

Nutrition and tuberculosis in Ethiopia: The role of vitamin D₂ derived from sun exposed oyster mushroom on the treatment outcomes of tuberculosis

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Chapter 1

Preface

CHAPTER 1.1. ACKNOWLEDGMENT

1.1 Acknowledgment

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CHAPTER 1.2. CO-AUTHORS

1.2 Co-authors contribution

All the scientific works included in this doctoral dissertation thesis were designed, performed and published with the knowledge and approval of the main supervisor, **Prof. Dr. med. H. K. Biesalski**.

Tibebeselassie Seyoum Keflie undertook all the practical and analytical works. He conceived, designed and performed the experiments; collected, analysed and interpreted data; prepared the draft of the manuscript; and followed all aspects of the publication processes.

Co-authors from different institutes were partially involved in the scientific works of this thesis.

Prof. Dr. Donatus Nohr, Dr. Christine Lambert and Nils Nöle from University of Hohenheim; Dr. Markos Abebe and Dr. Adane Mihiret from Armauer Hansen Research Institute; Dr. Aregash Samuel from Ethiopian Public Health Institute; and Dr. Ashagrie Zewdu Woldegiorgis from Centre for Food Science and Nutrition, Addis Ababa University were critically reviewed and approved the manuscripts.

CHAPTER 1.3. PUBLICATION LISTS

1.3. Publication lists

Peer-reviewed publications

1. **Tibebeselassie Seyoum Keflie**, Aregash Samuel, Christine Lambert, Donatus Nohr and Hans Konrad Biesalski. **2018**. Dietary Patterns and Risk of Micronutrient Deficiencies: their Implication for Nutritional Intervention in Ethiopia. *J Nutrition Health Food Sci* 6(1):1-16 DOI: 10.15226/jnhfs.2018.001120.
2. **Tibebeselassie Seyoum Keflie**, Aregash Samuel, Ashagrie Zewdu Woldegiorgis, Adane Mihret, Markos Abebe, Hans Konrad Biesalski. 2018. Vitamin A and zinc deficiencies among tuberculosis patients in Ethiopia. *J Clin Tuberc Other Mycobact Dis* 12 27–33.
3. **Tibebeselassie Seyoum Keflie**, Nils Nölle, Christine Lambert, Donatus Nohr, Hans Konrad Biesalski. 2015. Vitamin D deficiencies among tuberculosis patients in Africa: A systematic review. *Nutrition* 31: 1204–1212.
4. **Tibebeselassie Seyoum Keflie**, Nils Nölle, Christine Lambert, Donatus Nohr, Hans Konrad Biesalski. 2018. Impact of the natural resource of UVB on the content of vitamin D₂ in oyster mushroom (*Pleurotus ostreatus*) under subtropical settings. *Saudi Journal of Biological Sciences* <https://doi.org/10.1016/j.sjbs.2018.07.014>.
5. **Tibebeselassie Seyoum Keflie**, Aregash Samuel, Ashagrie Zewdu Woldegiorgis, Adane Mihret, Markos Abebe, Christine Lambert, Donatus Nohr, Hans Konrad Biesalski. Vitamin D₂ from sun-exposed Oyster Mushroom: Its impact on the treatment outcomes of tuberculosis. Submitted manuscript.

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3. **Tibebeselassie Seyoum Keflie**, Hans Konrad Biesalski. The role of vitamin D derived from sun-exposed oyster mushroom on the treatment outcomes of tuberculosis. Organized by Humboldt reloaded Interdisciplinary Summer school 2018 under the theme of ‘‘Is health nature or nurture. Bridging WHO and evidence-based research in alcoholism, depression and nutrition. September 20, 2018. University of Hohenheim, Germany.
4. **Tibebeselassie Seyoum Keflie**, Nils Nölle, Christine Lambert, Donatus Nohr, Hans Konrad Biesalski. Effects of sun-treatment on the quantities of vitamin D and minerals in oyster mushroom (*Pleurotus ostreatus*) cultivated in Ethiopia. Third international conference on food chemistry and technology (FCT-2017). November 2-4, 2017, Baltimore, MD, USA.

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5. **Tibebeselassie Seyoum Keflie**, Hans Konrad Biesalski. Approaches of young scientists from food security centre, University of Hohenheim and Centre for Development Research (ZEF), University of Bonn. October 19, 2017. Stuttgart, Germany.
6. **Tibebeselassie Seyoum Keflie**, Hans Konrad Biesalski. Dietary patterns and nutritional status: their implication for nutritional intervention in North Shewa, Ethiopia. March 16, 2017. DAAD Alumni Food Security Networks Seminar. Session: Current research on food security issues. Stuttgart, Germany.
7. **Tibebeselassie Seyoum Keflie**, Nils Nölle, Christine Lambert, Donatus Nohr, Hans Konrad Biesalski. Vitamin D deficiencies among TB patients in Africa: A Systematic Review. National Nutrition Program (NNP) related research finding dissemination workshop. Theme: Evidence-Based Information to Support the Current and Future NNP. October 23-25, 2014. Executive Hotel – Adama, Ethiopia.

Poster presentations

1. **Tibebeselassie Seyoum Keflie**, Christine Lambert, Donatus Nohr, Hans Konrad Biesalski. Vitamin D₂ from sun-treated oyster mushroom as an adjunctive therapy of tuberculosis. Fourth international congress of hidden hunger. February 27- March 1, 2019. Stuttgart, Germany.
2. **Tibebeselassie Seyoum Keflie**, Sarah Triller, Julian Philipp Wald, Christine Lambert, DonatusNohr, Hans Konrad Biesalski. Stinging nettle (*Urtica simensis*). An indigenous but unrecognized micronutrient potential for combatting hidden hunger in Ethiopia. September 20-22, 2017. University of Bonn, Germany.

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3. **Tibebeselassie Seyoum Keflie**, Christine Lambert, Donatus Nohr, Hans Konrad Biesalski. Dietary patterns and nutritional status: Their implication for nutritional intervention in North Shewa, Ethiopia. 11th European Nutrition and Dietetics Conference. June 29-July 01, 2017 Madrid, Spain.
4. **Tibebeselassie Seyoum Keflie**, Nils Nölle, Christine Lambert, Donatus Nohr, Hans Konrad Biesalski. Effects of sun-treatment on the quantities of vitamin D and minerals in oyster mushroom (*Pleurotus ostreatus*) cultivated in Ethiopia. Third international congress hidden hunger. March 20-22, 2017. Stuttgart, Germany.
5. **Tibebeselassie Seyoum Keflie**, Hans Konrad Biesalski. Does sun-exposure have impact on the contents of vitamin D₂ and minerals in oyster mushroom? CLIFOOD conference organized by Food Security Centre of Hohenheim University. March 8, 2017. Stuttgart, Germany.

CHAPTER 1.4. SUMMARY

1.4. Summary

Tuberculosis (TB) is an old infectious disease which causes ill-health among millions of people each year. Effective anti-TB drugs are available since 1950's, but still the global burden of TB remains enormous. The disease is very complex and there is a need to look for supportive treatment to the standard anti-TB drugs. Cognizant of this, the present doctoral study was undertaken by giving emphasis on nutrition and TB in Ethiopia. The aim of this doctoral dissertation thesis was to deal with the nutritional situation of people with and without TB and come-up with solutions that could support the effort of combatting TB. In this thesis, five papers (four published and one submitted) were included.

The first paper encompassed the study of dietary and nutritional assessment. In this study, dietary inadequacy, poor nutritional quality and high risk of micronutrient deficiencies were identified. The main dietary pattern included cereals, vegetables and legumes. About one-third of the population consumed animal source food (ASF). Malnutrition was the common problem in people with and without TB. This suggested that malnutrition may pave the way for TB.

The case-control study in the second paper revealed that more than one-half of TB patients had vitamin A and zinc deficiencies. More than three-fourth of TB patients had below half of the energy fulfilment. The protein intake was above the average fulfilment, but most TB patients relied on cereal-based diets. Patients with TB used a larger proportion of proteins from oral feeding for oxidation and hence for energy production. About half of the patients were undernourished. Thus, vitamin A and zinc deficiencies along with protein-energy malnutrition need to be addressed in the management programme of TB.

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The third paper included systematic review which explored the existence of vitamin D deficiency (VDD). Sunshine, which is very important for the synthesis of vitamin D under the skin, is widely available in Africa throughout the year. Surprisingly, more than three-fourth of TB patients in Africa had VDD and vitamin D insufficiency (VDI). Statistically significant variables such as use of sun protection (lack of sun-exposure), inadequate dietary intake, low body mass index (BMI), high skin pigmentation, use of drugs (anti-retroviral and /or anti-TB), low socioeconomic status, rainy season, covering body skin with clothes , old age and comorbidities were identified as the main predictor variables that hampered the status of vitamin D.

Vitamin D can be obtained from dietary intakes apart from endogenous synthesis after sun-exposure. Mushroom as such, is a potential non-animal source of vitamin D. The experimental study in the fourth paper revealed that sun-exposure significantly increased the content of vitamin D₂ in oyster mushroom. Increasing the surface area for sun-exposure enhanced the production of vitamin D₂. Other factors such as duration of sun-exposure and moisture content determined the production of vitamin D₂. Exposing slices of oyster mushroom to direct sun for brief period provided enough vitamin D₂ that could satisfy the current recommended dietary allowance (RDA) of vitamin D without any visible changes in colour and texture.

The study in the fifth paper was a randomized controlled trial and demonstrated for the first time the role of mushroom-derived vitamin D₂ on the treatment outcomes of TB. Intervention with vitamin D₂ derived from sun-exposed oyster mushrooms brought significant

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improvement in vitamin D status, clinical outcomes and immunological responses, but not in sputum smear and culture conversion. The intervention corrected VDD in more than one-third of TB patients. About one-third of the variability in TB score in the intervention group was accounted for by the change in the serum 25 hydroxy (OH) vitamin D level. There were also significant improvements in the serum IFN- γ and cathelicidin LL-37 peptide levels after intervention. The balance of cytokines was skewed to TH₁ responses due to high level of IFN- γ . Thus, mushroom-derived vitamin D₂ could serve as potential, safe, easily available and cost-effective adjunctive therapy for TB. Taken collectively, foods enriched with vitamin D need to be included in the national TB control programme to support the first line anti-TB drugs, increase the cure rate and reduce the infectiousness of TB.

CHAPTER 1.5. ZUSAMMENFASSUNG

1.5. Zusammenfassung

Tuberkulose (TB) ist eine alte Infektionskrankheit, an der jedes Jahr Millionen von erkranken. Wirksame Medikamente gegen Tuberkulose sind seit den 1950er Jahren erhältlich, aber die enorme Bürde durch Tuberkulose ist weltweit immer noch groß. Die Krankheit ist sehr komplex und es besteht die Notwendigkeit, eine unterstützende Behandlung für die Standard-Anti-TB-Medikamente zu suchen. In Anbetracht dessen legte die vorliegende Doktorarbeit ihren Fokus auf die Ernährung von Tuberkulosepatienten in Äthiopien. Ziel dieser Dissertation war es, sich mit der Ernährungssituation von Menschen mit und ohne Tuberkulose zu befassen und Lösungen zu finden, die den Kampf gegen Tuberkulose unterstützen können. Die Ergebnisse dieser Arbeit resultierten in fünf Artikel (vier veröffentlicht und einer eingereicht).

Das erste Artikel befasste sich mit der Bewertung der Ernährung und des Ernährungsstatus. In dieser Studie wurden unzureichende Ernährung, eine schlechte Ernährungsqualität und ein hohes Risiko für Mikronährstoffmangel festgestellt. Das Hauptnahrungsmuster beinhaltete Getreide, Gemüse und Hülsenfrüchte. Etwa ein Drittel der Bevölkerung konsumierte tierische Nahrung (TN). Unterernährung war das häufigste Problem bei Menschen mit und ohne TB. Dies deutet darauf hin, dass Mangelernährung den Weg für TB ebnen könnte.

Die Fall-Kontroll-Studie in der zweiten Veröffentlichung ergab, dass mehr als die Hälfte der TB-Patienten einen Mangel an Vitamin A und Zink aufwies. Mehr als drei Viertel der TB-Patienten nahmen weniger als die Hälfte der empfohlenen Energie zu sich. Die Proteinaufnahme lag über dem Durchschnitt, aber die meisten TB-Patienten wiesen ein getreidebasiertes Ernährungsmuster auf. Patienten mit TB verwendeten einen größeren Anteil an Nahrungsprotein zur Oxidation und damit zur Energiegewinnung. Etwa die Hälfte der

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Patienten war unterernährt. Daher, müssen Vitamin A – und Zinkmängel zusammen mit Protein-Energie Mangelernährung im Behandlungsprogramm für TB angegangen werden.

Der dritte Artikel beinhaltete eine systematische Übersicht über die Prävalenz eines Vitamin-D-Mangel (VDD) bei TB-Patienten. Sonnenschein, der für die Synthese von Vitamin D in der Haut sehr wichtig ist, ist in Afrika das ganze Jahr verfügbar. Überraschenderweise hatten mehr als drei Viertel der TB-Patienten in Afrika eine VDD- und Vitamin-D-Insuffizienz (VDI). Statistisch signifikante Variablen wie Sonnenschutz (fehlende Sonnenexposition), unzureichende Nahrungsaufnahme, niedriger Body Mass Index (BMI), hohe Hautpigmentierung, Medikamenteneinnahme (antiretroviral und / oder anti-TB), geringer sozioökonomischer Status, Regenzeit, Bedeckung der Körperhaut durch Kleidung, Alter und Begleiterkrankungen wurden als Hauptprädiktorvariablen identifiziert, die den Status von Vitamin D beeinträchtigten.

Vitamin D kann neben der körpereigenen Synthese durch Sonnenexposition auch über die Nahrung aufgenommen werden. Pilz stellen eine potenzielle nicht-tierische Vitamin-D-Quelle dar. Die experimentelle Studie in der vierten Veröffentlichung ergab, dass die Sonnenexposition den Vitamin-D₂-Gehalt in Austernpilzen signifikant erhöht. Eine Steigerung der bestrahlten Oberfläche erhöhte die Produktion von Vitamin D₂. Andere Faktoren wie die Dauer der Sonneneinstrahlung und der Feuchtigkeitsgehalt beeinflussen die Produktion von Vitamin D₂ ebenfalls. Wenn Austernpilzscheiben für kurze Zeit direkter Sonneneinstrahlung ausgesetzt wurden, war der Vitamin D₂ – Gehalt ausreichend um die derzeit empfohlene Tagesdosis (RDA) für Vitamin D ohne sichtbare Änderungen der Farbe und Textur zu erreichen.

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Die Studie in der fünften Veröffentlichung war eine randomisierte kontrollierte Studie und zeigte zum ersten Mal die Rolle von aus Pilzen stammendem Vitamin D₂ für die Behandlungsergebnisse bei TB. Die Intervention mit Vitamin D₂ aus sonnenexponierten Austernpilzen führte zu einer signifikanten Verbesserung des Vitamin D-Status, der klinischen Ergebnisse und der immunologischen Reaktionen, jedoch nicht zu einer Verbesserung des Sputumabstrichs und der bakteriellen Kulturumwandlung. Die Intervention korrigierte VDD bei mehr als einem Drittel der TB-Patienten. Etwa ein Drittel der Variabilität des TB-Scores in der Interventionsgruppe war auf die Veränderung des Serum-25-Hydroxy (OH) -Vitamin-D-Spiegels zurückzuführen. Es gab auch signifikante Verbesserungen bei den Serum-IFN- γ und Cathelicidin-LL-37-Peptidspiegeln nach der Intervention. Das Gleichgewicht der Zytokine wurde aufgrund des hohen IFN- γ -Spiegels auf TH₁ – Antworten verzerrt. Somit könnte aus Pilzen gewonnenes Vitamin D₂ als potenzielle, sichere, leicht verfügbare und kostengünstige Zusatztherapie für TB dienen. Zusammengenommen müssen mit Vitamin D angereicherte Lebensmittel in das nationale TB-Bekämpfungsprogramm aufgenommen werden, um die ersten Medikamente gegen TB zu unterstützen, die Heilungsrate zu erhöhen und die Infektiosität von TB zu verringern.

CHAPTER 1.6. ABBREVIATIONS

1.6. Abbreviations

25(OH)D - 25-hydroxy vitamin D

AFB - Acid Fast Bacilli

AHRI - Armauer Hansen Research Institute

ALERT - All Africa Leprosy Rehabilitation and Training Centre

ASF – Animal Source Foods

BMI - Body Mass Index

CI - Confidence Interval

CRP - C- Reactive Protein

CYP27B1 – Cytochrome P 27 B1

DDS – Diet Diversity Score

DOTS - Directly Observed Treatment Short Course

ELISA - Enzyme Linked Immunosorbent Assay

FAAS - Flame Atomic Absorption Spectrometry

FAO – Food and Agriculture Organization

FFQ - Food Frequency Questionnaire

FVS – Food Variety Score

HIV- Human Immuno-deficiency Virus

HPLC - High Performance Liquid Chromatography

IFN- γ - Interferon gamma

IL - Interleukin

IQR - Inter Quartile Range

IZiNCG - International Zinc Nutrition Consultative Group

LL – Lower Limit

MUAC - Mid Upper Arm Circumference

OR - Odds Ratio

RAE – Retinol Activity Equivalent

RDA - Recommended Dietary Allowance

SC-I - Severity Class I

SD - Standard Deviation

sVDD - Severe Vitamin D Deficiency

T regs - Regulatory T cells

TB - Tuberculosis

TLR – Toll-Like-Receptors

TN - Tierische Nahrung

TNF- α - Tumor necrosis factor alpha

UL – Upper Limit

UNICEF – United Nations Children’s Fund

UVB - Ultraviolet B ray

VDD - Vitamin D deficiency

VDR - Vitamin D Receptor

VDS - Vitamin D Sufficiency

VIF - Variance Inflation Factor

WHO – World Health Organization

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Chapter 2

General Introduction

CHAPTER 2.1. INTRODUCTION

2.1. Introduction

2.1.1. Epidemiology of tuberculosis

Tuberculosis (TB) is an old infectious disease which is caused by mycobacteria belonging to *Mycobacterium tuberculosis* complex [1] and has affected humans for thousands of years [2]. The global burden of TB remains enormous. About one-third of the global populations were infected with TB [3] and nearly 10 million people developed TB disease in 2017 [4]. Most cases of active TB occur in males [4], adults [1] and during economically productive years of people's lives (19-49 years) [5]. Undernutrition, smoking and HIV infection linked to poverty are the factors that have considerable impact on TB incidence [1, 6]. In addition, the emergence of drug resistant strains of *M. tuberculosis* complicates the management of TB [7].

2.1.2. Burden of TB in Ethiopia

Of the thirty high TB burden countries in 2017, Ethiopia ranked 25th with the incidence rate of 164 per 100,000 population [4]. According to the Ministry of Health reports, TB was the leading cause of hospital admissions and the third leading cause of hospital deaths [8]. More than half of TB patients were males and young adults between the age of 15 and 34 years [9, 10]. The high proportion of TB in young adults seems to suggest the rapid rate of TB circulation in the community [11]. Rural and pastoral communities were highly affected by TB [10]. The occurrence of chronic undernutrition, widespread poverty, overcrowding, and HIV infection increased the risk of TB infection and disease progression [12].

