy, currently available studies on the DM and DHF/DSS association do not provide sufficient clinical details to differentiate between effects of DM vs. DHF/DSS.

The potential role of altered innate immunity in mediating the association between DM and a severe clinical presentation of dengue obtains indirect support by observations that the latter may also have a higher prevalence of asthma and allergies. Patients with asthma and allergy also exhibit altered Th1 and Th2 responses (Figueiredo et al., 2010, Mahmood et al., 2013). But in DM, factors beyond the altered cytokine profile may additionally put people at risk. Diabetic patients express high rates of hypertension and impaired renal function which are both themselves associated with endothelial dysfunction (Dharmashankar and Widlansky, 2010, Lin et al., 2010). Hypertension was also observed to be more common in patients with dengue (Figueiredo et al., 2010, Mahmood et al., 2013, Pang et al., 2012, Karunakaran et al., 2014, Lye et al., 2010), but often exhibited no independent effect in regression models adjusting for diabetes (Mahmood et al., 2013, Pang et al., 2012). Pang and colleagues (2012) reported a higher risk for a severe clinical presentation of dengue in hypertensive when compared to non-hypertensive diabetic patients (Pang et al., 2012).

Conclusion

Understanding factors increasing the likelihood of dengue patients with severe clinical symptoms would help the physician to decide in a timely fashion on the need for close observation, adequate treatment, or hospitalization. The available

evidence points to DM as a potentially important co-factor. Additional prospective studies among DM and non-DM patients are needed to assess the impact of pre-existing DM as well as of hyperglycemia at the time of dengue diagnosis on the risk of a severe clinical presentation of dengue and of related deaths. Even in the absence of causal inference, it seems justified that fever episodes in patients with DM and living in a dengue endemic region are confirmed for dengue as soon as possible and that they remain under close surveillance if an acute dengue infection is confirmed. Future studies need to also address whether better control of glycemia level in dengue patients with DM can improve the outcome of the patient and decrease the risk of a severe clinical presentation. As the fluid management in diabetic patients with a severe clinical presentation of dengue poses a particular challenge, this issue should be taken into consideration by these studies. Diabetic patients in dengue endemic regions should consistently receive recommendations to protect against dengue infection by taking preventive measures against indoor mosquito breeding and against mosquito bites.

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13 Curriculum Vitae

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Born February 22th 1977, married, 1 child
Mother tongue French, fluent in English, understanding German, Spanish and Kiswahili
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CURRENT POSITIONS

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Senior registrar at the Travel Clinic (70%)	Department of Ambulatory Care and Community Medicine University of Lausanne Switzerland
Research scientist, clinical epidemiologist (30%)	Infectious Diseases Service Department of medicine University Hospital of Lausanne Switzerland

QUALIFICATIONS

Academic titles

2002	University of Lausanne, Switzerland	Graduation in Medicine
2012	University of Lausanne, Switzerland	MD thesis, Doctor of Medicine Title: *"Impact of a nurse vaccination program on hepatitis B immunity in a Swiss HIV Clinic"*
2015	Swiss Tropical and Public Health Institute	PhD in Epidemiology University of Basel, Switzerland Title:*"Comorbidity between communicable and non-communicable diseases: The example of the dual burden of tuberculosis and diabetes in Dar es Salaam, Tanzania"* Distinction: *Summa cum laude*

Professional titles

2007	Swiss Medical Association (FMH)	Post-graduate title in Internal Medicine
2010	Swiss Medical Association (FMH)	Post-graduate title in Infectious Disease

PROFESSIONAL EXPERIENCE

2003-2005	Resident, Department of Internal Medicine, Hospital of Neuchâtel, Switzerland, Prof. R. Malinverni
2005-2006	Resident, Department of Ambulatory Care and Community Medicine, University Hospital of Lausanne, Switzerland, Prof. A. Pécoud
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2012-2015 Research scientist, Department of Epidemiology, Swiss Tropical and Public Health Institute, Basel, Switzerland and Ifakara Health Institute, Dar es Salaam, Tanzania

RESEARCH ACTIVITIES

2008-2012 *Transplant infectious diseases*: i) Retrospective study on cytomegalovirus prophylaxis in kidney transplant recipients, ii) European survey on the management of tuberculosis in solid-organ transplant recipients and candidates

Swiss HIV Cohort Study: Three retrospective studies using the database of the Swiss HIV Cohort Study addressing relevant questions for patients' management

Microbiology: Evaluation of a new method for susceptibility testing of mycobacteria

2012-2016 Principal investigator of a 3-year project: "Co-occurrence of tuberculosis and diabetes in Tanzania: an epidemiological and immunological study". *Manuscripts submitted*