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2.1.3. Nutrition and TB interaction

Nutrition has an important role in preventing illness and reducing morbidity and mortality in people living with TB and other infectious diseases. A healthy diet can support to manage symptoms, maximize the benefits of medications, and enhance their quality of life [13]. Little was known about the link between nutrition and infection during the 1950s. Until that time, it was believed that protein deficiency (kwashiorkor) was the predominant basis of nutrition problems more than total calorie deficiency (marasmus). Because severe protein deficiency bore a definite relationship to antibody formation and the development of the immune system. The focus then changed to energy, with the assumption that if a person consumed enough kilocalories of energy, all nutrient needs would be met [14]. After 1959, the research on the interaction of nutrition, immunity, and infection was advanced [15] and showed extensive, synergistic, antagonistic, and cyclical interactions between malnutrition and infection [14].

The complex interaction between malnutrition and infection creates a hostile environment that perpetuates a vicious circle and leads to the two entities benefiting from each other. There are two effects that can occur in the presence of both malnutrition and infection. The first one is the synergistic effect, and it happens when an infection worsens the malnutrition or when the malnutrition contributes to decreasing the immune response to infection. The second effect is an antagonistic mechanism, which occurs less frequently than synergistic effect. The antagonistic mechanism happens when malnutrition decreases the multiplication of the infectious agent [13].

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TB and undernutrition are the major problems of public health in developing countries. They are linked in a complex relationship and have synergistic effects. TB may cause undernutrition through increased metabolic demands and decreased intake. Whereas, undernutrition may predispose people to clinical disease development or delay recovery by depressing important immune functions [16, 17]. The reactivation of latent or previously subclinical TB infection perhaps related to deteriorated nutritional status [18].

Most of health facilities in Ethiopia frequently report TB and undernutrition in the list of top ten diseases. However, only few studies done in the past considered TB and undernutrition. A cross-sectional study conducted in Addis Abeba demonstrated that more than one-third of adult TB patients were undernourished [19]. Another study conducted in Sidama, the southern region of the country, found a high prevalence of undernutrition in TB patients [20]. A study done in Gondar, the northern region of the country, also reported a higher magnitude of undernutrition in patients with TB [21].

2.1.4. Micronutrients and TB

Micronutrients have diverse metabolic characteristics and functions [18]. For instance, zinc is essential for the activities of over 300 enzymes, carbohydrate and energy metabolism, protein synthesis and degradation, nucleic acid production, heme-biosynthesis, cell differentiation, and immune function [14, 22, 23]. Vitamin A is involved in lymphocyte proliferation, macrophage activity, and maintenance of mucosal surfaces and epithelial function [24, 25]. Previous studies

have suggested that active TB is associated with low serum levels of zinc [26], vitamin A and vitamin D [17, 27, 28]. Scrimshaw et al. [15] underlined that no nutritional deficiency is more consistently synergistic with infectious disease than that of vitamin A. Zinc has essential roles in vitamin A metabolism and its deficiency impairs the synthesis of retinol binding proteins and reduces plasma retinol concentration [18, 29].

2.1.5. Immune response to TB

The variety of genetic and other risk factors for developing active TB underscore the complex relationship between the host immune system and TB infection [30]. The host protective immune mechanism depends critically on the interaction and cooperation between monocytes, macrophages and T-lymphocytes and their cytokines [31]. Following TB exposure, healthy individuals mount cell-mediated immune response. The immune response initially involves the uptake of bacterium into macrophages as part of the non-specific innate immune response, and later recruitment of T-lymphocytes, the cellular immune response [6, 32].

The lifetime risk of conversion from latent to active TB is around 5% to 10% in healthy population [33], but this can rise to around 50% in immunocompromised population [34]. Dietary deficiency causes thymic atrophy and impairs the generation and maturation of T-lymphocytes [18]. Undernutrition may selectively compromise portions of the cell-mediated immune response by reducing the expression of interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) [35].

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2.1.6. Vitamin D and TB

A role of vitamin D in resistance to active TB has been known for more than 100 years [6]. Vitamin D was used to treat TB in the pre-antibiotic era, when special sanatoria were built in sunny locations, such as the Swiss Alps [14]. Vitamin D is required for macrophage activation and blocks *M. tuberculosis* proliferation [35, 36]. Resistance to infection may be determined genetically through the ability of vitamin D to activate macrophages. Effects of vitamin D are mainly determined through a vitamin D receptor (VDR), which has common polymorphisms to influence VDR activity. Polymorphisms are potentially the means of genetic susceptibility to active TB [37]. Genetic variations in the VDR were identified as a major determinant of the risk for TB [38].

Vitamin D can be formed mainly via exposure to sun. It can also be consumed as cholecalciferol (vitamin D₃) in oily fish and vitamin D fortified foods [39] or ergocalciferol (vitamin D₂) in ultraviolet B (UVB) treated mushroom [40]. Both cholecalciferol and ergocalciferol are activated by the liver and kidney [6, 39] into 25-hydroxy (OH) vitamin D and 1,25(OH)₂ vitamin D, respectively.

Vitamin D deficiency (VDD) occurs when there is a lack of sun-exposure combined with low dietary intake of vitamin D. The deficiency of vitamin D contributes to the acquired susceptibility to TB infection and allows the disease to progress to the active form [6, 41]. The combination of a specific genotype with VDD is suggested to strongly predispose a person with latent TB to active disease [30]. Rifampin and isoniazid treatment may alter vitamin D metabolism and influence the polymorphisms during treatment as well [42]. Thus,

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supplementation with vitamin D may be necessary where sun-exposure and diets are insufficient. However, further research on the impact of vitamin D supplementation during TB treatment is needed [6].

2.1.7. Mushrooms and vitamin D

Mushrooms are fungi and belong to the division of Basidiomycota [43]. They have different biological entities compared to plants and animals, despite being considered a vegetable from a culinary perspective [44]. Consumption of mushrooms is rapidly increasing worldwide. The most commonly consumed mushrooms are *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes* which together comprise approximately three-quarters of all mushroom consumed [45]. In Ethiopia, wild mushrooms are harvested in forests during rainy season. However, cultivation of oyster mushroom is a new activity in the country in which it is used as a means of income [46].

The presence of both ergosterol and vitamin D₂ in mushrooms was first reported in the early 1930s [44, 47]. Retail fresh mushrooms have negligible amount of vitamin D₂ [48]. But when exposed to UVB radiation, ergosterol in the mushroom is transformed to pre-vitamin D₂, which is then thermally isomerised in a temperature-dependent process to ergocalciferol, commonly known as vitamin D₂ [43, 44]. Vitamin D₂ from mushrooms is as effective as supplemental vitamin D₂ [44] as well as vitamin D₃ [49] in raising and maintaining the serum 25(OH)D concentration. Vitamin D₂ is relatively stable during storage and cooking processes [44].

CHAPTER 2.2. SCOPE OF THE WORK

2.2. Scope of the work

The emergence of drug resistant strains of *M. tuberculosis*, mainly due to poor adherence to lengthy treatment, complicated the control of TB even more. TB affects mainly males and young adults who are the productive power of economy and have major contribution for food and nutrition security particularly in developing countries. There is bidirectional interaction between nutrition and TB. Nutrition plays a big role in the immune system which is critical to combat TB. TB, in turn, leads to undernutrition by increasing nutritional requirements, changing metabolic processes, causing loss of appetite, and decreasing food intake. Protein-energy undernutrition and micronutrient deficiencies increase the risk of TB. Despite such interaction, the current management program for TB has given less attention to nutrition. In the case of Ethiopia, there is a wide gap of research on nutritional problems of TB patients. Thus, understanding the underlying nutritional situation has immense value in designing appropriate interventional strategies that can support the treatment of TB. To put in nutshell, this doctoral thesis has given due consideration on nutrition and TB where the scope of the work has been concisely described by formulating the following research questions. These questions were the bases for the general discussion and conclusion of the thesis.

Research Questions

Nutritional situation- What are the dietary patterns of the communities in the study area?

Micronutrients- Which micronutrients are predominantly deficient among TB patients?

VDD – Why do TB patients in Africa face the challenge of vitamin D deficiency albeit year-round-sunshine?

Mushroom – Could sun-exposed mushroom be used as an alternative nutritional resource of vitamin D?

Vitamin D-TB – What are the roles of vitamin D on the treatment outcomes of TB?

CHAPTER 2.3. AIMS AND OUTLINES

2.3. Aims and outlines of the thesis

The aim of this doctoral thesis was to deal with the nutritional situation of the people with and without TB and come-up with solutions that could support the effort of fighting against TB. In order to understand the relationship between nutrition and TB, in fact there is a need to study the nutritional situation of patients with TB before and after infection. However, it is unethical to let the people, who are involved in the study, get the infection of TB. Rather we prefer to assess the dietary patterns of the people with and without TB and identify the risks of micronutrient deficiencies in the communities (**Paper 1**). We identified vitamin A and zinc deficiencies, protein-energy malnutrition and low nutritional status in TB patients (**Paper 2**). The problem of vitamin D provoked our attention. Sunshine, which is very important to synthesize vitamin D under the skin, is available in Africa through-out the year. But we wonder why TB patients in this continent suffer from VDD. For this reason, we systematically reviewed the extent of VDD together with the major risk factors among TB patients in the continent (**Paper 3**). To address the deficiency of vitamin D, we opt to look for locally available food resources. Having the potential for vitamin D content, cheap price and easily available in the local market of Ethiopia, we undertook a study on mushroom. In this study, the impact of sun-exposure on the content of vitamin D₂ in oyster mushroom was assessed (**Paper 4**). Moreover, a study on the effects of vitamin D₂ on the treatment outcomes of TB was performed by providing sun-exposed oyster mushroom for patients with TB (**Paper 5**).

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Chapter 3

Publications

CHAPTER 3.1. DIETARY PATTERN AND RISK OF
MICRONUTRIENT DEFICIENCIES: THEIR IMPLICATION FOR
NUTRITIONAL INTERVENTION IN ETHIOPIA

**3.1. Dietary pattern and risk of micronutrient deficiencies:
their implication for nutritional intervention in Ethiopia**

By Tibebelessie Seyoum Keflie, Aregash Samuel, Christine Lambert, Donatus Nohr
and Hans Konrad Biesalski.

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Dietary Patterns and Risk of Micronutrient Deficiencies: their Implication for Nutritional Intervention in Ethiopia

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Abstract

Background: Dietary patterns are the quantities, proportions, variety or combinations of different foods and beverages in diets, and the frequency with which they are habitually consumed. In Ethiopia, information on the dietary patterns and association of the proxies of dietary adequacy and quality with nutritional outcomes is scarce. The aims of this paper were to assess the interactions between dietary patterns, nutritional adequacy, nutritional quality and the risk of micronutrient deficiencies, and to highlight their implications in nutritional interventions.

Methods: A community based cross-sectional study was carried out in North Shewa zone of Amhara Regional State, central Ethiopia from December 2014 to February 2015. Multistage sampling techniques were employed to recruit participants and 640 subjects involved in the study. Data were collected using structured and seven-day recall questionnaires. Chi-Square test, Kruskal-Wallis test, Spearman correlation, multiple linear and multinomial regression models were used for inferential analyses.

Results: The main dietary patterns included cereals, vegetables and legumes. Animal Source Foods (ASF) was consumed by 35.4% of participants. The median (range) of Food Variety Score (FVS) and Diet Diversity Score (DDS) were 16 (8-25) and 3.43 (1.14-5.57), respectively. About 28 % of subjects were malnourished. FVS had a positive correlation with DDS ($r=0.502, p<0.001$) and Body Mass Index (BMI) ($r=0.145, p<0.001$). DDS had also a positive correlation with BMI ($r=0.19, p<0.001$). Family size and educational status were identified as determinant factors for FVS, but the later had a significant influence on DDS. The risks of vitamin A and iron deficiencies were 60.3% and 86.3%, respectively. The consumption of food groups rich in vitamin A and haem iron were significantly different across FVS and DDS ($p<0.05$).

Conclusions: Dietary inadequacy, poor nutritional quality and high risk of micronutrient deficiencies were identified. These underlined the implications of nutritional interventions in central Ethiopia.

Keywords: dietary patterns; micronutrients; FVS; DDS; BMI; Ethiopia

Abbreviations: ASF – Animal Source Foods; BMI – Body Mass Index; DDS – Diet Diversity Score; FVS – Food Variety Score; LL – Lower Limit; OR – Odds Ratio; UL – Upper Limit; UNICEF – United Nations Children’s Fund; WHO – World Health Organization.

Background

Dietary patterns are the quantities, proportions, variety or combinations of different foods and beverages in diets, and the frequency with which they are habitually consumed. The dietary patterns’ approach considers the inherent interactions between foods and nutrients in promoting either health or increasing disease risk [1]. Until quite recently, there has been extensive focus on the quantity of food produced and consumed in food security rhetoric and in policy and decision-making arenas and much less attention given to the nutritional quality of foods and diets [2].

Nutritional deficiencies are not only the result of inadequate food consumed, but also of poor dietary quality and diversity despite adequate calories in many cases [3]. The prevalence of diseases associated with a poor-quality diet is increasing in Ethiopia. Even though most people consume plant based foods, diets low in fruits and vegetables are found to be the most common risk factors contributing to a large portion of diet-related Non-Communicable Diseases (NCD) [4, 5, 6, 7]. In 2013, more than a third (35.1%) of all deaths in Ethiopia was caused by NCDs [7]. The emergence of NCDs imposes another burden on the country’s health system while it is still striving to address infectious diseases and under nutrition.

Understanding the dietary patterns and evaluating their qualities are essential for nutritional intervention. The quality of diet can be assessed using a simple score of foods variety and dietary diversity [8]. Food variety is expressed as the number of biologically distinct foods eaten over a designated period. It minimizes the adverse consequences of food on health; and reduces the risk of NCDs [9]. It is usually quantified by the number of food items compared with the number of nutritious food groups known as dietary diversity [10, 11].

Assessing Food Variety Score (FVS) is a quick, simple and low-cost method of determining the nutritional adequacy of a diet. It is believed that a nutritionally adequate diet is best achieved by consuming a diverse range of foods [12]. Likewise, individual Diet Diversity Score (DDS) is a simple proxy measure of the nutritional quality of individual's diets, particularly that of micronutrient adequacy of a diet [13]. Both FVS and DDS reflect the quality of the diet. Scores of dietary diversities have been positively correlated with macro and micronutrient adequacy of the adolescents and adults [9, 14, 15]. Savy et, al. described the importance of studying the association between proxies of overall dietary quality and nutritional outcomes [8]. Workicho et, al. also highlighted the need of tracking dietary quality and progress in nutritional outcomes in a population to develop timely interventions [16].

Until recent time, very few studies have been conducted in Ethiopia in relation to balanced and diversified diets. Of these studies, none has attempted to point out the implication of dietary patterns and risks of micronutrient deficiencies on nutritional intervention. Therefore, the aims of the present study were:

- to assess the dietary patterns, nutritional adequacy and nutritional quality of the populations;
- to examine their relationship with nutritional status; and
- to describe the implications of the outcomes in nutritional interventions in Ethiopia.

Materials and Methods

Study Area and Subjects

This study was conducted as a community based cross-sectional study in North Shewa zone of Amhara Regional State, Central Ethiopia from December 2014 to February 2015. Based on the 2007 census, 928,694 men and 908,796 women with a total of 1,837,490 people inhabited in this area [17]. Multistage sampling techniques were used to recruit study subjects. First, all kebeles, which are the smallest administrative unit, were stratified into urban and rural settings. Second, four kebeles from urban and three kebeles from rural settings were selected by simple random sampling technique. Third, households were included from each kebele by systematic random sampling technique. The first household was selected by a lottery system and then, every third household was included in the study. Lastly, one study subject was randomly selected from each household.

The criteria to include study subjects were age above 18 years,

living in the house for at least 6 months and willing to participate in the study. Subjects who were absent during the survey, disabled, seriously ill or had some difficulty of communication were excluded. Single population proportion formula was used to determine the sample size. Taking the assumptions of 50% dietary intake below average DDS with 95% confidence interval, 4% margin of error and 10% drop out rate, a sample size of 660 was obtained. However, we recruited 100 study subjects from each kebele with a total of 700 study subjects to participate in the study.

Data collection

The data were collected by seven health extension workers who have been graduated with diploma (2 to 3 years' college training) in nursing. Questionnaires which comprised of different parts were translated into Amharic, the local language. In the structured questionnaire, there were socio-demographic and anthropometric parts, whereas; in the dietary diversity questionnaire, the dietary intake assessment part was included. All interviewers were given one-day training on the content of the questionnaires and on the techniques, how to ask the list of ingredients for composite dishes and how to probe for meals and snacks not indicated in the list of 7-day recall. Before instigating the interview, each of them practiced on the questionnaire to alleviate ambiguity and minimize errors as much as possible.

Dietary Assessment

Seven-day recall of dietary intake was carried out on the study subjects. The dietary diversity questionnaire, which was adapted from the guidelines for measuring household and individual dietary diversity, was employed for face-to-face interview [12]. Each subject was asked to describe what he or she ate and/or drank for breakfast, snack, lunch, snack and dinner whether at home or outside the home for the past seven days. The interviews included a detailed description of foods consumed, the ingredients used, the cooking method, and brand names (for packed foods). The food items were subsequently transformed into food groups and their frequencies of consumption were computed and used for further analyses Table 1.

Food Variety Score (FVS)

FVS was measured using simple count of individual food items consumed during the seven days. Food variety checklist developed by Savige et, al. was used to score the food items [12]. Each type of food consumed is scored once over a week time. The maximum score would be 57. Quantities smaller than 1-2 tablespoons (except for fats and oils) do not represent a sufficient quantity to rate FVS. The results of FVS were categorized into five dietary adequacy groups: very poor (<10 FVS per week), poor (10-19 FVS per week), fair (20-24 FVS per week), good (25-30 FVS per week) and very good (>30 FVS per week) [12].

Diet diversity score

Individual DDS was calculated as the number of food groups consumed during the first day recall. These food groups were

Table 1: Food groups used for dietary diversity score*

Number	Food groups	Subgroups	Scores (if consumption is: yes=1, otherwise: no=0)
1	Starchy staples	Cereals, grains, white roots and tubers	1 or 0
2	Dark green vegetables	Locally available vitamin A rich vegetables such as kale, lettuce, spinach and wild forms such as samma (stinging nettle)	1 or 0
3	Other vitamin A rich fruits and vegetables	vitamin A rich fruits (mango), vegetables (carrot) and tubers (vitamin A blended sweet potatoes)	1 or 0
4	Other fruits and vegetables	Fruits: such as avocados, banana, dates, etc.	1 or 0
		Vegetables: such as cabbage, onion, garlic, green pepper, tomatoes, etc.	
5	Organ meat	Red organ meats such as liver, kidney, heart and any processed organ meats	1 or 0
6	Meat and fish	Beef, lamb, goat meat, chicken and fresh fish	1 or 0
7	Eggs	Chicken eggs, quail eggs	1 or 0
8	Legumes, nuts and seeds	Legumes/pulses: such as beans, peas, lentils, peanuts, etc.	1 or 0
		Seeds: such as oil seeds and pumpkin seeds	
9	Milk and milk products	Dairy products such as milk, butter, sour milk, butter milk, cheese and whey	1 or 0

*Adapted from the guidelines for measuring household and individual dietary diversity [13].

based on the guidelines for measuring household and individual dietary diversity Table 1. The score for individual diet diversity goes from 0 to 9 [13]. The percentages of the consumption of food groups rich in micronutrients such as vitamin A or iron were calculated using the food groups of DDS

Anthropometric Measurements

The weights of the subjects were measured while they were dressed in light clothes to the nearest of 100g. SECA personal weighing scale (used by UNICEF) was employed for measurement. The heights of the subjects were measured using tape meter fixed on the wall without shoes to the nearest of 0.1 cm. For the calculation of Body Mass Index (BMI), the weight of the subject (in kg) was divided by the height (in meter) squared of the subject. BMI was described as underweight (<18.50 kg/m²), normal weight (18.50-24.99 kg/m²), overweight (25-30 kg/m²) and obese (>30 kg/m²) [18].