Co-Principal investigator of a 2-year project: "Etiologies of acute febrile illness among adults attending outpatient departments in Dar es Salaam, Tanzania". *Manuscripts in preparation*

TEACHING ACTIVITIES

Pre-graduate (Faculties of Biology and Medicine)

Epidemiology and Surveillance: Tropical viral diseases (B3 and M1, University of Lausanne)

Public Health: Tuberculosis as a public health problem (B2, University of Lausanne)

General Medicine: Fatigue syndrome and infectious diseases (M1, University of Lausanne)

Tropical Health: HIV in Africa (B3, University of Lausanne)

From Basics to Clinics: Tropical medicine (B3, University of Lausanne)

Post-graduate

Consultant of Infectious Diseases, Focus on HIV and Tuberculosis, Médecins sans Frontières (MSF), Kinshasa, Congo

Travel medicine to pharmacists, University of Lausanne

Rapid Diagnostic Tests to general practitioneers, University of Lausanne

AWARDS

2008	Revue Médicale Suisse	Price of the redaction group for *"Rickettiosis a clinical approach"* Best publication of the year 2007
2012-2013	Swiss National Science Foundation	Postdoctoral training award
2015-2017	Leenards Foundation	Junior Clinical Scientist award

GRANTS

2012	Société Académique Vaudoise	Research grant (CHF. 10'000.00)
2012-2013	SICPA Foundation	Research grant (CHF. 30'000.00)
2012-2013	MSD Merck Sharp & Dohme AG	Research grant (CHF.50'000.00)
2016	*Swiss National Science Foundation*	*Application submitted National Research Program 72 "Antimicrobial Resistance"*

PUBLICATIONS LIST

Original articles in peer-reviewed journals

1. **Boillat N**, Greub G. Rickettiosis : a clinical approach. ***Rev Med Suisse*** 2007; 3: 1222-7. ***Awarded "best publication of the year"***
2. **Boillat N**, Greub G. Cat scratch disease and other human infections caused by Bartonella species. ***Rev Med Suisse*** 2008; 4: 901-7.
3. **Boillat N**, Bally F, Peter O, Praz G, Troillet N. Occupational exposure and rapid HIV test. ***Arch Intern Med*** 2008; 168: 1468.
4. **Boillat N**, Frochaux V. Animal bites and infection. ***Rev Med Suisse*** 2008; 4: 2149-55.
5. **Boillat N**, Genton B, D'Acremont V, Raoult D, Greub G. Fatal case of Israeli spotted fever after Mediterranean cruise. ***Emerg Infect Dis*** 2008; 14: 1944-6.
6. Longtin Y, Troillet N, Touveneau S, **Boillat N**, Rimensberger P, Dharan S, Gervaix A, Pittet D, Harbarth S. Pseudomonas aeruginosa outbreak in a pediatric intensive care unit linked to a humanitarian organization residential center. ***Pediatr Infect Dis J*** 2010; 29: 233-7.
7. **Boillat Blanco N**, Pascual M, Venetz JP, Nseir G, Meylan PR, Manuel O. Impact of a preemptive strategy after three months of valganciclovir cytomegalovirus prophylaxis in kidney transplant recipients. ***Transplantation*** 2011; 91: 251-5.
8. **Boillat Blanco N**, Kuonen R, Bellini C, Manuel O, Estrade C, Mazza-Stalder J, Aubert JD, Sahli R, Meylan P. Chronic norovirus gastroenteritis in a double hematopoietic stem cell and lung transplant recipient. ***Transpl Infect Dis*** 2011; 13: 213-5.
9. **Boillat Blanco N**, Probst A, Waelti Da Costa V, Giulieri S, Bernasconi E, Calmy A, Elzi L, Rauch A, Weber R, Vernazza P, Cavassini M, Bochud PY. Impact of a nurse vaccination program on hepatitis B immunity in a Swiss HIV Clinic. ***JAIDS*** 2011; 58: 472-4.
10. Balaskas K, Vaudaux J, **Boillat-Blanco N**, Guex-Crosier Y. Azithromycin in toxoplasmic retinochoroiditis. ***Med Sci Monit*** 2012; 18: 296-302.
11. **Boillat-Blanco N**, Manuel O, Vaudaux B. Hepatitis B immunization in the neonate and the immunocompromised: time to resort to dedicated teams? ***Rev Med Suisse*** 2012; 8: 901-4.
12. **Boillat-Blanco N**, Darling K, Taffé P, Osih R, Fehr J, Strahm C, Adami M, Elzi L, Daou S, Wandeler G, Matthias Cavassini. Impact of recommendation updates in well-controlled patients on non-recommended antiretroviral therapies: the Swiss HIV Cohort Study. ***JAIDS*** 2013; 1: 180-9.
13. **Boillat-Blanco N**, Aguado JM, Aubert JD, Sester M, Grossi P, Kamar N, Pascual M, Manuel O. European survey on the management of tuberculosis in solid-organ transplant recipients and candidates. ***Transplant Int*** 2013; 26: e69.
14. **Boillat-Blanco N**, Darling K, Schoni-Affolter F, Vuichard D, Rougemont M, Fulchini R, Bernasconi E, Aouri M, Clerc O, Furrer HJ, Gunthard HF, Cavaasini M. Virological outcome and management of persistent low-level viraemia in HIV-1-infected patients: 11 years of the Swiss HIV Cohort Study. ***Antivir Ther*** 2015; 20: 1655-75.
15. Htun NS, Odermatt P, Eze IC, **Boillat-Blanco N**, D'Acremont V, Probst-Hensch N. Is Diabetes a Risk Factor for a severe clinical presentation of Dengue? – Review and Meta-analysis. ***PLoS Negl Trop Dis*** 2015; 24: e0003741.
16. **Boillat-Blanco N**, Furustrand Tafin U, Jaton K, Trampuz A. Susceptibility Testing of Mycobacterium abscessus by Isothermal Microcalorimetry. ***Diagn Microbiol Infect Dis*** 2015; 83: 139-43.
17. **Boillat-Blanco N**, Kaushik LR, Mganga M, Mrangu NS, Minja LT, Bovet P, Schindler C, Von Eckardstein A, Gagneux S. Daubenberger C, Reither K, Probst-Hensch N. High Frequency of Transient Hyperglycemia in Patients with Tuberculosis in Sub-Saharan Africa: Implications for diabetes screening algorithms. ***J Infect Dis*** 2016; 213:1163-72.
18. Moulin E, Selby K, Cherpillod P, Kaiser L, **Boillat-Blanco N**. Simultaneous outbreaks of dengue, chikungunya and Zika virus infections: diagnosis challenge in a returning traveller with nonspecific febrile illness. ***New Microbes New Infect*** 2016; 11: 6-7.