Statistical Analysis

Statistical analysis was carried out using IBM SPSS version 23 statistical program. Continuous data were checked for normality using Kolmogorov-Smirnov test. When data were not normally distributed (p>0.05), nonparametric tests were used. In descriptive summaries, median (range) and percentages were used to present the data. Inferential statistics were also employed. The difference between proportions of categorical variables was examined by Chi-Square test. Kruskal-Wallis test was used to determine the differences between socio demographic variables on FVS, DDS and BMI.

Bivariate analyses were carried out to test the links between socio demographic variables and dietary scores. The socio demographic variables which were significantly linked to either dietary scores or BMI were selected as potential confounders (P < 0.15). Significant variables subsequently included into the

multivariate analysis in order to better identify the collinearities between variables ($P < 0.05$). Multiple linear regression model was employed to differentiate the independent predictors of FVS and DDS after adjusting for confounding factors.

Spearman correlation was used to examine the association between FVS, DDS, BMI, age and average meal frequency. The relationships between the groups of FVS, DDS and BMI were analysed using a multinomial logistic regression model. Odds Ratio (OR) was used to report the strength of association between the proportions of vitamin A and haem iron rich foods consumption between urban and rural areas. Unless specified, p value < 0.05 was considered as statistically significant.

Results

Socio-demographic study

The study was undertaken in seven kebeles in urban and rural areas of North Shewa zone of Amhara Regional State, Central Ethiopia. A total of 700 participants were recruited. But, 640 were involved in the study with a response rate of 91.4%. The remaining 8.6% were excluded because of their refusal and absence during the study period. Table 2 presents the socio-demographic characteristics of the study participants. Most participants belonged to Amhara ethnic group (93.5%) and Orthodox Tewahido Christian (89.8%). The median age

Table 2: Socio demographic characteristics

Variables		Percent
Living place (n=640)	Urban	54.8
Gender (n=640)	Female	44.7
Age	Median: 35, Range (18, 76)	
Religion (n=616)^a	Orthodox Tewahido	89.8
	Muslim	3.7
	Protestants	4.4
	Others	2.1
Ethnicity (n=631)^b	Amhara	93.5
	Oromo	3.2
	Gurage	0.8
	Tigre	2.5
Occupations (n=613)^c	Farmers	34.6
	Government Employees	21.7
	Non-Government Employees	3.9
	Private	25
	House wife	10.6
	Retired	0.8
Education (n=620)^d	Students	3.4
	Illiterate	15
	Primary	37.9
	Secondary	17.9
	Tertiary	27.3
Marital Status (n=610)^e	Religious teaching	1.9
	Single	23.1
	Married	68.7
	Divorced	5.4
Family size (n=640)	Widowed	2.8
	1 to 3	57.5
	4 to 6	37.2
	7 to 10	5.3

Missed participants: 24 (a), 9 (b), 27 (c), 20 (d) and 30 (e)

was 35 years (range= 18 to 76 years). A little below half of the participants were females (44.7%), 54.8% were urban dwellers, 34.6% were farmers, 62.1% had at least primary education, 68.7% were married and 57.5% had 1 to 3 family size.

Dietary patterns

About 130 food items were identified in the study areas (See additional file 1). The main dietary patterns were included cereals, vegetables and legumes. Almost all subjects consumed starchy staples (99.7%) and about 58% consumed legumes

cooked with oils and fats (99.2%) for the whole week Table 3. In every day meal, 95.6% consumed the food group of other fruits and vegetables Table 4. Dairy products (62.4%), dark green vegetables (49.69%), meat (37.9%) and, other vitamin A rich fruits and vegetables (30.35%) were consumed at least once in a week Table 4, 5. On the other hand, organ meat (2.6%) and fish (3.6%) were seldom consumed by the subjects. The food consumption patterns were significantly different between urban and rural areas ($P < 0.05$).

Additional file 1: Food items identified in North Shewa, Ethiopia		
Cereals and grains		Legumes/ pulses
Ambasha, circular flat bread dough	Kinche, boiled splitted barley served with butter	Ashuk, roasted and boiled faba bean
Anebabero, double injera covered with butter and chilli in the middle	Kinche, boiled splitted wheat served with butter	Bokolt, germinated faba bean
Atmit, very thin barley porridge	Kita, unleavened flat barley bread	Endushdush, soaked and roasted faba bean
Atmit, very thin wheat porridge	Kita, unleavened flat teff bread	Fool, pureed stewed faba bean
Besso, roasted and milled barleyflour served with butter	Kita, unleavened flat wheat bread	Nifro, boiled chickpea
Biscuit, homemade fried dough bread	Kolo, roasted and mixed barley, chickpea and pea	Nifro, boiled faba bean
Bonbolino, homemade fried dough bread containing sugar	Kolo, roasted and mixed wheat, chickpea and sunflower	Nifro, boiled faba bean and maize
Bread, wheat	Kolo, roasted barley	Nifro, boiled faba bean and wheat
Cake	Kolo, roasted chickpea	Shorba, lentil, carrot, and macaroni soup
Chechebisa, pieces of barley bread mixed with butter	Kolo, roasted pea	Shorba, lentil, pea and carrot soup
Chechebisa, pieces of wheat bread mixed with butter	Kolo, roasted wheat	Siljo, fermented faba bean, sunflower and mustard slurry
Cukis	Macaroni	Wot, beyeyinet -varieties of stews
Dabo-kolo, very small size roasted bread dough	Nifro, boiled wheat	Wot, faba bean, chili, onion and oil stew
Fetira, fried filo dough cooked with egg and covered with honey	Pizza	Wot, pea flour, onion, chili and oil stew
Firfir, pieces of barley injera with stew	Porridge, barley served with butter and chili	Wot, splitted lentil, chili, onion and oil stew
Firfir, pieces of bread with stew containing butter	Porridge, wheat served with butter and chili	Wot, splitted pea, onion, chili and oil stew
Firfir, pieces of teff injera with stew containing beef	Sambusa	Wot, splitted pea, onion, oil and turmeric stew
Firfir, pieces of teff injera with stew containing butter	Sandwich, sliced bread with fried egg in the middle	Wot, tegabino - pea flour, onion, chili, garlic and oil stew
Firfir, pieces of teff injera with stew containing dried beef	Spaghetti, pasta	Wot, whole lentil, onion and oil stew
Fitfit, pieces of teff injera mixed with beef broth	Steamed rice	
Fitfit, pieces of teff injera mixed with pea flour, onion and oil sauce	Tirosho, flat barley bread dough covered with butter	

Fitfit, pieces of teff injera mixed with sunflower sauce	Tirosho, flat wheat bread dough covered with butter	
Injera, barley		
Injera, teff		
Injera, wild oat		
Vegetables and tubers	Meat	Sugar / Confectionary
Atkilt, mixed vegetables and fruits	Beef with steamed rice	Sugar
Bula - false banana porridge served with butter	Dulet, semi roasted organ meat (sheep and goat) with butter	Honey
Ethiopian kale	Kikil, boiled beef	Sugar cane
Fried potatoes	Kikil, boiled egg	Salts and spices
Kariya, green pepper	Kikil, boiled goat meet	Salt
Kariya, sinig - green pepper stuffed with onion and oil	Kikil, boiled mutton	Bird's eye chili
Kikil, boiled potatoes	Kitfo, raw or sautéed minced beef mixed with chili and clarified spicy butter	Bishop's weed
Kikil, boiled sugar beet	Milas na senber, roasted cow tongue and rumen	Black cumin
Lettuce with onion, oil and aceto vinegar	Raw beef	Cardamom
Raw tomatoes with onion, green peppers and oil	Roasted beef	Cinnamon
Samma, Stinging nettle	Roasted goat meat	Cloves
Shorba, vegetables soup	Roasted mutton	Coriander seeds
Sils, roasted tomatoes with onion, oil and green pepper	Shoriba, beef broth	Rue
Swiss chard	Wot, beef with kale	Alcohol beverages
Wot, beetroot, onion and oil stew	Wot, minced beef and egg stew	Tela, local beer
Wot, cabbage, onion and oil stew	Wot, red beef stew	Keribo, hops free local drinks
Wot, cabbage, potatoes, carrot, onion and oil stew	Wot, red chicken stew	Tej, mead honey or sugary wine
Wot, carrot, onion and oil stew	Wot, red dried beef stew	Areke, homemade hard liquour
Wot, kale, garlic, onion, and oil stew	Wot, red mutton stew	Wine
Wot, potatoes, onion, chili and oil stew	Eggs	Beer
Wot, pumpkin, chili, onion and oil stew	Chicken eggs	
Wot, stinging nettle and barley flour stew	Dairy products	
Wot, Swiss chard, onion and oil stew	Aguat, whey	
Wot, tomatoes, chili, onion and oil stew	Arera, butter milk	
Fruits	Ayib, Cheese	
Avocado	Butter	

Banana	Milk	
Juice, mixed	Yoghurt	
Juice, avocado	Fish	
Juice, mango	Fresh fish	
Mango	Fats and oil	
Orange	Oil	
Temir, dates	Butter	
Tringo, citron		

Table 3: Percentages of the consumption frequencies of starchy staples, legumes and others per week

Food groups		Urban (n=347)	Rural (n=289)	Total (n=636)	P-value
Starchy staples	1-3 days	0	0.7	0.3	0.206
	The whole week	100	99.3	99.7	
Legumes, nuts and seeds	None	0.6	0	0.3	<0.001*
	1-3 days	6.1	1.4	3.9	
	4-6 days	50.1	23.2	37.9	
	The whole week	43.2	75.4	57.9	
Oils and fats	None	0.9	0	0.5	0.086
	1-3 days	0	0.7	0.3	
	The whole week	99.1	99.3	99.2	
Spices, condiments and beverages	None	0.3	0	0.2	0.285
	1-3days	0	0.7	0.3	
	4-6days	0.3	0.7	0.5	
	The whole week	99.4	98.6	99.1	

*Statistically significant

Table 4: Percentages of the consumption frequencies of fruits and vegetables per week

Food groups		Urban (n=347)	Rural (n=289)	Total (n=636)	P-value
Dark green vegetables	None	46.4	57.1	51.3	0.001*
	1-3 days	42.9	39.4	41.4	
	4-6 days	8.6	3.5	6.3	
	The whole week	2	0	1.1	
Other vitamin A rich fruits and vegetables	None	58.5	83	69.7	<0.001*
	1-3 days	35.2	15.9	26.4	
	4-6 days	4.6	0.7	2.8	
	The whole week	1.7	0.3	1.1	
Vegetables	None	0.6	0	0.3	<0.001*
	1-3 days	0.6	0.7	0.6	
	4-6 days	3.4	26	13.6	
	The whole week	95.4	73.4	85.5	

Fruits	None	85.1	97.2	90.6	<0.001*
	1-3 days	13.4	2.8	8.6	
	4-6 days	0.9	0	0.5	
	The whole week	0.6	0	0.3	

*Statistically significant

Table 5: Percentages of the consumption frequencies of meat, organ meat, eggs and dairy per week

Food groups		Urban (n=347)	Rural (n=289)	Total (n=636)	P-value
Meat	None	47.6	79.6	62.1	<0.001*
	1-3 days	40.3	19.4	30.8	
	4-6 days	11	1	6.5	
	The whole week	1.2	0	0.6	
Organ meat	None	95.7	99.7	97.5	0.003*
	1-3 days	4	0	2.2	
	4-6 days	0.3	0	0.2	
	The whole week	0	0.3	0.2	
Eggs	None	73.2	93.8	82.5	<0.001*
	1-3 days	25.9	6.2	11	
	4-6 days	0.9	0	0.5	
Dairy products	None	44.4	29.4	37.6	<0.001*
	1-3 days	34.3	43.6	38.5	
	4-6 days	19	26	22.2	
	The whole week	2.3	1	1.7	

*Statistically significant

Meal Frequency

The median meal frequency was 3 (range= 1 to 5). Comparing its distribution, average meal frequency had statistically significant difference across urban and rural areas ($P<0.001$).

Food variety score

The median FVS was 16 (range= 8 to 25) per week. Urban and rural dwellers had a significant difference in FVS ($P<0.001$) Table 6. More than 98% of participants had poor and fair FVS in the areas Figure 1. There was also a significant FVS difference between socio demographic variables such as occupation ($P<0.001$), educational status ($P<0.001$) and family size ($P<0.001$). Spearman's rank correlation showed that FVS had a positive association with DDS ($r= 0.502$, $P<0.001$) and BMI ($r= 0.145$, $p < 0.001$). However, it had a negative correlation with average meal frequency ($r= -0.102$, $P = 0.01$). The results of regression model revealed that educational status and family size had significant influences on FVS ($P< 0.05$) Table 7.

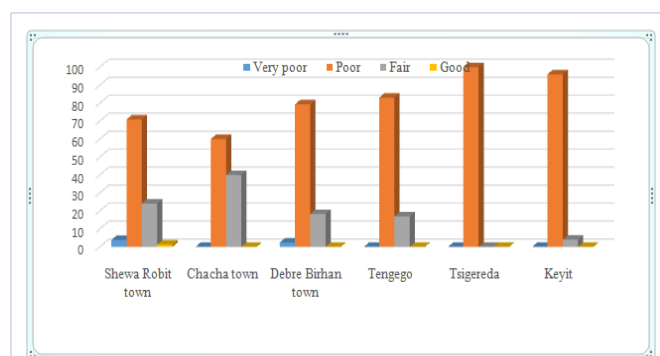


Figure 1: Food variety scores in different locations

Diet diversity score

The median DDS was 3.43 score (range = 1.14 to 5.57). The DDS of urban and rural dwellers was significantly different ($P=0.004$) Table 6. Nearly, 41% of subjects had 2.50-3.50 scores

Table 6: FVS, DDS and BMI distribution across urban and rural settings

Variables	Urban			Rural			P-value
	N	Mean	SD	N	Mean	SD	
FVS	348	17.09	3.35	289	15.31	2.51	<0.001*
DDS	348	3.63	0.63	289	3.45	0.38	0.004*
BMI	340	22.96	3.86	250	22.31	3.2	0.028*

*Statistically significant

Table 7: Prediction of the effect of explanatory variables on dietary scores and BMI using multiple linear regression model

Explanatory variables	FVS		DDS		BMI	
	β (standardized)	P-value	β (standardized)	P-value	β (standardized)	P-value
Occupations	0.046	0.247	0.01	0.811	0.075	0.082
Educational status	0.198	<0.0001*	0.122	0.004*	0.054	0.230
Family size	-0.171	<0.0001*	-0.045	0.292	-0.009	0.836
R²	0.094		0.021		0.01	

*Significant contribution

Table 8: Vitamin A rich food groups used in vitamin A analysis

Pro-vitamin A			Pre-vitamin A		
Food group	Food item	RAE ($\mu\text{g}/100\text{g}$)*	Food group	Food item	RAE ($\mu\text{g}/100\text{g}$)*
Vitamin A rich vegetables or tubers	Carrot, raw	835	Organ meat	Liver (cattle), raw	4968
	Carrot, cooked	852		Liver (cattle), cooked	9442
	Sweet potato (orange or dark yellow), raw	709		Liver (sheep), raw	7391
	Sweet potato (orange or dark yellow), cooked	1043		Liver (sheep), cooked	7491
	Pumpkin, raw	426		Kidney (cattle), raw	419
	Pumpkin, cooked	288		Kidney (cattle), cooked	0
Dark green leafy vegetables	Kale, raw	500	Eggs	Kidney (sheep), raw	95
	Kale, cooked	681		Kidney (sheep), cooked	137
	Spinach, raw	469		Chicken eggs, raw	160
	Spinach, cooked	524		Chicken eggs, cooked, fried	219
	Lettuce, raw	370		Quail eggs	156
Vitamin A rich fruits	Apricots	96	Milk and milk products	Milk, low fat	71
	Mango	54		Butter	684
	Papaya	47		Sour milk, cultured	124
				Butter milk, whole	47
				Cheese, fat free	11
				Whey, acid fluid	2

*USDA [42].

Table 9: Haem iron rich food groups used in iron analysis

Food groups	Food items	Haem iron (mg/10)*	Food groups	Food items	Haem iron (mg/100g)*
Organ meat	Liver (cattle), raw	4.9	Flesh meat	Beef (meat and by products), raw	5.67
	Liver (cattle), cooked	6.54		Beef, chuck for stew, all grades, cooked	2.96
	Liver (sheep), raw	7.37		Goat, raw	2.83
	Liver (sheep), cooked	8.28		Goat roasted	3.73
	Kidney (cattle), raw	4.6		Lamb, ground, raw	1.55
	Kidney (cattle), cooked	5.8		Lamb, ground, cooked	1.79
	Kidney (sheep), raw	6.38		Chicken meat, stewed	1.17
	Kidney (sheep), cooked	12.4		Fish and seafood	Catfish, raw
	Heart (cattle), raw	4.31	Catfish, cooked		1.43
	Heart (cattle), cooked	6.38	Tilapia, raw		0.56
	Heart (sheep), raw	4.6	Tilapia, cooked, dry heated		0.69
	Heart (sheep), cooked	5.52	Mixed species, raw		0.89
			Mixed species, cooked		1.14

*USDA [42].

and 55% had 3.51-4.50 scores. Altogether, 96% of subjects had scores below average DDS (4.5 out of 9 DDS). The bivariate analysis showed that there was statistically significant mean DDS difference between age group ($P=0.028$), occupation ($P=0.027$), educational status (<0.001), marital status ($P=0.018$) and family size (0.004). DDS had also a positive correlation with BMI ($r=0.190$, $P<0.001$) and average meal frequency ($r=0.219$, $P<0.001$). After controlling for occupations and family size, educational status had a significant influence on DDS Table 7.

Vitamin A and haem iron indicators

Table 8 and 9 indicate the food groups that were used for the analyses of the consumption of vitamin A and haem iron rich foods. Vitamin A can be obtained from foods, either as preformed retinoids (with biological activity of retinol) in animal products or as pro-vitamin A Carotenoids, mainly β -carotene in plant products. Vitamin A and haem iron intake are usually expressed as retinol activity equivalent (RAE) and mg/100g, respectively. The recently accepted conversion factor for pro-vitamin A such as β -carotene is 12, for β -cryptoxanthin and α -carotene is 24, meaning that $12\mu\text{g}$ β -carotene and $24\mu\text{g}$ β -cryptoxanthin or α -carotene, respectively, supposedly exert the activity of $1\mu\text{g}$ vitamin A [19, 20].