Original articles submitted to peer-reviewed journals

19. ***Boillat-Blanco N**, Bovet P, Kaushik LR, Mganga M, Minja LT, Saleh L, Imboden M, Schindler C, Gagneux S. Daubenberger C, Reither K, Probst-Hensch N. Association between Tuberculosis, Diabetes Mellitus and Vitamin D in Tanzania: a longitudinal case control study.*
20. ***Boillat-Blanco N**, Tumbo A, Amelio P, Perreau M. Kaushik LR, Mganga M, Schindler C, Gagneux S., Reither K, Probst-Hensch N, Pantaleo G, Portevin D, Daubenberger C. Glycemia is inversely correlated with BCG-specific CD4 T cell immunity in Tanzanian adults with latent and active tuberculosis.*

Book chapters

Boillat Blanco N, Prod'Hom G, Connell J, De Gascun C, Gilligan P, Greub G. Diagnostic Microbiology for Infectious Diseases in Cardiothoracic Transplant and Mechanical Circulatory Support Recipients**.** *ISHLT Monograph* 2011; Vol 5; Chap 24.

***Boillat Blanco N**, Jaton K, Greub G. Medical importance of mycobacteria in human medicine. Free-living amoebae: an evolutionary crib for emerging pathogens 2016; Chap IV. XIII.*

SCIENTIFIC MEETINGS

Boillat Blanco N, Pascual M, Venetz JP, Nseir G, Meylan PR, Manuel O. Usefullness of a preemptive strategy after three months of antiviral prophylaxis for Cytomegalovirus in kidney transplant recipients, poster presentation. **American Transplant Congress** (Boston,USA) 2009.

Boillat Blanco N, Furustrand U, Jaton K, Trampuz A. Detection and susceptibility testing of mycobacteria: a comparison of conventional and microcalorimetric methods, oral presentation. **European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)** (Milan, Italy) 2011.

Boillat Blanco N, Jaton K, Nseir G, Yerly P, Aubert JD, Pascual M, Manuel O. Prevalence and clinical characteristics of mycobacterial infection in a cohort of solid organ transplant recipients over a 13-year period, poster presentation. **European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)** (Milan, Italy) 2011.