Percentages for consumption of vitamin A and haem iron rich food groups were estimated using the first day data. Of 640 participants, 39.7% consumed either plant or animal source of vitamin A and 13.7% consumed organ meat, flesh meat or fish source of haem iron. In other words, the risk of vitamin A deficiency was 60.3% and about 86% of the consumers did not

obtain animal source of iron. The consumption of Animal Source Food (ASF) was 35.4%.

The odds of consuming all types of vitamin A rich food groups in urban settings were 1.69 times higher than the odds of rural settings. Similarly, haem iron rich food groups were 9 times more likely to be consumed in urban settings than rural settings Table 10. As indicated in Table 11, the consumption of food groups rich in vitamin A and haem iron were significantly different across FVS and DDS ($P<0.05$).

Vitamin D rich foods and iodized salt consumptions

Vitamin D rich food sources such as fish, organ meat and sun treated mushroom were consumed by 3.6%, 2.6% and 4.0% of participants, respectively. This indicated that more than 95% of the study participants were at risk of vitamin D deficiencies. Salt iodization is basically a safe and effective strategy for the prevention and control of iodine deficiency disorders. Salt consumption rate was very high, however, only 69.5% of subjects used iodized salt in their diet. This was far below the target of WHO iodine coverage (90%) [21].

Food taboos

About 20% of participants avoided one or more food items from their diet. Bread (3.1%), milk (2.8%), fermented injera (1.7%), raw beef (1.7%) and tomatoes (1.3%) were the major food items reported as food taboos. Table 12 shows the percentages of avoided food items from consumption.

Nutritional status

Anthropometric measurements showed that the median

Table 10: Indicators for the consumption of vitamin A and haem-iron rich food groups

Food groups		Percent (n=640)	Urban vs Rural		Pearson Chi-Square	P-value	Odds Ratio	95% CI	
			Urban	Rural				LL	UL
Plant Vitamin A (Pro-vitamin A)	Yes	15.8	81	20	30.78	<0.0001*	4.03	2.39	6.71
	No	84.2	270	269					
Animal Vitamin A (Pre-vitamin A)	Yes	29.5	109	80	0.78	0.377	1.18	0.83	1.65
	No	70.5	242	209					
All Vitamin A	Yes	39.7	159	95	9.84	0.002*	1.69	1.21	2.31
	No	60.3	192	194					
Animal Source Food	Yes	35.5	142	85	8.13	0.004*	1.63	1.16	2.25
	No	64.5	209	204					
Haem Iron	Yes	13.7	79	9	49.86	< 0.001*	9.04	3.61	18.2
	No	86.2	272	280					

*Statistically significant

Table 11: Vitamin A and haem iron rich food groups consumption across FVS and DDS

Scores	Categories	Vitamin A consumption			P-value	Haem iron consumption			P-value
		Yes n=251	No n=386	Chi-square test		Yes n=88	No n=549	Chi-square test	
Food variety score	< 10 FVS/week	0.8	1.3	13.15	0.004*	0.0	1.3	16.01	0.001*
	10-19FVS/week	75.3	85.5			68.2	83.6		
	20-24FVS/week	23.5	13.2			31.8	14.9		
	25-30FVS/week	0.4	0.0			0.0	0.2		
Diet diversity score	< 2.50 DDS	0.8	0	44.09	< 0.001*	2.3	0	17.6	0.001*
	2.50-3.50 DDS	27.1	50.5			29.5	43.2		
	3.51-4.50 DDS	64.9	47.9			63.6	53.2		
	4.51-5.50 DDS	7.2	1.6			4.5	3.6		

*Statistically significant

body weight, height and BMI were 60 (range= 37 to 89) kg, 1.65 (range= 1.2 to 1.9) m and 22.05 (range= 13.49 to 40.21) kg/m², respectively. Men and women had statistically significant difference in body weight and height measurements ($P < 0.001$). Our study revealed that 6.9% of subjects were underweight, 17.1% were overweight and 4.1% were obese. This implied that the proportion of malnutrition in the area was 28.1%. Unlike body weight and height, men and women did not have a significant difference in BMI ($P= 0.164$). But, there was a statistically significant difference in BMI across urban and rural settings ($P=0.028$) Table 6. The link between BMI and DDS was limited.

Discussion

Dietary Patterns

We identified about 130 food items. The major dietary patterns composed of cereals (teff, wheat and barley), vegetables (onion, green pepper, tomato and cabbage), legumes (peas, faba bean and lentils), oils (cooking oil) and spices (salt). All these food items are the ingredients of injera and thick stew made from flour of roasted legumes ('shiro wot'). The trend of taking hot beverages (coffee and tea) with sweets (sugar) was habituated by almost all people. In line with this finding, other studies also reported cereals, vegetables, legumes and oils as the main staples in Ethiopia [16, 22].

Relying on such dietary patterns implied that starchy staples and legumes are the predominant sources for energy and protein, respectively. Energy-dense foods, especially mixtures of sugars and fat, tend to be more palatable than foods of low energy density and high-water content [23]. Excessive intake of beverages and sweets containing added sugar could be a driving force behind obesity epidemic [24].

Less frequently, food groups containing dairy products (milk, butter, butter milk, yogurt and cheese), dark green vegetables (kale, spinach and lettuce), meat (beef, lamb, goat meat and chicken) and other pro-vitamin A rich fruits and vegetables (apricots, mango, carrots and pumpkins) were included in the dietary patterns. This was substantiated by the reports of Workicho, et al. and Amare, et al. in which they indicated that fruits and animal products were less frequently consumed in Ethiopia [16, 25]. Although the country has a very large livestock population, the availability of meat and other animal products for local human consumption is limited mainly due to economic reasons [26].

We also identified that the dietary patterns rarely entailed food groups containing fish (fresh fish) and organ meat (liver, kidney, heart and tripe). Despite abundant resources, fish consumption in Ethiopia is very limited. This is due to cultural factors and poor connections between production areas and markets. Fish is mostly consumed in large towns during periods of religious fasting [26]. There was also a limited access to organ meats. One of the reasons could be infection. Most of the time, livers from cattle and sheep are infected by internal parasites and as the result they are condemned from consumption.

Our results showed that dietary patterns were significantly different in urban and rural settings ($P < 0.05$). The difference could be attributed to availability and accessibility of food groups. Urban people are very close to the market where much variety of foods could be available. Although all people could not have equal access to food varieties because of affordability, the case for rural people is even worse. Large numbers of rural people are living at subsistence level and far away from the market so that they have less access and economic power to purchase food varieties with high price.

Meal frequency

The median meal frequency per day was 3 with a range of 1 to 5. But, another study reported a range of 2 to 3 meal frequency per day [27]. This implied that the lowest consumption rate was 1 and the highest was 5. The consumption rate significantly varied in urban and rural areas ($P < 0.001$). This could be justified by the difference in food groups availability and accessibility in urban and rural settings.

Food varieties

The median FVS was 16 per week but the range varied from 8 to 25 per week. Urban dwellers had a higher FVS as compared to rural dwellers. Based on the classification made by Savige, et al. 98% of people had FVS between poor and fair dietary adequacy

[12]. This result is prone to seasonal fluctuation. The scenario could be even worse during low production season in rural settings as the present study was conducted during harvesting season. FVS had a positive correlation with DDS ($r = 0.502$, $P < 0.0001$) and BMI ($r = 0.145$, $P < 0.0001$). But, it had a negative correlation with average meal frequency ($r = -0.102$, $P = 0.01$). This inverse association suggested a monotonous type of diet. Besides, Kant explained that consuming some food items more frequently means that other food items are being consumed less frequently [28].

Socio demographic characteristics such as educational status and family size were linked to FVS, but their impacts were different. Educational status had a positive influence on FVS. This implied that educated persons better understand the health benefit of consuming nutritious foods and spend much budget on food varieties. This was corroborated by other studies done in Ethiopia and in Tanzania [16, 29]. On contrary, family size had a negative influence on FVS. Increasing the members of family without increasing income could deter the access to food varieties. Income and supply of foods had great impacts on the dietary diversity of food consumed [30].

Dietary diversity

Dietary diversity is a qualitative measure of food consumption which evaluates the dietary quality of the individuals. Based on the guideline for individual DDS nine food groups were used for assessment and the median DDS was 3.43 (range = 1.14 to 5.57). This was comparable with the mean DDS of 3.4 reported by Weldehaweria, et al. among lactating women in Tigray, Ethiopia [13]. In the same region, another study reported a median of 5 DDS, but 14 food groups were used for evaluation [22]. Hence, it is very difficult to compare the three results owing to the difference in study subjects and number of food groups used for counting. The implication of the median 3.43 DDS was that half of the people at least included 3 food groups in their diet. Mostly, starchy staples, vegetables and legumes were involved in the dietary patterns.

About 96% of people in the study areas had DDS below an average 4.5. A diet of at least 4 DDS was valid as nutritionally adequate, but below 4 DDS represented poor diversity [32, 33]. DDS was directly associated with BMI and average meal frequency. After controlling for age group, occupation, marital status and family size, the influence of educational status on DDS was significant. This was supported by Workicho, et al. and Mbwana, et al. [16, 29]. However, there were reports that showed the inverse relationship between education and DDS [25, 34, 35]. The explanation given was that although some people particularly women were educated; their employment rate was very low and have less income as the result they relied on poor nutrition.

Vitamin A and iron indicators

DDS can be used as a proxy indicator to assess the likelihood of achieving micronutrient requirements [16]. Micronutrients can be obtained from vegetables, fruits and Animal Source Foods

(ASF). Failing to consume such kind of foods regularly may impair the immune systems and prone to infectious diseases. In the present study, consumption of vitamin A and haem iron rich food groups were assessed using the first day data of food consumption.

The results showed that 39.7% of people consumed either plant or animal source of vitamin A and 13.7% consumed organ meat, flesh or fish as source of haem iron. This implied that the remaining 60.3% was at risk of vitamin A deficiency and 86.3% was unable to get haem iron sources of foods. According to WHO definition, when food groups with high vitamin A content are consumed less than three times in a week by three fourth or more of vulnerable groups, there is a high risk of inadequate vitamin A status [36]. Given this definition, the result of the present study revealed that there were high risks for vitamin A and iron deficiencies in Central Ethiopia.

Table 12: Food items avoided from the diet

Food group	Food item	Percent
Vegetables	Lettuce	0.94
	Kale	0.63
	Tomatoes	1.26
	Beetroot	0.47
Fruits	Avocado juice	0.47
Cereals	Bread	3.14
	Porridge	1.1
	Roasted wheat	1.1
	Fermented injera	1.73
Legumes and seeds	Lentils	0.47
	Linseeds	0.47
Meat and Fish	Raw beef	1.73
	Goat meat	0.47
Eggs	Fried egg	0.47
Dairy products	Milk	2.83
	Yogurt	0.63
	Cheese	0.47
Salts and sugar	Salt	0.94
	Sugars	0.47

We compared the consumption of vitamin A and haem iron rich food groups in urban and rural settings. The results showed that urban people were nearly 1.7 times and 9 times more likely to consume vitamin A and haem iron rich food groups than rural people, respectively. This, in turn, implied that the risks of vitamin A and iron deficiencies would be higher among rural communities than urban communities. This was corroborated by the report of Alaofe, et al. that vitamin A and iron deficiencies were high among rural communities particularly women and their children

in Northern Benin [37]. The consumption of vitamin A and

haem iron rich food groups was significantly different across FVS and DDS ($P < 0.05$). This suggested that people who are consuming less variety of food items and diversity of food groups are less likely to incorporate food groups rich in vitamin A and haem iron in their diet.

Vitamin D and iodine indicators

Low intake of vitamin D was realized in the study areas. Very few people consumed fish (3.6%), organ meat (2.6%) and sun treated mushrooms (4%). Although vitamin D rich foods are the second sources of vitamin D next to sunshine Keflie, et al. in their review paper reported the predictors of vitamin D deficiencies in tropical African countries. Taking the predictor factors into account, the risk of vitamin D deficiencies could be anticipated to be very high. Iodine consumption was also assessed, and the result revealed that 69.4% of people consumed iodized salt albeit high salt consumption rate. This implied that salt iodization coverage did not reach to at least three fourth of the communities. However, WHO suggested 90% coverage for the prevention and control of iodine deficiency disorders [21]. In other words, more than one fourth of the people are at risk of iodine deficiency disorders.

Food taboos

Food taboos were another concern. One fifth of people avoided one or more food items from their diet. The major reasons ascribed to food taboos were culture and health related problems. Some people usually avoided consumptions of raw foods such as tomatoes, vegetables, beef and milk due to their fear for infectious diseases; fermented injera and bread for stomach ache; and eggs and goat meat for cultural reasons.

Nutritional status

The results of BMI (median=22.05; range= 13.49-40.21kg/m²) illustrated that 28.1% of people were malnourished. Of whom, 6.9% were underweight, 17.1% were overweight and 4.1% were obese. The proportion of overweight people was larger than the proportions of underweight and obese. This suggested that overweight has become a serious public health concern followed by underweight and obesity. There is a perception among many Ethiopian communities that overweight and being obese are indicators for wealth and health status. BMI had a statistically significant difference across urban and rural settings. The proportion of overweight and obesity were like the report of Amare, et al. in Gondar town of Ethiopia (21.3%) [25]. But, the proportion of overweight alone was higher than those reported among adolescent girls in rural Southern Ethiopia (13.8%) [27]. This suggested in general that malnutrition is the major public health problem in Ethiopia.

After the socio demographic characteristics were controlled, the link between BMI and DDS became insignificant. Savy, et al. described that the socio demographic and economic context could reduce the strength of the link between nutritional status and DDS [8]. This was explained that nutritional status was not

only determined by the quality of food but also the quantity of the foods consumed.

In several studies done elsewhere, height less than 1.45m was used as a cut-off point for determination of stunting in women [39-41]. Based on this threshold, the anthropometric results indicated that 4.6% of people were stunted, of whom three fourth were women. The proportion of stunted women was slightly comparable to the report of 2.2% from a study among lactating women in Tigray, Ethiopia [22]. Even though, the proportion of underweight women was higher than that of men, the nutritional status was not significantly different. This showed that both men and women were affected by malnutrition without any difference. And hence, the nutritional intervention measures should give emphasis on both genders.

Limitations

Although this study has the strength of dealing with dietary patterns, nutritional adequacy and nutritional quality, the dietary assessment instruments used to define the dietary patterns are based on self-report and may inflict some levels of report bias. This study does not contain data on seasonal variations of food consumption as it is a community based cross-sectional study.

Conclusions

In conclusion, the people of North Shewa, Central Ethiopia have cereal and legume based dietary patterns. Almost all of them have poor dietary adequacy and nutritional quality, and 28.1% are malnourished. Overweight and obesity are the upcoming nutritional problems besides under nutrition. Family size and educational status determine FVS. But, the later determines DDS. Related with low frequency of consumptions, the risks for vitamin A, vitamin D, iron and iodine deficiencies are very high. All these nutritional problems underlined the implications of nutritional interventions. Therefore, by giving emphasis on the improvement of food and nutrition security, and considering the real situations of the area, the following recommendations are made:

1. Improving meal frequency, food varieties and diet diversities;
2. Creating awareness of the nutritional benefits of consumption of locally available food items including edible wild plants like stinging nettle (*Urticasimensis*);
3. Demonstrating the idea of balanced diet in the garden or kitchen garden;
4. Developing food based dietary guidelines;
5. Promoting nutrition sensitive agricultural practices; and
6. Promoting micronutrient enriched staple food.

Declarations

Competing interests

The authors declare that they have no competing interest related with this study.

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There was no specific grant for this study.

Authors contributions

Design of the study: TSK, CL and HKB.

Data collection, analysis, interpretation and draft of manuscript: TSK

Critical review of the manuscript: AS, CL, DN and HKB. All the authors read and approved the manuscript.

Ethical approval and consent to participate

This study is a part of our project which has been ethically approved by Armauer Hansen Research Institute (AHRI) – All Africa Leprosy Rehabilitation Training (ALERT) Centre ethical approval committee in Ethiopia. It has also obtained permissions from zonal and district level health bureaus. The purposes and objectives of the study were explained to the study subjects. After informing about their right to withdraw from the study at any time, informed consent was obtained from each study subject

Availability of data and material

All data supporting the conclusions of this study are included in this article [and its additional file].

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CHAPTER 3.2. VITAMIN A AND ZINC DEFICIENCIES AMONG TUBERCULOSIS PATIENTS IN ETHIOPIA

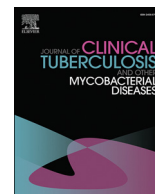
3.2. Vitamin A and zinc deficiencies among tuberculosis patients in Ethiopia

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Vitamin A and zinc deficiencies among tuberculosis patients in Ethiopia

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ABSTRACT

Background: The link between tuberculosis (TB) and malnutrition has long been recognized. Vitamin A and zinc deficiencies may reduce the host defenses and increase the risk for diseases.

Objective: The aim of the present study was to estimate the difference in vitamin A and zinc deficiencies together with dietary intakes among pulmonary TB patients and controls.

Materials and methods: A case-control study design was employed to undertake this study in North Shewa, Ethiopia. Sputum smear examination, high-performance liquid chromatography (HPLC), flame atomic absorption spectrometry (FAAS), and enzyme-linked immunosorbent assay (ELISA) were used to analyse acid fast bacilli (AFB), vitamin A, zinc, and C-reactive protein (CRP), respectively. Dietary intake was assessed using a 24-h recall questionnaire. Mann–Whitney U test, Kruskal–Wallis test, Chi-square, odds ratio (OR), Spearman correlation, and multinomial logistic regression model were computed for data analyses.

Results: In this study, 62 TB cases and 59 controls were included. The proportions of vitamin A deficiency among TB cases and controls were 56.4% and 39.0%, respectively. All TB cases and 92.5% controls were zinc deficient. The odds of TB cases with deficiencies of vitamin A and zinc was 2.3 (95% CI: 1.1 to 4.8) times more likely as compared to the controls. More than 80% of all participants had below average fulfilment of energy and vitamin A intakes.

Conclusion: Vitamin A and zinc deficiencies are severe problems among TB patients. Moreover, undernutrition determines the development of TB. Therefore, the management programs of TB need to address the problems of vitamin A and zinc deficiencies together with protein-energy malnutrition.

Introduction

Tuberculosis (TB) is an ancient disease caused by *Mycobacterium tuberculosis*. Despite the applied efforts for its control, TB is still one of the major public health challenges. According to the global TB report in 2017, 10.4 million TB cases and 1.67 million TB deaths occurred worldwide in 2016. Of whom, about 70% of the new cases and more than 85% of the deaths were reported from South East Asia and Africa [1]. The report also showed that Ethiopia is one of the top 30 TB burden countries globally with 182,000 new cases and 30,000 deaths in 2016 [1].

The link between TB and malnutrition has long been recognized;

malnutrition may predispose people to the development of clinical disease, and TB can contribute to malnutrition [2]. Malnourished people are also susceptible to a new infection since their immune systems are debilitated [3]. As key contributors to immune function and cytokine kinetics, micronutrients such as vitamin A and zinc play a major role in combating TB [4].

Vitamin A is a fat-soluble vitamin which is needed in small quantities for several metabolic activities in the body [5]. Vitamin A and its active metabolites are important for growth and differentiation of a variety of cells, mainly in mucosa-associated epithelia [6], T and B lymphocytes, macrophages, and generation of antibodies [7]. Zinc is also an essential trace element with diverse physiologic and metabolic

Abbreviations: AFB, Acid Fast Bacilli; BMI, Body Mass Index; CI, Confidence Interval; CRP, C-Reactive Protein; DDS, Dietary Diversity Score; ELISA, Enzyme-Linked Immunosorbent Assay; FAAS, Flame Atomic Absorption Spectrometry; HPLC, High Performance Liquid Chromatography; IQR, Inter Quartile Range; IZiNCG, International Zinc Nutrition Consultative Group; MUAC, Mid Upper Arm Circumference; SD, Standard Deviation; TB, Tuberculosis; VIF, Variance Inflation Factor

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functions [8] such as maintaining immunological integrity, cellular immunity, and antioxidant activity [9]. Because of the absence of specialized zinc storage in the body, a daily intake is required to achieve its steady-state [10]. In the prechemotherapeutic era, cod liver oil rich in vitamin A was used regularly for the treatment of TB to strengthen the host defense system [11].

Vitamin A and zinc deficiencies are common features of pulmonary TB. Deficiencies of both micronutrients can reduce host defenses and immune responses [12]. A study done in Rwanda revealed that 29% of adult TB patients had serum vitamin A levels consistent with deficiency (less than 0.7 $\mu\text{mol/L}$) [13]. Deficiency in zinc (below 10.7 $\mu\text{mol/L}$) is thought to be one of the primary causes of morbidity in developing countries and yet little is known about the status of the world [14].

Until recent time, many of the epidemiological studies conducted on vitamin A and zinc deficiencies in Ethiopia were focusing on children and pregnant women [5,15,16]. However, there is a paucity of information on the magnitude of the deficiencies of these micronutrients in TB patients. The available studies were very few [17,18] and conducted long years ago in some pocket areas of North West part of Ethiopia. Therefore, the present study was designed to estimate the difference in vitamin A and zinc deficiencies together with dietary intakes among pulmonary TB patients and non-TB controls.

Materials and methods

Study design, area and population

A facility-based case-control study was conducted in North Shewa Zone of Amhara Regional State, Central Ethiopia. In this study, one referral hospital and five health centers were involved. The study population included TB patients and non-TB controls who were living in the same geographic area. All TB cases who visited the health facilities between March and August 2015 were recruited.

Selection of TB cases and controls

The diagnosis of TB was performed microbiologically and radiologically as per the national standard diagnostic algorithm of Ethiopia [19]. As per the diagnostic algorithm, smear positive pulmonary TB is defined by at least two initial sputum smear examinations positive for acid fast bacilli (AFB) by direct microscopy, or a patient with one initial smear examination positive for AFB by direct microscopy and culture positive, or a patient with one initial smear examination positive for AFB by direct microscopy and radiographic abnormalities consistent with active TB as determined by a clinician.

Smear-negative pulmonary TB is also defined by a patient having symptoms suggestive of TB with at least three initial smear examinations negative for AFB by direct microscopy, and no response to a course of broad-spectrum antibiotics; radiological abnormalities consistent with pulmonary TB; decision by a clinician to treat with a full course of anti-TB; or a patient whose diagnosis is based on culture positive for *M. tuberculosis* [19,20].

In this study, both smear positive and negative pulmonary TB cases who were willing to participate in the study were included. All selected TB cases were used as a point of contact to recruit controls. Individuals who were apparently healthy, non-TB, living in the same geographic areas with TB cases and interested in participating in the study were selected as controls. As vitamin A and zinc concentrations in the serum are affected by many physiological and pathological states and drugs, TB cases and controls with pregnancy, lactation, chronic or degenerative diseases, and who were taking vitamin A and zinc supplements, corticosteroid drugs and oral contraceptives were excluded from the study.

Duration of treatment

The treatment of TB lasts for a total of six months for newly diagnosed TB patients or for those who had taken previously the anti-TB drugs for less than one month as per the standard treatment guidelines for the general hospital in Ethiopia. The treatment regimen consists of 2 months treatment with Rifampicin (R), Isoniazid (H), Pyrazinamide (Z) and Ethambutol (E) during the intensive phase, followed by four months with Rifampicin and Isoniazid in the continuation phase (2RHZE/4RH) [21].

Data collection

A structured questionnaire, comprised of socio-demographic characteristics, health status and diet factors, was prepared, translated into Amharic (the local language) and administered to each of the study participant. Medical records were also used to collect clinical characteristics.

Anthropometric measurements

Standardized procedures were employed to measure body weight, height, and mid upper arm circumference (MUAC). All study participants were weighed while wearing light clothes using an electronic platform weighing scale to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm by means of a seca stadiometer.

Body mass index (BMI) was calculated as body weight (kg) divided by height (m) squared (kg/m^2). BMI values of 18.5, 17.0, and 16.0 kg/m^2 were used as the cut-off values below which patients were classified as having mild, moderate, or severe malnutrition [21]. MUAC was measured half way between the olecranon and acromion processes of the left arm using a flexible non-stretch measuring tape to the nearest 0.1 cm while the arm is hanging relaxed, without compressing the tissues. MUAC less than 23 cm for male and 22 cm for female is used to define undernutrition as per FANTA III [22].

Dietary intake assessment

The dietary intake was assessed by means of a 24-h recall to estimate the intake of energy, protein, vitamin A and zinc. Each 24-h recall was performed using a standardized four-stage protocol [23,24]. First, a complete list of all food and beverages consumed over the 24 h was obtained. Second, detailed descriptions of the food and beverages consumed, including the cooking methods and brand names were recorded, together with the time and place of consumption. Third, estimates of the portion sizes of all consumed food items were determined by referring to household measuring and serving utensils (e.g., spoons, plates or cups), and food packages. Finally, the food recall was reviewed to ensure that all items had been recorded correctly. The amount of nutrient intake from dietary recalls was calculated using nutrisurvey software (Dr. Juergen Erhardt SEAMEO-TROPMED RCCN-University of Indonesia copy right[©] 2007).

After the dietary intake assessment, the individual dietary diversity score (DDS) was calculated as the number of food groups consumed over the 24-h recall. These food groups were based on the guidelines for measuring the household and individual dietary diversity. The score for individual diet diversity goes from 0 to 9 [25,26]. Considering four food groups as the minimum acceptable dietary diversity, the individual DDS less than four was classified as having a poor dietary diversity [26,27].

Blood collection and serum separation

After overnight fasting, 10 mL of venous blood was withdrawn from antecubital fossa vein into a non-heparinized vacutainer tube between 8:00 and 10:00am at the selected health facilities. Blood samples were allowed to clot for about 1 h in the dark and subjected to centrifugation

at 4000 rpm for 10 min at room temperature. Samples with visible haemolysis were discarded.

The sera were separated immediately into aliquots of sterile Eppendorf tubes by means of sterile Pasteur pipettes and stored at -20°C in the laboratory of the health facility. The sera were later transported in the ice box to Armauer Hansen Research Institute (AHRI) where they were stored at -80°C until analysis. Aliquots of the sera were transferred to Ethiopian Public Health Institute (EPHI) for the analysis of serum vitamin A concentration and to Natural Sciences College of Addis Ababa University for the determination of the serum zinc concentration.

Measurements of vitamin A, zinc and C-reactive protein (CRP)

Vitamin A

Serum vitamin A concentration was determined using high performance liquid chromatography (HPLC) as explained in the method of Arroyave et al. [28] with slight modifications. In brief, 100 μL of ethanol and an equal volume of retinyl acetate (reconstituted in ethanol) as internal standard were added into a 2 mL Eppendorf tube containing 100 μL of serum sample. The solution was then mixed for 1 min using vortex mixer. In the first round, 750 μL of n-hexane was added into the solution, vortex mixed for 1 min and centrifuged at 3000 rpm for 10 min. The supernatant was transferred into a new Eppendorf tube. In second round, 750 μL of n-hexane was added and the whole steps were repeated. The pooled supernatant was then evaporated under a stream of nitrogen, reconstituted in 200 μL of methanol, and transferred into the injection vial.

A Shimadzu HPLC system (Shimadzu, Tokyo) which composed of a reverse phase Supelco LC-18 column (250×4.6 mm, 5 μm particle size) and UV–vis detector was used to separate and detect the retinol at 325 nm with a column temperature set at 40°C . Series of standards (having a concentration of 60, 40, 20, 10 and 5 $\mu\text{g}/\text{dL}$) and samples were loaded into the autosampler tray of the HPLC. Methanol was used as a mobile phase with injection volume of 40 μL , flow rate of 1 mL/min and retention time of 15 min.

Retinol was determined by comparing the retention times with the external standard and quantified based on externally drawn calibration curve using the series concentrations of standards. All extraction procedures were performed under dim light to avoid oxidation of the compound. Vitamin A concentration below 0.7 $\mu\text{mol}/\text{L}$ was considered as deficient.

Zinc

Serum zinc concentration was determined by means of flame atomic absorption spectrometry (FAAS) with a micro-sampling technique in AA-7000 Atomic Absorption Spectrophotometer (Shimadzu, Japan) using the method described in Sepehri et al. [29]. In brief, 10 mL of concentrated nitric acid was added to 1 mL of serum in a beaker and heated for 3 h, below boiling point, on a hot plate. When the volumes of the samples reduced to about one-third, 5 mL of 30% hydrogen peroxide solution was added. The samples were further heated almost to dryness at the same temperature. Finally, the residues were dissolved in 50 mL of 1% nitric acid and filtered. The prepared samples were transferred into 50 mL polyethylene tubes for zinc analysis. The concentration of 10.7 $\mu\text{mol}/\text{L}$ was used as a cut-off value below which was considered as zinc deficient.

C-reactive protein (CRP)

CRP was measured at AHRI, Ethiopia using enzyme-linked immunosorbent assay (ELISA) (Human CRP ELISA Kit, HK 358, Hycult biotech) method according to the manufacturer's instruction as indicated in the manual of the kit. For each TB patient, CRP was measured in duplicate. The mean value ≥ 10 mg/L was considered as CRP positive.

Ethical consideration

This study is a part of our project entitled "effect of micronutrients on the treatment outcome of TB", which has been ethically approved by the ethical approval committee of AHRI- ALERT (All Africa Leprosy Rehabilitation and Training) Centre. Supportive letters were also obtained from the zonal and districts' health bureau of Amhara Regional State. This study was carried out in accordance with the principle of Helsinki declaration. After explaining the purpose and objective of the study, written informed consent was obtained from each of the study participant.

Statistical analysis

Statistical analysis was conducted using IBM SPSS version 23 statistical program. All continuous data were checked for a normal distribution using a Shapiro-Wilk test. Descriptive statistics, including frequency, proportion, and median (IQR-Inter Quartile Range) were employed to summarize the study variables. Mean and standard deviation (SD) were used to describe concentrations of vitamin A and zinc, BMI, and MUAC. Odds ratio (OR) together with 95% confidence interval (CI) was computed to assess the strength of association.

The significance of the difference in continuous and categorical variables between groups was compared using Mann–Whitney U test and Chi-square test, respectively. Kruskal–Wallis test was used to assess the difference in the serum vitamin A and zinc concentrations of TB patients at different duration of treatment. The association between MUAC, BMI, and DDS was examined using Spearman correlation test.

Bivariate analysis was done to explore the crude association between different predictor variables and TB. To control for possible confounding factors and identify factors that are independently associated with TB, multinomial logistic regression analysis was performed for those variables with p-value of less than 0.2 in the bivariate analysis. The major assumptions of logistic regression model were multicollinearity and interaction among independent variables. The absence of multicollinearity was checked using variance inflation factor (VIF)/tolerance and the fitness of logistic regression model was checked using Hosmer–Lemeshow statistical test. Unless specified, p-value < 0.05 was considered as statistically significant.

Results

Socio-demographic characteristics

The present study was conducted on 62 TB patients and 59 controls. The median (IQR) age was 26 (14) years with the minimum and maximum age of 14 and 77 years. Most of the participants were Orthodox Tewahido Christians in religion (89.3%) and farmers in occupation (31.1%) (Table 1).

There was statistically significant difference in sex between TB patients and the controls ($P < 0.05$). The odds of males with TB was 2.2 (95% CI: 1.05 to 4.48) times more likely as compared to the odds of females. The most (85%) TB afflicted age group was found between 19 and 50 years.

Clinical signs and symptoms of TB

TB patients showed various clinical signs and symptoms. The identified clinical signs and symptoms at the time of diagnosis were fatigue (100%), cough (83.3%), malaise (83.3%), night sweating (83.3%), fever (75%), anorexia (75%), productive cough (75%), chest pain (70.8%), dyspnoea (54.2%), haemoptysis (29.2%) and tachycardia (25%).

Table 1
Socio-demographic characteristics of TB patients and Controls.

Type	Variables	TB-cases (n)	Control (n)	P-value
Sex	Female	26	36	0.036
	Male	36	23	
Residence	Urban	24	22	0.391
	Rural	29	37	
Age (years)	14–18	9	9	0.805
	19–30	37	32	
	31–50	12	15	
	51–70	3	3	
	More than 70	1	0	
Marital status	Single	31	26	0.807
	Married	30	32	
	Widowed	1	1	
Educational level	Illiterate	8	8	0.644
	Primary	20	16	
	Secondary	23	21	
	Tertiary	11	12	
Religion	Religious Teaching	0	2	0.176
	Orthodox Tewahido	56	52	
	Christian			
	Muslim	1	5	
	Protestant	3	2	
Occupation	Catholic	2	0	0.002
	Student	9	15	
	Private	12	19	
	Government employees	8	7	
	Non-Government employees	0	1	
	Farmer	29	8	
	Housewife	2	8	
Retired	1	0		

Vitamin A and zinc deficiencies

The proportions of vitamin A, zinc and the combination of the two micronutrient deficiencies were 47.9%, 96.5% and 47.8%, respectively. The mean serum retinol and zinc concentrations in females ($0.80 \pm 0.47 \mu\text{mol/L}$ and $2.10 \pm 1.20 \mu\text{mol/L}$, respectively) were not significantly different from that of males ($0.77 \pm 0.60 \mu\text{mol/L}$ and $2.50 \pm 2.50 \mu\text{mol/L}$, respectively) ($p > 0.05$). The proportions of vitamin A deficiencies among TB patients and the controls were 56.4% and 39.0%, respectively.

All TB patients and 92.5% of the controls were identified as zinc deficient. There were statistically significant differences in all proportions of the deficiencies between TB patients and the controls at $p < 0.1$. The odds of TB patients who were vitamin A deficient was 2.03 (90% CI: 1.1 to 3.7) times greater than the odds of the controls. In the same line, TB patients had vitamin A and zinc deficiencies 2.3 (95% CI: 1.1 to 4.8) times more likely as compared to the controls.

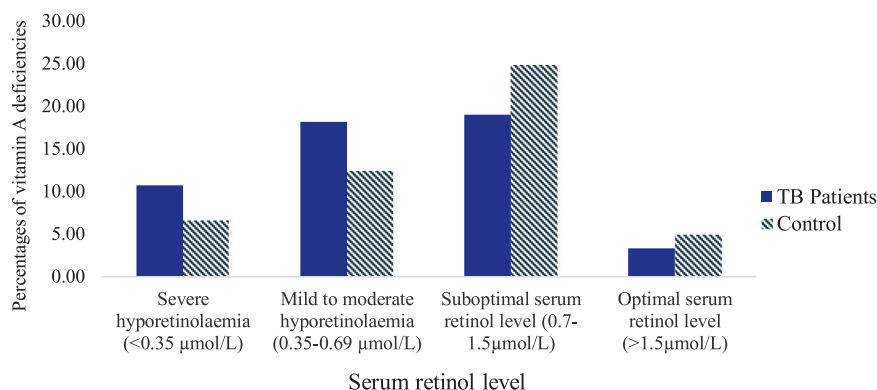


Fig. 1. Percentages of vitamin A deficiencies in TB patients and controls. The categories of vitamin A deficiencies were indicated in WHO global report [30], Olang et al. [31] and Kassaye et al. [32].

Fig. 1 shows a wide-ranging of serum retinol levels. The proportions of severe and, mild to moderate hyporetinolaemia were higher in TB patients than the controls. About 19% of TB patients and 25% of the controls had suboptimal retinol levels, whereas the optimal level in both groups was below 5%.

CRP

About 62% of TB patients were CRP positive. The mean \pm SD of retinol and zinc concentration in CRP positive TB patients were $0.62 \pm 0.50 \mu\text{mol/L}$ and $2.05 \pm 0.74 \mu\text{mol/L}$, respectively. Whereas, these values in CRP negative TB patients were $0.75 \pm 0.69 \mu\text{mol/L}$ and $2.48 \pm 1.20 \mu\text{mol/L}$, respectively. Excluding CRP positives, the prevalence of vitamin A deficiencies in CRP negative TB patients became 66.7%. Both CRP negative and positive TB patients did not have statistically significant differences in serum retinol ($p = 0.25$) and zinc ($p = 0.09$) concentrations.

Durations of TB treatment

Fig. 2 shows the mean serum retinol and zinc concentrations in different durations of TB treatment. During the first month of TB treatment, the retinol level was below the cut-off value of $0.70 \mu\text{mol/L}$ but increased slightly afterward. Similarly, the zinc level increased slightly up to 2 months of treatment but its concentration throughout the entire courses of the treatment was below the cut-off value of $10.70 \mu\text{mol/L}$. Per the result of the analysis of variance, the retinol and zinc concentrations were not significantly different in both within and between the durations of the treatment.

Dietary intakes

The mean DDS was 3.56. Most (84%) of TB patients and the controls (86%) had below average DDS (4.5 out of 9 food groups). DDS had statistically significant correlation with BMI ($\gamma = -0.22$; $p = 0.019$). There was also a significant difference in DDS between males and females ($p = 0.031$). While analysing the nutrients intake, about 87% of TB patients and 80% of the controls had below 50% fulfilment of energy intake. Almost all participants had below 50% fulfilment of vitamin A intake. But, less than 30% of participants had protein and zinc intakes below 50% fulfilments. In other words, many participants had above the average fulfilments of protein and zinc intakes, albeit much cereal-based diet.

Nutritional status

More than 29% of the participants had BMI below 18.50 kg/m^2 . The mean BMI of female TB patients ($18.80 \pm 3.80 \text{ kg/m}^2$) was

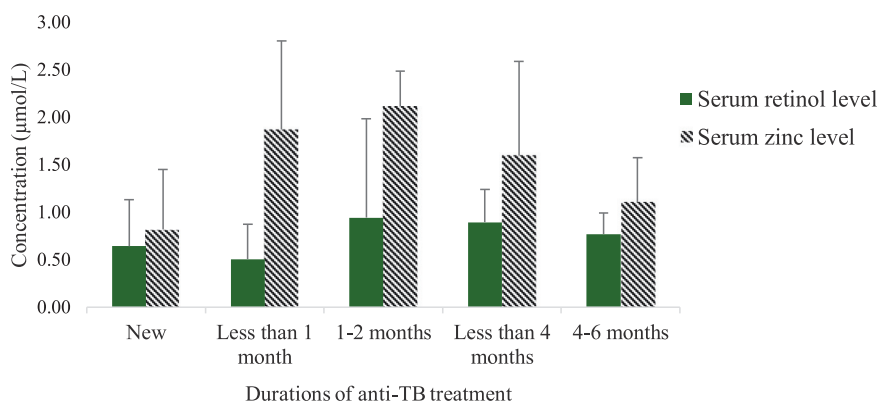


Fig. 2. Mean concentrations of serum retinol ($\mu\text{mol/L}$) and zinc ($\mu\text{mol/L}$) in different duration of anti-TB treatment.

significantly lower than females in the controls ($20.40 \pm 2.92 \text{ kg/m}^2$) ($p < 0.05$). There was a statistically significant difference in the proportions of undernutrition ($\text{BMI} < 18.50 \text{ kg/m}^2$) between TB patients (40.3%) and the controls (19.3%) ($P < 0.05$). Undernourished individuals had 2.8 times the odds of TB disease compared to the controls. As defined by MUAC, undernutrition for both male and female was 44.2%. The mean MUAC of females in TB patients ($20.60 \pm 2.20 \text{ cm}$) was significantly different from those females in the controls ($21.80 \pm 2.20 \text{ cm}$) at $p < 0.10$. MUAC was significantly correlated with BMI ($\gamma = 0.56$, $P < 0.001$).