Boillat Blanco N, Darling K, Taffé P, Osih R, Fehr J, Strahm C, Adami M, Elzi L, Daou S, Wandeler G, Matthias Cavassini. Impact of International Aids Society-USA (IAS-USA) guideline updates in well-controlled patients on three non-recommended combination antiretroviral therapies: the Swiss HIV Cohort Study, poster presentation. **XIX International AIDS Conference** (Washington D.C., USA) 22-27 July 2012.

Boillat Blanco N, Darling K, Schöni-Affolter F, Günthard H, Fulchini R, Rougemont M, Bernasconi E, Vuichard D, Furrer H, Clerc O, Cavassini M. Determinants and consequences of persistent low-level viraemia in HIV-1 infection: Swiss HIV Cohort Study, poster presentation. **International Conference on Antimicrobial Agents and Chemotherapy** (**ICAAC)** (San Francisco, USA) 9-12 September 2012.

Boillat-Blanco N, Gagneux S, Minja LT, Kelemani A, Daubenberger C, Reither K, Probst-Hensch N. Association of tuberculosis and diabetes mellitus in urban Tanzania, oral presentation. **44th Union World Conference on Lung Health** (Paris, France) 30 October-3 November 2013.

Boillat-Blanco N, Daubenberger C, Gagneux S, Minja LT, Kelemani A, Mganga M, Reither K, Probst-Hensch N. Alcohol use is a strong independent risk factor for tuberculosis in urban Tanzania, oral presentation. **45th Union World Conference on Lung Health** (Barcelona, Spain) 28 October-1 November 2014.

Boillat-Blanco N, Zainab M, Samaka J, Mlaganile T, Klaassen B, Mamin A, Genton B, Kaiser L, D'Acremont V. Detection of a dengue outbreak through systematic screening of febrile patients in Dar es Salaam, late breaker oral presentation. **63rd Annual Meeting American Society of Tropical Medicine and Hygiene (ASTMH)** (New Orleans, USA) 2-6 November 2014.

Boillat-Blanco N, Samaka J, Zainab M, Mlaganile T, Kasimoto T, Mamin A, Genton B, Kaiser L, D'Acremont V. Etiologies of acute febrile illness among adults attending outpatient clinics in dar es

Salaam, Tanzania, poster presentation. **63rd Annual Meeting American Society of Tropical Medicine and Hygiene (ASTMH)** (New Orleans, USA) 2-6 November 2014.

Boillat-Blanco N, Klaassen B, Franco Narvaez L, Zainab M, Samaka J, Mlaganile T, Masimba J, Mamin A, Genton B, Kaiser L, D'Acremont V. Dengue fever outbreak in the native and expatriate communities of Dar es Salaam, Tanzania, oral presentation. **9th European Congress on Tropical Medicine and International Health (ECTMIH)** (Basel, Switzerland) 6-10 September 2015.

Boillat-Blanco N, Zainab M, Samaka J, Mlaganile T, Kasimoto T, Mamin A, Genton B, Kaiser L, D'Acremont V. Etiologies of acute febrile illness among adults attending outpatient clinics in Dar es Salaam, Tanzania, poster speed talk. **9th European Congress on Tropical Medicine and International Health (ECTMIH)** (Basel, Switzerland) 6-10 September 2015.

Boillat-Blanco N, Mganga M, Kaushik LR, Mrangu NS, Minja LT, Schindler C, Von Eckardstein A, Gagneux S, Daubenberger C, Reither K, Probst-Hensch N. Longitudinal evolution of diabetes during tuberculosis in Dar es Salaam, Tanzania, oral presentation. **9th European Congress on Tropical Medicine and International Health (ECTMIH)** (Basel, Switzerland) 6-10 September 2015.

Boillat-Blanco N, Zainab M, Samaka J, Mlaganile T, Kasimoto T, Mamin A, Genton B, Kaiser L, D'Acremont V. Etiologies and Outcome of Adult Sepsis in Urban Outpatient Clinics in Tanzania, poster presentation. **IDWeek** (San Diego, USA) 7-11 October 2015.

Boillat-Blanco N, Minja LT, Ramaiya KL, Schindler C, Gagneux S, Daubenberger C, Reither K, Probst-Hensch N. Diabetes among patients with tuberculosis in Africa: to test or not to test?, oral presentation. **46th Union World Conference on Lung Health** (Cape Town, South Africa) 2-6 December 2015.

Boillat-Blanco N, Zainab M, Samaka J, Mlaganile T, Kasimoto T, Mamin A, Genton B, Kaiser L, D'Acremont V. Composite rather than single-test based diagnosis of acute dengue, malaria and typhoid should be used in febrile patients, poster presentation. **European Congress of Clinical Microbiology and Infectious Diseases (*ECCMID)*** *(Amsterdam, Netherlands) 9-12 April 2016.*