Determinants of TB

The multinomial logistic regression model in Table 2 indicated that sex and BMI had statistically significant association with TB patients ($p < 0.05$). Adjusting for BMI, serum retinol level, religion, and occupation, females had 0.2 times less the odds of TB compared to males. Similarly, undernourished individuals had 3.3 times the odds of TB compared to well-nourished individuals holding sex, serum retinol level, religion and occupation constant.

Discussion

In most countries, TB notification is twice as high in men as in women [33]. The study done in central part of Ethiopia also showed a high rate of TB notification in male (60.9%) and people with the age group of 14 to 54 years (87.3%) [20]. In line with this, the odds of males with TB in the present study was 2.2 times more likely as

compared to the odds of females. The most (85%) TB afflicted age group was also found between 19 and 50 years. The reason why males and the productive age groups are highly afflicted by TB could be associated with their sociocultural, behavioral and biological components [33].

Micronutrient deficiencies are rampant in Ethiopia. More than 47% of TB patients and controls were affected by the deficiencies of either vitamin A, zinc or their combination. This was corroborated by the report of Keflie et al. [26] who identified the presence of a high risk of micronutrient deficiency in central part of Ethiopia ($> 60\%$). According to WHO and International Zinc Nutrition Consultative Group (IZiNCG), the risks of vitamin A and zinc deficiencies are of public health concern when the prevalence of low serum retinol and zinc concentrations are greater than 20% [34]. This implied that the results of the present study were more than twice of the indicator of the public health concern.

Comparing with the control, the odds of TB patients who were vitamin A deficient was 2 times higher. The proportion of vitamin A deficiency in TB patients was 56.4% which was closer to the report of nearly 60% among TB patients in Gondar, Northwest Ethiopia [18]. Severe and mild to moderate types of vitamin A deficiency was more common in TB patients than the controls. The low concentration of vitamin A in the serum of TB patients could be resulted from TB induced anorexia that lead to low consumption of vitamin A rich food items; reduced absorption of dietary vitamin A owing to parasites co-infection; decreased mobilization of hepatic reserves of retinol; rapid utilization of vitamin A by target tissues; and increased urinary losses of vitamin A associated with fever [18,35]. This justification was strengthened by the occurrence of fever and anorexia together with

Table 2
Multinomial logistic regression model for different variables in TB patients.

Variables	β	Wald	DF	P-value	Odds Ratio	95% CI	
						Lower Limit	Upper Limit
Sex							
Females	-1.64	10	1	0.002*	0.19	0.07	0.53
Males							
BMI							
< 18.50 kg/m^2	1.2	5.06	1	0.024*	3.33	1.17	9.50
$\geq 18.50 \text{ kg/m}^2$							
Retinol							
< 0.70 $\mu\text{mol/L}$	0.62	1.77	1	0.184	1.86	0.74	4.64
$\geq 0.70 \mu\text{mol/L}$							
Religion							
Orthodox Tewahido	0.7	0.22	1	0.64	2.01	0.10	38.56
Muslim	-0.48	0.07	1	0.796	0.62	0.02	23.34
Protestant							
Occupations							
Students	-0.92	2.01	1	0.16	0.40	0.11	1.42
Private workers	-0.84	1.77	1	0.183	0.43	0.13	1.48
Government employees	-0.97	1.59	1	0.207	0.38	0.09	1.71
Housewives	1.73	2.91	1	0.088	1.26	0.35	4.51
Farmers							

NB: The reference category is the controls.

*Statistically significant at $p < 0.05$.

fatigue, cough, malaise, and night sweating in more than 74% of TB patients in the present study.

Likewise, there was a significant difference in the proportion of zinc deficiency among TB patients and controls. All TB patients were identified as zinc deficient. This result was much higher than the report of the previous study done in Northwest Ethiopia (32.1%) [17]. Such a proportional difference could be owing to variations in the dietary patterns and seasons together with the availability and accessibility of food items rich in zinc. In line with the present study, the low serum zinc concentration in TB patients was also reported in Indonesia [24], Ecuador [36], Malaysia [37], and Korea [38].

The lowered concentration of zinc among TB patients could probably be associated with reduction in hepatic production of a zinc carrier protein (α 2-macroglobulin); redistribution of zinc from blood circulation to other tissues; and/or a rise in the production of metallothionein, a protein that transports zinc to the liver [17,37,39]. The concomitant deficiencies of vitamin A and zinc were very high in TB patients as compared to the control. This could be due to the high magnitude of zinc deficiency in TB patients. Vitamin A and zinc deficiencies are usually occurred together, as zinc deficiency impairs the synthesis of retinal binding proteins and reduces plasma retinol concentration [40].

Several studies indicated the essence of measuring CRP in TB patients to get a clear picture on the serum retinol and zinc concentrations [18,36,41]. CRP is a tissue protein which is produced at the time of acute phase response and its concentration changes rapidly as a result of infection or inflammation. Visser et al. [41] showed the relationship between the low retinol concentration in active TB and the acute phase response. Likewise, Cuevas and Koyanagi [9] described the low concentration of zinc in TB patients with and without a raised CRP.

In the present study, we did not observe any difference in the concentrations of vitamin A and zinc in the serum of TB patients with and without CRP. In line with our finding, Karyadi, et al. [24] reported that retinol concentration did not correlate significantly with markers of acute phase response. This suggested that CRP is not the best option to control the change in the acute phase response in the concentrations of vitamin A and zinc in the serum of TB patients.

We observed a fluctuation of vitamin A and zinc concentrations in TB patients at different durations of anti-TB treatment. During the first month, the retinol concentration was below the cut-off value but later increased slightly. The gradual increment of zinc concentration was also observed up to the second month of the treatment, but such a concentration was below the cut-off value throughout the whole course of anti-TB treatment. The improvement of vitamin A was in accordance with the reports of Mugusi et al. [7] in Tanzania and Kassu et al. [18] in Ethiopia. It is believed generally as patients improve and fever subsides, the loss of vitamin A declines, and its serum concentration resume to the reference ranges [18].

Despite the low improvement, our observation on the changes of zinc concentration was corroborated by the findings of Khanna et al. [42] and Kassu et al. [17]. On the contrary, Edem and others [39] revealed that trace elements and vitamins were lowest at 2 and 4 months of anti-TB drug therapy. This may be due to drug-micronutrient interactions or drug induced nutrient depletion. Further, the effects of anti-TB drugs on the absorption of zinc was presented by Karyadi et al. [11] in their study done in Indonesia. Ethambutol was shown in rats to increase not only zinc absorption but also urinary zinc losses, resulting in reduced circulating zinc concentrations [43].

In the comparison made on nutritional status as measured by BMI and MUAC, the results were significantly lower in TB patients than the controls. Undernourishment in TB patients was almost three times higher than that of the controls. In agreement with our results, several studies demonstrated undernutrition as a well-recognized clinical sign of active TB [24,44,45]. Pakasi et al. [46] described undernutrition as a reflection of two processes. One would be protein-energy malnutrition (which severely affects host defense) and the other was wasting due to the catabolism induced by the acute phase response. The causes of

undernutrition in patients with active TB could be anorexia, impaired absorption of nutrients, or increased catabolism associated with the inflammatory and immune response [24,44]. Our study identified undernutrition and sex as significant determinants of active TB.

The dietary intake assessment revealed that the mean DDS was 3.56, and above 80% of TB patients and the controls had below the average DDS. These results were in accordance with the previous study done in the same area [26]. A diet of at least 4 DDS was valid as nutritionally adequate, but below 4 DDS represented a poor dietary diversity [26,47]. This suggested that both TB patients and controls had poor dietary diversity. The intakes of energy and vitamin A rich food items were also poor. More than 85% of TB patients could not fulfil even half of their daily requirements of energy and vitamin A. Poor dietary intake of vitamin A rich food is an important predictor of vitamin A deficiency [16].

Although the intakes of protein and zinc looked better, the main dietary sources were cereal based. The previous study in the same area also indicated a high reliance on the consumption of cereals, vegetables and legumes with less animal source foods [26]. As our body does not store zinc, it requires a constant dietary intake. Most plant-based diets are not good sources of zinc owing to the presence of phytate, a component of plants that chelates zinc and prevents its absorption [48]. The low content of zinc in Ethiopian soil could be another factor for the inadequate intake of zinc [15]. Hence, plant-based diets with low animal source proteins were accounted for the high deficiency of zinc in both TB patients and controls.

Limitation

Although this study had the strength of dealing with the serum vitamin A and zinc concentrations in TB patients together with CRP, nutritional status and dietary intakes, it was a facility-based study and the participants could not represent the general population. In addition, the 24-h recall method, which was used to assess the dietary intake, could probably miss some of the feeding behaviours particularly in the case of altered dietary patterns.

Conclusion

In conclusion, vitamin A and zinc deficiencies are severe problems among TB patients and non-TB controls. The cause-effect relationship between undernutrition and TB is just like the puzzle of the hen or the egg, of the two which comes first. Our study underlines that undernutrition determines the development of TB. Most TB patients have DDS below average and, very poor dietary intakes of vitamin A, zinc, protein, and energy. During the duration of anti-TB treatment, the concentrations of vitamin A and zinc improve but not to the extent of curbing their deficiencies. Therefore, to claim success, TB management program needs to give emphasis on addressing the problems of vitamin A and zinc deficiencies together with protein-energy malnutrition. In the future, we recommend further community-based studies to be conducted at different parts of Ethiopia to substantiate our findings.

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Conflict of interest

The authors declared that they have no competing interest.

Author's contribution

TSK designed the study, collected the data, analyzed, interpreted and drafted the manuscript. AS, AZ, AM, MA and HKB critically reviewed the manuscript. All the authors read and approved the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jctube.2018.05.002.

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3.3. Vitamin D deficiencies among tuberculosis patients in Africa: A systematic review.

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Review

Vitamin D deficiencies among tuberculosis patients in Africa: A systematic review



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ABSTRACT

The aim of this study was to explore the existence of vitamin D deficiency (VDD) in tuberculosis (TB) patients living in Africa and to identify its predictor variables. PRISMA guidelines and checklists were used. The sources of the data were Medline/PubMed, Web of Science, Scopus, and Google Scholar databases. We identified 23 articles, of which 15 reported the status of vitamin D in TB with TB. The definition of serum vitamin D status was summarized as severe, deficient, and insufficient when the concentration of 25-hydroxyvitamin (OH)-D ≤ 25 , ≤ 50 , and ≤ 75 nmol/L, respectively. The reports showed that up to 88.9% and 96.3% of patients with TB tested by radioimmunoassay had VDD and vitamin D insufficiency, respectively. Statistically significant variables such as lack of sun exposure, inadequate dietary intake, season, clothing, comorbidities, low body mass index, age, skin pigmentation, use of antiretroviral therapy and anti-TB drugs, and socio-economic status were identified as the main predictor variables of vitamin D status. VDD and vitamin D insufficiency were highly prevalent in TB patients in Africa. Further case–control studies are warranted to clarify the cause–effect relationship between vitamin D and TB and thereby, design valuable strategies to manage VDD among TB patients in Africa.

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Introduction

Sun exposure as a therapeutic approach to treat tuberculosis (TB) was used more than 100 y ago, before the identification of *Mycobacterium tuberculosis* as the causative agent for TB [1]. Exposure of children suffering from rickets and TB to artificial ultraviolet (UV) light resulted in a positive effect on both diseases. The use of sanatoria was also based on the belief that fresh air and sun exposure led to a positive outcome in the treatment of TB [2]. Vitamin D is a known immune modulator [3] that can improve cell-mediated immunity [4] and the phagocytic capacity of macrophages [5]. It also increases the production of antimicrobial peptides such as cathelicidin [5], which is part of the innate immune system that plays a critical role in the fight against TB. Recent research highlighted the production of cathelicidin through toll-like receptor pathway [6].

Vitamin D is unique among hormones as it can be made in the skin from sun exposure [7]. Most (90%) vitamin D is synthesized in the skin under the influence of UV sunlight, and only 10% is obtained from food, mainly salmon and cod fish, and dairy products [8]. Although sunshine is abundant, TB is one of the most pernicious infectious diseases in Africa. Sub-Saharan Africa carries the greatest proportion of new cases per population, with >255 cases per 100 000 population in 2012 [9]. Almost 30% of TB cases and 34% of TB-related deaths occur in Africa [10].

Few community- and facility-based studies have been conducted with different population groups in Africa to assess the distribution of vitamin D deficiency (VDD) in patients with TB and their cause–effect relationship. These studies have provided insight into the status of vitamin D in patients with TB. However, four questions still need to be addressed:

1. Is VDD common in TB patients living in Africa?
2. If yes, which level of deficiency is highly predominant?
3. What are the reasons for VDD in TB patients?
4. What are the predictors of VDD?

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Therefore, the present study was designed to address these four questions through a comprehensive systematic review of all articles published in peer-reviewed journals.

Materials and methods

Data sources and search strategy

PRISMA guidelines and checklists were used to conduct this systematic review [11]. Data were collected from published articles without time restriction. Electronic searches of Medline/PubMed, Web of Science, Scopus, and Google Scholars were done through May 25, 2014. Details of the search criteria for each database were as follow:

- Medline/PubMed: (“vitamin d deficiency”[MeSH Terms] OR “vitamin d deficiency”[All Fields]) AND (“tuberculosis”[MeSH Terms] OR “tuberculosis”[All Fields]) AND (“patients”[MeSH Terms] OR “patients”[All Fields]) AND (“africa”[MeSH Terms] OR “africa”[All Fields])
- Web of Science: (“vitamin d deficiency” OR “cholecalciferol” OR “ergocalciferol”) AND (“tuberculosis” OR “tuberculosis patients”) AND (“africa”)
- Scopus: “vitamin d deficiency” AND “tuberculosis patients” AND “Africa”
- Google Scholars: “vitamin D deficiency” OR “cholecalciferol” OR “ergocalciferol” OR “calcidiol” OR “calcitriol” AND “tuberculosis” AND “Africa”

Eligibility criteria

Eligibility for inclusion focused on studies reporting VDD among TB patients in Africa without restricting for age, sex, or ethnicity. We included all original articles in English published in peer-reviewed journals. However, reviews, commentaries, letters, and theses were excluded. We also excluded articles on African immigrants, as we only focused on TB patients living in Africa.

Definitions

The cutoff values of serum 25-hydroxyvitamin (OH)-D ≤ 75 , ≤ 50 , and ≤ 25 nmol/L were used to define insufficiency (VDI), deficiency, and severe vitamin deficiency (sVDD), respectively [12,13]. To convert values of 25-OH-D concentration from conventional (ng/mL) to the International System of Unit (nmol/L), we multiplied by factor 2.496 [14].

Data extraction and processing

The following data were extracted from each selected study: author(s); publication year; country/city; latitude; study type; sample size; TB cases; age; laboratory test; predictor variables; percentages of males and females; and serum 25-OH-D ≤ 75 , ≤ 50 , and ≤ 25 nmol/L. Detailed descriptions of the extracted data can be found in Table 1. Factors that have statistically significant differences in each article were considered predictor variables.

Results

Search results

The literature search and selection process are schematically indicated in Figure 1. Initially, the search in Medline/PubMed, Web of Science, Scopus, and Google Scholars yielded 2919 articles. After looking through their titles and abstracts, 80 potential studies were identified; however, only 23 were included in the systematic review analysis. Fifty-seven studies were excluded because of they were duplicates (44); reviews (3); or commentaries, letters, or thesis (1 each); they included African immigrants (3) or they did not measure 25-OH-D (4).

Study characteristics

Table 1 summarizes the characteristics of each study. Of the 23 studies, 11 were conducted in eastern Africa (latitude $06^{\circ}50'S/10^{\circ}N$), 5 in southern Africa (latitude $13^{\circ}30'S/33^{\circ}S$), 4 in

western Africa (latitude $06^{\circ}25'N/12^{\circ}N$) and 3 in northern Africa (latitude $26^{\circ}34'N/36^{\circ}43'N$). Most of the studies were cross-sectional and prospective. Considering all studies reviewed, 15 reported the vitamin D status in TB patients.

In general, all the studies were heterogeneous in reporting age and vitamin D status. Age was reported either as mean \pm SD or median (range or interquartile range). Similarly, in some articles, vitamin D levels were described by ng/mL but this unit was converted into nmol/L by multiplying by factor 2.496 [14]. Additionally, there differences were observed in the laboratory tests used to measure the level of vitamin D in the blood of patients with TB, as indicated in Table 2. For measuring vitamin D in both TB and non-TB cases, eight studies used immunoassay [8,15–21], five used high-performance liquid chromatography [22–26], four used liquid chromatography-tandem mass spectrometry [12,27–29], and six used other tests [17,31–35] (Table 1).

Vitamin D deficiency

There were inconsistencies in the threshold levels of 25-OH-D concentration when defining sVDD, VDD, and VDI, as described in Table 3. Half of the studies defined sVDD as ≤ 25 nmol/L; 77.3% (17 of 22) defined VDD as ≤ 50 nmol/L, and 60% (12 of 20) defined VDI as ≤ 75 nmol/L. Based on these results and those of another study [13], we summarized the definition of serum vitamin D status as follow: sVDD ≤ 25 nmol/L; VDD ≤ 50 nmol/L, and VDI ≤ 75 nmol/L. Subsequently, we used these definitions to determine the extent of deficiencies in TB patients living in Africa.

The prevalence of sVDD was reported in up to 45.7% of patients using radioimmunoassay in TB and non-TB cases. Twenty-two studies in non-TB cases and 15 studies in TB cases reported the prevalence of VDD in the range of 1.2% to 88.9%. The minimum prevalence of VDD was measured using a Cobas E601 Analyser and the maximum was measured using radioimmunoassay. Likewise, the prevalence of VDI was reported using different laboratory tests by 20 studies in non-TB cases and 13 in TB cases in the range of 15% and 96.3% (Table 1). However, 16 studies did not report the prevalence of sVDD in TB patients. Considering the regions, the highest prevalence of VDI (74.5%–96.3%) and VDD (42%–88.9%) in patients with TB was reported in southern Africa.

What are the reasons for VDD in TB patients?

There is paucity of data that explain the reasons for VDD in patients with TB. TB causes weight loss and micronutrient deficiencies by increasing nutritional requirements, changing metabolic processes, or decreasing appetite and causing a reduction in food intake [36]. Vitamin D is required for immune modulation and infection with *Mycobacterium tuberculosis* binds to toll-like receptors (TLR 2/1) on macrophages, leading to upregulation of 1- α -hydroxylase gene expression to promote greater conversion of 25-OH-D to 1,25-(OH)₂-D. This phenomenon accounts for the drop in 25-OH-D, an indicator for VDD [24].

TB may lead to changes in dietary vitamin D intake and sun exposure through a reduction in appetite and by restricting outdoor physical activities. Patients with TB who have very little adipose tissue are unable to store vitamin D, so they have no reserves when external sources are lacking [20]. TB and its treatment also may have direct biological effects on the

Table 1
Summary of reviewed studies

Author (s), year [ref. No.]	Country, city	Latitude	Study type	Sample size	TB cases	Sex		Age (y)	Laboratory test	Serum concentration of 25-OH-D (nmol/L or ng/mL × 2.5)						Predictor (s)
						Male (%)	Female (%)			Non-TB cases			TB cases			
										≤25 (%)	≤50 (%)	≤75 (%)	≤25 (%)	≤50 (%)	≤75 (%)	
Kibirige et al. 2013 [19]	Uganda, Kampala	00°20'N	Cross-sectional	260	260	56.2	43.8	Mean: 34.7 ± 9.5	ECLIA	NA	NA	NA	13.5	44.2	85.4	Inadequate diet, age, BMI
Banda et al. 2011 [20]	Malawi, Blantyre	13°30'S*	Cross-sectional	161	161	50.9	49.1	Median: 30–50	EIA	NA	NA	NA	20.4	42	74.5	Not identified
Mahmoud and Ali 2014 [12]	Egypt, Sohag	26°34'N*	Case-control	65	40	44	21	Mean: 35 ± 8 [†] 36 ± 6 [‡]	ELISA kit	NA	0	0	NA	50	72.5	Dietary, sun exposure, genetic
Conesa-Botella et al. 2012 [34]	Uganda, Kampala	00°20'N*	Prospective	162	119	53.1	46.9	Median: 34 (28–39)	CL	NA	11.63	51.16	NA	24.37	40.34	Comorbidities: TB, HIV
Tostmann et al. 2010 [35]	Tanzania, Sanya Juu	3°10'60S	Prospective	81	81	79	21	Median: 31 (26–41)	Cobas E601 Analyser	NA	NA	NA	NA	1.23	17.28	Diet, sun exposure
Wejse et al. 2007 [16]	Guinea-Bissau, Bissau	12°N	Case-control	856	362	53.7	46.3	Mean: 37.4 ± 13.7 [†] ; 37.3 ± 12.9 [‡]	ID-LC-MS/MS on API 3000 MS	4.9	13.15	39.07	0.28	8.56	46.13	Sex, ethnicity, season, sun exposure
Mehta et al. 2013 [31]	Tanzania, Dar es Salaam	06°50'S	Cohort	677	677	67.9	32.1	Mean: 32.3 ± 8.9	LC-MS	NA	NA	NA	NA	15.66	61.74	Season, money spent on food, comorbidities: TB and HIV
Conesa-Botella et al. 2012 [21]	South Africa, Cape Town	33°55'S*	Longitudinal	81	81	40.7	59.3	Median: 33.5 (27–43)	RIA	NA	NA	NA	45.7	88.9	96.3	Use of ART and anti-TB therapy
Sudfeld et al. 2012 [26]	Tanzania, Dar es Salaam	06°50'S*	Cohort	1103	43	31.2	68.8	Median: 30–40	HPLC-MS using API-5000	NA	9.2	52.8	NA	NA	NA	Comorbidities: HIV, wasting
Sudfeld et al. 2013 [27]	Tanzania, Dar es Salaam	06°50'S*	Prospective cohort	1103	43	31.2	68.8	Median: 30–40	HPLC-MS using API-5000	NA	9.2	52.8	NA	NA	NA	Use of ART, comorbidities: oral thrush, wasting,
Friis et al. 2008 [22]	Tanzania, Mwanza	2.28 S	Cross-sectional	653	508	58.7	41.3	Median: 25–35	RIA	NA	NA	NA	0.8	10.6	41.2	Comorbidities: TB, season, marital status, dietary intake,
Mastala et al. 2013 [23]	Malawi, Blantyre	13° 30' S*	Cross-sectional	157	161	52.9	47.1	Mean: 38.9 (18–80)	EIA	0	16.6	47.8	11.2	42.2	74.5	Lower BMI, dietary intake, use of ART, comorbidities: heart failure, TB
Friis et al. 2013 [24]	Tanzania, Mwanza	2.28 S	Cross-sectional	1570	1250	58.1	41.9	Median: 25–35	CLIA	NA	4.3	39.6	NA	2.5	15	BMI, season, sun exposure, dietary intake, comorbidity: HIV
Nansera et al. 2011 [28]	Uganda, Mbarara	0.6132 S	Cross-sectional	150	50	51.3	48.7	Mean: 33 ± 9	SPE –RE-HPLC	0	20	NA	12	38	NA	Weight loss, comorbidity: HIV
Steenhoff et al. 2012 [36]	Botswana, Gaborone	22° 00'S*	Case-control	38	19	36.8	63.2	Median: 34 (31–38)	Diasorin Liaison assay	NA	78	NA	NA	78	NA	None
Martineau et al. 2011 [32]	South Africa, Cape Town	33°S	Cross-sectional	370	370	44.6	55.4	Median: 31.7 (25.8–42.3)	ID-LC-MS/MS	NA	NA	NA	14.05	62.7	88.1	Season, comorbidities: TB, HIV, lower BMI

Wejse et al. 2009 [33]	Guinea-Bissau, Bandim	11°50'60N*	RCT	365	365	60.8	39.2	Mean: 37 ± 14	ID-LC-MS/MS on API 3000 MS	NA	NA	NA	NA	8.2	44.7	Not identified
Glew et al. 2010 [29]	Nigeria, Gombe	10°17'22N*	Prospective	51	NA	43.1	56.9	Mean: 51.5 ± 10.8	UPLC-MS	NA	21.7	66.7	NA	NA	NA	Sex, sun exposure, clothing, BMI, dietary intake
Allali et al. 2009 [37]	Morocco, Rabat	34°02'N*	Cross-sectional	415	NA	-	100	Mean: 50 ± 9.3	CL	4	43	91	NA	NA	NA	Age, clothing, time spent outdoors, dietary intake
Feleke et al. 1999 [30]	Ethiopia, Addis Ababa	10°N	Prospective	61	NA	39.3	60.7	Median: 21 (20–22) [§] ; 25 (22–28)	HPLC	NA	97	77	NA	NA	NA	Clothing, dietary intake, skin color
Oduwole et al. 2010 [25]	Nigeria, Lagos	06°25'N*	Case-control	34	NA	70.6	29.4	Mean: 1.7 (0.5–2.5); 1.75 (0.5–2.5)	RIA	NA	5.88	47.06	NA	NA	NA	Comorbidity: pneumonia, clothing, sun exposure
Djennane et al. 2014 [38]	Algeria, Tizi-Ouzou	36°43' N	Prospective	435	NA	46.7	53.3	Median: 10 (8–13)	ECL-CPB	8.1 [¶] 17.4 [#]	29.9 [¶] 41.4 [#]	58.4 [¶] 65.2 [#]	NA	NA	NA	Season, skin color, dietary intake, SES, sun exposure
Mehta et al. 2009 [39]	Tanzania, Dar es Salaam	06°50'S*	Prospective	884	NA	-	100	Mean: 24.6 ± 5 y	CL	NA	NA	39.25	NA	NA	NA	Comorbidity: HIV

API, atmospheric pressure chemical ionization; ART, antiretroviral therapy; BMI, body mass index; CL, chemoluminescence; CLIA, chemiluminescent immunoassay; ECL-CPB, electrochemiluminescence competitive protein-binding assay; ECLIA, electrochemiluminescence immunoassay; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; HPLC-MS, high-performance liquid chromatography tandem mass spectrometry; ID-LC-MS/MS, isotope-dilution liquid chromatography-tandem mass spectrometry; NA, not available; RIA, radioimmunoassay; SES, socioeconomic status; SPE-RE-HPLC, solid-phase extraction reverse-phase high-performance liquid chromatography assay; TB, tuberculosis; UPLC-MS, ultra-performance liquid chromatography coupled with mass spectrometry

* Not indicated in the literature.

† TB case.

‡ Control.

§ Nonpregnant.

|| Pregnant.

¶ September.

March.

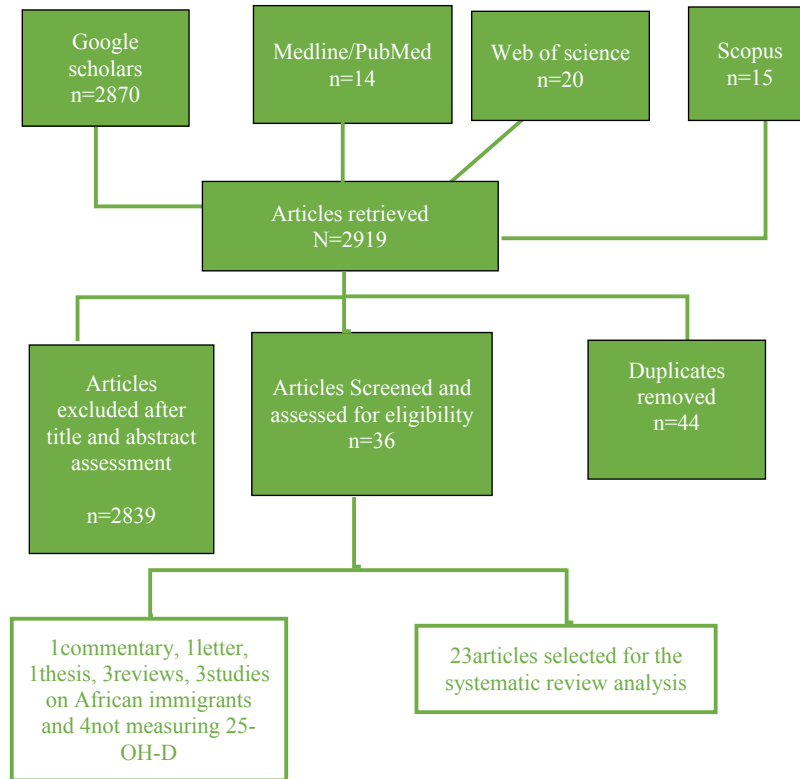


Fig. 1. Search strategy and selection process.

metabolism of vitamin D (e.g., isoniazid and rifampicin have been reported to reduce serum 25-OH-D), which will contribute to an association between low serum 25-OH-D and TB [20]. However, there further studies are needed on the cause–effect relationship between VDD and TB.

Predictor variables of vitamin D deficiency

In the present study, 22 articles identified predictor variables associated with VDD. Based on the location of the studies done, we summarized the variables into eastern, southern, western and northern Africa (Table 1). Variables such as season and

inadequate diet intake were identified in all regions. However, clothing and sun exposure were not identified in southern Africa. Several studies showed comorbidities and low body mass index (BMI; <18.5 kg/m²) to be variables in eastern, western, and southern Africa.

An independent association between VDD and increased age (>55 y) and dark skin photo-type were reported in eastern and northern Africa. Studies on the use of antiretroviral therapy (ART) in eastern and southern Africa and anti-TB drugs in southern Africa indicated that ART drugs reduce vitamin D status in the body [17,23]. The interactions of sex, ethnicity, and religion with vitamin D status and TB were also reported in western

Table 2
Laboratory tests used to measure the level of vitamin D in TB patients

Laboratory test	Author(s) year [ref.no]	≤25 nmol/L	≤50 nmol/L	≤75 nmol/L
ECLIA	Kibirige et al. 2013 [19]	13.5	44.2	85.4
EIA	Banda et al. 2011 [20]	20.4	42	74.5
	Mastala et al. 2013 [23]	11.2	42.2	74.5
ELISA	Mahmoud and Ali 2014 [12]	NA	50	72.5
CL	Conesa-Botella et al. 2012 [34]	NA	24.37	40.34
Cobas E601 Analyser	Tostmann et al. 2010 [35]	NA	1.23	17.28
ID-LC-MS/MS on API 3000 MS	Wejse et al. 2007 [16]	0.28	8.56	46.13
	Martineau et al. 2011 [32]	14.05	62.7	88.1
	Wejse et al. 2009 [33]	NA	8.2	44.7
LC-MS	Mehta et al. 2013 [31]	NA	15.66	61.74
RIA	Conesa-Botella et al. 2012 [21]	45.7	88.9	96.3
	Friis et al. 2008 [22]	0.8	10.6	41.2
CLIA	Friis et al. 2013 [24]	NA	2.5	15
SPE-RE-HPLC	Nansera et al. 2011 [28]	12	38	NA
Diasorin Liaison assay	Steenhoff et al. 2012 [36]	NA	78	NA

API, atmospheric pressure chemical ionization; CL, chemoluminescence; CLIA, chemiluminescent immunoassay; ECLIA, electrochemiluminescence immunoassay; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; ID-LC-MS/MS, isotope-dilution liquid chromatography-tandem mass spectrometry; LC-MS, liquid chromatography tandem mass spectrometry; NA, not available; RIA, radioimmunoassay; SPE-RE-HPLC, solid-phase extraction reverse-phase high-performance liquid chromatography assay; TB, tuberculosis

Table 3
Threshold levels

Categories of vitamin D deficiency	Reported threshold values		Studies reporting these values (n)	References
	ng/mL	nmol/L		
Severe deficiency	5	12.5	2	19,37
	<8	<20	1	25
	<10	<25	6	16,19–23
	<11	<27.5	1	32
	<12	<30	2	30,38
Deficiency	10–20	25–50	2	20,22
	11–16	27.5–40	1	25
	12–19	30–47.5	1	28
	<15	37.5	1	37
	<20	<50	17	12,16,19,21,23,24,26,27,29–36,38
Insufficiency	<30	<75	12	12,16,21,23,24,29,31–34,37,38
	11–28	27.5v70	1	25
	20–30	50–75	5	20,22,26,27,35
	21–29	52.5–72.5	1	19
	<32	<80	1	39

Africa [12]. Other predictor variables such as time spent outdoors <30 min/d and socioeconomic status (SES) were reported in northern Africa [33,34] and money spent on food per person per day was also reported in eastern Africa [27]. Compared with TB patients who were married, single patients had 10.4 nmol/L (95% confidence interval [CI], 4.0–16.9) lower concentrations [18].

Discussion

The relationship between vitamin D and TB may be mediated through the mechanisms of increased cathelicidin production and enhancement of the capacity of macrophages [8]. Vitamin D is essential for an interferon- γ -mediated pathway in macrophages that leads to autophagy, phagosomal maturation, and other antimicrobial activities against *M. tuberculosis* [22].

Vitamin D deficiency

We have systematically reviewed all publications in peer-reviewed journals. Some variability was observed in the definition of VDD. More than half of the studies agreed on serum 25-OH-D concentration ≤ 25 nmol/L as an indicator of sVDD; ≤ 50 nmol/L as an indicator of VDD; and ≤ 75 nmol/L as an indicator of VDI. In fact, this definition is in agreement with a previously described definition [13].

Our systematic review also identified that VDD is common in TB patients living in Africa. The highest prevalence of VDD (42%–88.9%) and VDI (74.5%–96.3%) were reported in southern Africa [16,17,19,28,32]. All the studies reported the prevalence of VDD $\leq 88.9\%$. In relation, 86.7% (13 of 15) of the studies showed a prevalence of VDI that reached $\leq 96.3\%$. These findings imply that VDD and VDI are very common among patients with TB in Africa. In contrast, few studies reported the prevalence of sVDD in patients with TB, ranging from 0.2% to 45.7%. This is very low compared with the prevalence of VDD and VDI.

Predictors of vitamin D deficiency

The deficiency of sunshine-derived vitamin in the sunny continent was not expected. However, VDD and VDI were identified as a common problem among patients with TB in Africa.

But what are the predictors of these deficiencies in Africa? The present study summarized the major predictors as discussed hereafter.

Lack of sun exposure and inadequate diet intake

Almost all eligible studies reported lack of sun exposure and inadequate diet intake as predictors of VDD among patients with TB in Africa. The major source of vitamin D for most humans comes from sun exposure, typically between 1000 and 1500 h in spring, summer, and fall [7]. However, data on duration of sun exposure in Africa were missed. During sun exposure, the UVB (290–315 nm) portion of sunlight photolyzes 7-dehydrocholesterol (7-DHC) in the epidermis to previtamin D₃ [37]. Once formed, previtamin D₃ undergoes thermal isomerization to form vitamin D₃.

The amount of solar UVB radiation reaching the biosphere is a function of the solar zenith angle and depends on latitude, season, and time of the day [38]. Only brief daily sun exposure is required to produce adequate vitamin D, and excess sun exposure converts cholecalciferol to inactive metabolites [18]. Other predictor variables affect vitamin D status through impeding the intensity of sun exposure and affecting dietary intake. It was initially thought that both vitamin D₂ and vitamin D₃ follow the same metabolic pathway. However, minor differences in the chemistry of side chains between the two forms of vitamin D result in differences in the site of hydroxylation and leads to the production of unique biologically active metabolites. Supplementation of vitamin D₂ produces appreciable amounts of serum 25-OH-D₂, which has a lower affinity for vitamin D-binding protein and results in a shorter circulating half-life than that of 25-OH-D₃ [39].

Few foods naturally contain vitamin D, including oily fish such as salmon, mackerel, and herring; and oils from fish, including cod liver oil [13]. Sufficient amounts of vitamin D are obtained from wild fish compared with those that are cultivated [38]. This may be due to the ergosterol (previtamin D₂) and 7-DHC (previtamin D₃) content of plankton and zooplankton in the biomass. Sundried mushroom is rich in ergocalciferol (vitamin D₂) [7] and small amounts of cholecalciferol (vitamin D₃) can be obtained from liver, meat, egg yolk, and dairy products [40], which are rarely consumed by people in Africa [2].

Season

Although seasonal variation in vitamin D status in Africa is not expected, the present systematic review identified season as the major predictor variables of VDD. A study done in Tanzania showed mean vitamin D concentration of 74.8 nmol/L for January through February, and 66.3 nmol/L for July through October [27]. Similarly, another study showed VDI is very common in Moroccan women during the summer season [34]. In Guinea Bissau, more cases of hypovitaminosis D were found during the rainy season [12].

An 8-y period study in South Africa showed the highest concentration of serum 25-OH-D from January through March and lowest from July through September (56.8 versus 30.7 nmol/L, respectively; $P < 0.001$) [28]. A study conducted on reciprocal seasonal variation in TB notifications also indicated the highest number of TB cases from October through December (4222 versus 5080; $P < 0.001$) and the lowest from April through June [28]. These findings imply that seasonal and year-to-year variations reflect true differences in vitamin D status over time, due to variation in sun exposure and dietary intake [20].

Clothing, comorbidities, and low BMI

Studies conducted in eastern, western, and northern Africa identified clothing as a predictor of VDD [25,26,33]. Findings of low vitamin D status in Addis Ababa, Ethiopia were explained by clothing habits, leaving little skin open to sun exposure [26]. A study done in Nigeria revealed that pregnant women using veils for religious reasons had significantly lower 25-hydroxyvitamin D levels compared with nonveiled women [41]. Similarly, clothing was identified as the most important factor influencing hypovitaminosis D in Morocco [34]. Muslim custom dictates females to cover most of their skin surface in the public. One study described that because they are Muslim, the women do not derive much benefit from sunlight [25]. Likewise, the associations between ethnicity, clothing, and hypovitaminosis D were also reported in western Africa [12].

Although the cause–effect relationship has not yet been ruled out, comorbidities with TB [18–20,28,32], HIV [17,20,22,24,27,28,35], pneumonia [21], oral thrush [23], and heart failure [19] were identified as predictors of VDD in eastern, western, and southern Africa. These infectious and noninfectious diseases may attribute to VDD and VDI by a reduction in appetite and by restricting outdoor physical activities.

In line with this, a positive relationship between BMI (<18.5 kg/m²) and VDD was identified in Africa [15,16,18–20,22–25,28]. In fact, BMI is usually a measure of underweight in developing countries. A lack of adipose tissues in underweight patients leaves them unable to store vitamin D, meaning these patients have no reserves when external sources are lacking.

Use of ART and anti-TB drugs

A cross-sectional study conducted in Malawi identified the use of ART as significant predictor variable of VDD [19]. Another longitudinal study done in South Africa demonstrated that the use of nucleoside reverse transcriptase inhibitors decreases 25-OH-D concentration [17]. Additionally, VDD during ART initiation had significantly increased the risk for pulmonary TB and oral thrush infections [23]. It was previously reported that the use of anti-TB drugs can attribute to decreases in serum 25-OH-D levels [30].

In contrast, it has been shown that 25-OH-D concentrations increased during the first 2 mo of TB treatment [31]. The association between the use of drugs and 25-OH-D concentration is ascribed to the pharmacology of the drugs [42]. Two of the standard first-line anti-TB drugs, isoniazid (INH) and rifampicin (RMP), are known for inhibiting and inducing cytochrome P450 (CYP) activity, respectively, and can affect vitamin D metabolism. INH reduces 25-OH-D and 1,25-(OH)₂-D concentrations by the inhibition of 25-hydroxylase, as has been shown in *in vitro* and animal studies and in humans [43–46].

RMP is a strong inducer of CYP3A4 [47], which is a vitamin D 24- and 25- hydroxylase [48]. Induction of these enzymes increases the enzymatic conversion of 25-OH-D to the inactive metabolite 24,25-(OH)₂-D and results in decreased 25-OH-D and 1,25(OH)₂ D concentrations, as shown in studies in humans [46]. Combined use of INH and RMP reduces 25-OH-D and 1,25-(OH)₂-D concentrations in healthy individuals and patients with TB [31].

Age, sex, and marital status

The mean and median ages of the majority of study participants were >30 y. Studies done in Uganda [15] and Morocco [33] revealed that increasing age was independently associated with VDD. Additionally, it has been noted half of the population of

Europe >60 y of age have VDD [49]. This is justified by the inverse relationship between age and previtamin D₃ concentration in the epidermis [50]. Moreover, older individuals are not frequently exposed to sunlight.

The difference between sex and vitamin D status was also observed in western Africa. Most (83%) Fulani women have poor 25-OH-D concentration [25]. This was explained by the difference in skin exposure between men and women in the tribe. Sex was also identified as a significant risk factor of VDD in the Middle East and North African [51]. A study conducted in eastern Africa reported that unmarried patients have lower serum 25-OH-D concentration compared with married patients, possibly due to behavioral variations leading to work-related differences in sun exposure [18].

Skin pigmentation, SES, time spent outdoors, and money spent on food

This systematic review encompassed solely studies done in Africa and hence almost all participants had pigmented skin. The production of previtamin D₃ is dependent on the concentration of 7-DHC and melanin pigmentation [50]. To synthesize the same amount of vitamin D, dark-skinned individuals require three to four times longer sun exposure than light-skinned persons because melanin efficiently absorbs UVB radiation [52]. In relation, two studies reported skin pigmentation as a significant predictor of VDD in eastern and northern Africa, respectively [26,34].

A study conducted in northern Africa showed a significant association between poor SES and VDD [34]. Hypovitaminosis D was also associated with the time spent outdoors <30 min/d (odds ratio, 2.8; 95% CI, 1.4–5.7; *P* = 0.003) [33]. One study indicated that a 10-min exposure of the head and uncovered arms three times per week would be sufficient to prevent VDI [33]. Similarly, money spent on food per person per day has an interaction with low 25-OH-D concentrations [27].

Limitations of the analysis

The first limitation of this systematic review was the lack of representative studies from central Africa. Four databases were searched to retrieve all published articles but we could not find any studied from central Africa. Second, there was heterogeneity in study designs, sample sizes, and laboratory tests. Although four studies used case–control study design, three did not report vitamin D status in detail. The remaining study was also unmatched, thus making it difficult to draw conclusions about the direction of a potential cause–effect relationship between VDD and TB. In some studies, the sample size was small and the probability of detecting small differences would be very low. Selection bias was also observed in volunteer and hospital-based studies as most studies focused on smear-positive TB patients and rarely on smear-negative TB patients. In relation, there could be some variation in measurements as many studies used different laboratory tests.

Conclusion

VDD and VDI were highly prevalent among TB patients in Africa. These were attributed to the existence of predictors such as a lack of sun exposure, inadequate dietary intake, season, clothing, comorbidities, age, low BMI, skin pigmentation, use of ART and anti-TB drugs, SES, time spent outdoors, money spent on food, sex, and marital status. Understanding the problems with their predictors enable us to further question the association

between vitamin D status and TB and what should be done to address the problem. In fact, the options are vitamin D supplementation, food fortification, and biofortification, but these are economically less feasible. Could we suggest sun exposure for free to lessen the burden of VDD and TB? Therefore, further case-control studies are warranted to clarify the cause-effect relationship between vitamin D and TB to understand the interaction between vitamin D status and disease progression, and thereby, to design valuable strategies to manage VDD among TB patients in Africa.

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3.4. Impact of the natural resource of UVB on the content of vitamin D₂ in oyster mushroom (*Pleurotus ostreatus*) under subtropical settings

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Original article

Impact of the natural resource of UVB on the content of vitamin D₂ in oyster mushroom (*Pleurotus ostreatus*) under subtropical settingsTibebeslassie Seyoum Keflie^{a,b,*}, Nils Nölle^a, Christine Lambert^a, Donatus Nohr^a, Hans Konrad Biesalski^{a,b}^a Institute of Biological Chemistry and Nutrition (140a), University of Hohenheim, Garbenstr. 30, 70593 Stuttgart, Germany^b Food Security Centre, University of Hohenheim, Stuttgart, Germany

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ABSTRACT

Vitamin D deficiency is a pandemic problem. Non-animal source of vitamin D is obtained from edible mushrooms. Oyster mushroom (*Pleurotus ostreatus*) was sliced into the size of 1 cm³, 4 cm³ and 9 cm³, and treated with the sun as a natural resource of UVB under subtropical settings in Ethiopia. The content of vitamin D was measured by using high-performance liquid chromatography (HPLC). After sun treatment, there was a significant increment in the content of vitamin D₂ from nil to 67.4 ± 28.0 µg/g dry weight (DW). Based on the results of the overall pairwise comparisons, 1 cm³ size of slice group had the highest content of vitamin D₂. Duration of sun exposure, sizes of mushroom slices and moisture content were identified as determining factors for vitamin D₂ synthesis. Exposing slices of oyster mushroom to the sunlight for <30 min provides the amount that satisfies the current recommended dietary allowance (RDA) of vitamin D without any visible change in color and texture. Thus, sun treatment of oyster mushroom is an effective and economically cheap strategy in the fight against vitamin D deficiency.

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1. Introduction

Vitamin D deficiency is a pandemic and an ever-increasing problem in human nutrition and health. It is estimated that 30–50% of individuals in Europe and the U.S. have insufficient levels of vitamin D (Battault et al., 2013; Holick, 2009; Mansoor et al., 2010). In Africa, more than 80% of the people are prone to vitamin D deficiency (Keflie et al., 2015).

Oyster and other edible mushrooms have been recognized as a source for vitamin D since 1994 (Mattila et al., 1994). Mushrooms are the only non-animal food source that contains vitamin D

(Valverde et al., 2015). Ergosterol is the principal sterol and precursor for vitamin D₂ in mushrooms. It is converted via absorption of ultra violet B (UVB) light energy (290–320 nm). The absorption of light causes the isomerization of the molecule and bond cleavage between carbons 9 and 10, resulting in unstable intermediate “pre-vitamin D₂”. The conversion of pre-vitamin D₂ to vitamin D₂ then follows via a thermally catalysed process (Chen et al., 2010; Simon et al., 2011; Keegan et al., 2013).

The formation of ergocalciferol starts without delay after exposure to UVB light. As UVB light is not able to penetrate deeply into the fruiting body, the reaction rate must cease when the superficial ergosterol is used up. The yields of ergocalciferol are thus limited, but at the same time the risk of accumulating extreme doses is low (Krings and Berger, 2014). Prolonged exposure in the sun-exposed samples can significantly produce irreversible, over-exposed products by dimerization and ring cleavage of pre-vitamin D₂ and tachysterol (Keegan et al., 2013; Urbain et al., 2016).

Besides of the wavelength of UV irradiation, the efficiency of vitamin D₂ synthesis in mushrooms can be influenced by multiple parameters such as ambient temperature, exposure time and irradiation intensity. It is not known whether the effects of these factors are independent or interactive (Wu and Ahn, 2014).

So far, very few studies conducted until recent time focused on the natural resource of UVB irradiation for the synthesis of vitamin

Abbreviations: AI, adequate intake; DRI, dietary reference intake; IU, international unit; RDA, recommended dietary allowance; UVB, ultra violet ray B.

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D₂ (Simon et al., 2011; Urbain and Jakobsen, 2015; Urbain et al., 2016; Nölle et al., 2017). Of these studies, none of them addressed the potential effects of the sun on vitamin D₂ production under tropical and subtropical settings. There is also a scarce information on the content of vitamin D₂ in oyster mushroom. Nowadays, cultivated oyster mushroom is available in the markets of some areas in Ethiopia. Therefore, this study was designed to assess the impact of the natural resource of UVB irradiation on the content of vitamin D₂ in oyster mushroom under subtropical settings.

2. Material and methods

2.1. Chemicals

The internal standard (vitamin D₃) and external standard (vitamin D₂) were purchased from ENZO Life Sciences (Germany), whereas potassium hydroxide was purchased from Merck and ascorbic acid from AppliChem GmbH (Germany).

2.2. Experimental design

The experiment was undertaken on oyster mushroom. The samples of oyster mushrooms were categorized into sun treated and non-treated groups. Non-treated group was used as a control group of the experiment. The third group was added for further comparisons and irradiated with artificial UVB irradiation.

2.3. Mushrooms

Samples of oyster mushrooms for sun treatment and control groups were purchased from the local super market in Addis Abeba, Ethiopia on September 24, 2016. Those samples under the group of sun treatment were immediately processed and were either left whole or sliced manually using a knife and ruler into 1 cm³ (1 cm × 1 cm × 1 cm), 4 cm³ (2 cm × 2 cm × 1 cm) and 9 cm³ (3 cm × 3 cm × 1 cm) size of slice groups. The control group was placed into black plastic bag, knotted tightly, labelled and stored at minus 80 °C. The mean ± standard deviation (SD) of single mushroom stock was 32.7 ± 4.1 g with cap diameter of 8.2 ± 0.9 cm and stem length of 10.4 ± 0.5 cm (n = 12).

2.4. Sun treatment

Sun treatment was conducted between 8:00 am and 4:00 pm on 24 and 25 September 2016 at Aleltu town. This town is situated at 9° 11' 47 N, 39° 9' 12 E, and 2350 m above sea level in the north-eastern part of Addis Abeba, the capital city of Ethiopia. The minimum and maximum temperatures during the process of sun treatment were 21 °C and 34 °C, respectively. On both days, the sky was cloudless. Samples were divided into the above-mentioned size groups. Each of the samples was positioned horizontally and exposed to the sun light for the durations of either 30 min, 1 h, 3 h, 8 h or 16 h. In every 15 min for the first 1 h and in every 30 min later, the samples were turned around to expose all parts equally to the sun light. Changes in color and texture were checked every time during the process of turning. Like control group, the samples were placed into black plastic bags, knotted tightly, labelled and stored at minus 80 °C immediately after their treatment period was over.

2.5. Samples transportation

The samples were packed into an insulated plastic bags together with dry ice and transported to University of Hohenheim, Stuttgart, Germany where further laboratory analyses were

performed. Immediately after arrival, the samples were stored at minus 80 °C.

2.6. UVB irradiation

Samples for artificial UVB irradiation were purchased from a local supermarket in Stuttgart, Germany. Like the groups for sun treatment, the samples were divided into four groups: 1 cm³, 4 cm³, 9 cm³ size slices and whole size. All the samples including a sample of lyophilized powder were horizontally placed on the shelf of irradiation chamber equipped with UVB-lamps (Dr. Groebel, GmbH, Ettlingen, Germany). Each side of the samples was irradiated up to a UVB dose of 1.5 J/cm² at 22 °C.

2.7. Lyophilisation, pulverization and moisture content determination

All treated and non-treated samples were lyophilized with a Telstar LyoQuest freeze drier (Azbil Telstar Technologies SLU, LyoQuest-85 year 2014, Spain) and then, pulverized and homogenized into fine powder using a grinding mill (TH. Geyer, Germany) at Institute of Biological Chemistry and Nutrition, University of Hohenheim. The powders were kept in an airtight plastic bag and stored at minus 20 °C until used for laboratory analyses. The moisture content was determined using oven drying method at the Institute of Agricultural Engineering, University of Hohenheim.

2.8. Vitamin D extraction

Triplicate samples were taken from each group for the measurement of vitamin D₂. The extraction of vitamin D₂ was carried out based on the method of Nölle et al. (2017) with some modifications. Samples of pulverized and homogenized powder (1 g) were mixed with 19 mL of ethanol (with a chemical purity of 99.7%), 4 mL of 50% potassium hydroxide (500 g KOH in 1 L of H₂O), 1.333 mL of sodium ascorbate (1.75 g solved in 10 mL of 1 M sodium hydroxide) and 1 mL of vitamin D₃ (100 mg/L) as internal standard in a 50 mL falcon tube. The mixture was vortex mixed and subsequently saponified for 1 h in a water bath at 80 °C. The mixture was then cooled to ambient temperature in ice water. To promote a better separation of the layers, 10 mL of saturated sodium chloride solution was added. Subsequently, 15 mL of n-hexane was added, vortex mixed and centrifuged for 8 min at 4500g (Heraeus, Hanau, Germany). The n-hexane layer was transferred into a new falcon tube and the extraction processes were repeated twice, one time with 15 mL of n-hexane and later with 10 mL of n-hexane. The pooled organic layers were washed three times with deionised water until neutralized. The organic layer was then transferred into a 100 mL round bottom flask and rotary evaporated to dryness. The flask was rinsed with 6 mL of n-hexane and transferred into 10 mL of round bottom flask and, rotary evaporated again. Once evaporated to dryness, the sample was immediately re-dissolved in 1 mL of tetrahydrofuran and vortex mixed. Thereafter, the samples were centrifuged for 5 min at 1600g at 20 °C to remove impurities and then used for subsequent analyses by HPLC.

2.9. HPLC analysis

A system of HPLC (Shimadzu technologies) equipped with a DGU-20A3R degassing Unit, two LC-20AT pumps, a SIL-20A8HT auto sampler and a CBM-20A communication bus module (Shimadzu GmbH, Duisburg, Germany) was used to measure vitamin D₂ content at the Institute of Biological Chemistry and Nutrition. The column used was a Reprosil 80 ODS-2 analytical column, 4.6 × 250 mm, 3 μm particle size (Dr. Maisch GmbH, Ammerbuch, Germany). The mobile phase was composed of acetonitrile (77%),

deionized water (14%) and tetrahydrofuran (9%) at a flow rate of 2 mL/min with a total run time of 42 min. The injection volume was 10 μ L and detection was carried out by diode array detector at a wavelength of 265 nm. A set of six calibration standards of vitamin D₂ and D₃ were prepared with the contents of 10, 20, 100, 200, 300 and 400 μ g/mL, respectively. The Lab Solution software was used for HPLC control as well as acquisition and quantification of data.

2.10. Statistical analyses

Statistical analyses were performed using IBM SPSS statistics version 23. The normality of the data distribution was checked by Shapiro-Wilk's normality test. Graphs were generated with Microsoft Excel. The content of each analyte was described as mean \pm standard deviation (SD). Pre- and post-treatment comparisons were analysed by paired two sample *t*-test for means. The effects of two independent variables on the continuous dependent variable were examined using two-way analysis of variance (two-way ANOVA) and the estimated marginal means were compared with least significant difference (LSD) post hoc test. A *p* < 0.05 was considered as statistically significant.

3. Results and discussion

3.1. Moisture content, color and texture changes

In the present study, the overall moisture content of oyster mushroom was 92.5%. During the process of sun treatment, changes in colour, texture and moisture content were observed on the samples. Fig. 1 demonstrates the differences before and after sun treatment. The description in Table 1 shows the percentage of the loss of moisture and the extent to which the color and texture changed. In 1 cm³ size group, more than 67% of moisture content was lost within 3 h of sun exposure, followed by brown coloration and shrivelled texture. Within the same duration of sun exposure, the whole size group lost about 35% of moisture content with minor change in color and texture. In 9 cm³ size group, about 12% of the moisture content was lost within 30 min of sun exposure without any observable change in color and texture. With equal durations of sun exposure, about 19% of the moisture content was lost in 1 cm³ size group. This shows that increasing the surface area to volume ratio for sun exposure increases the loss of moisture with rapid change in color and texture.

The loss of moisture and shrivelled texture were due to evaporation of the moisture, whereas the color change was as the results of enzymatic reaction in the tissue of the mushroom. Kalac (2013) described that the color changes are usually caused by the presence of tyrosinase enzyme. This enzyme catalyses tyrosin to be oxidized to o-dihydroxyphenylalanine (DOPA), then to brown

quinonic pigments and eventually to melanins (Kalac, 2013). The reported tissue discoloration could affect the aesthetic value of mushrooms for consumption.

3.2. Vitamin D₂ as the result of sun exposure

The chromatograms in Fig. 2 shows the content of vitamin D₂ in non-treated and sun treated oyster mushrooms. The content of vitamin D₂ increased from nil to considerable levels with different slice sizes and durations of sun exposure. The maximum content of vitamin D₂ was produced in 1 cm³ size of slice group (67.4 \pm 28.0 μ g/g dw). Shapiro-Wilk's test with Lilliefors significance correction (*p* > 0.05) indicated that the contents of vitamin D₂ were normally distributed in 1 cm³, 4 cm³ and 9 cm³ size of slice groups. For all vitamin D₂ measurements, the coefficient of variation was <13.5%.

For further comparisons, the mean content of vitamin D₂ in 1 cm³ size of slice group was used. The mean of vitamin D₂ in 1 cm³ size in the present study was higher than the reports of Jasinghe and Perera (2005) on oyster mushrooms (45.1 \pm 3.1 μ g/g dw); Nölle et al. (2017) on brown button mushrooms (36 μ g/g dw); and Simon et al. (2011) on white button mushrooms (3.7 \pm 0.9 μ g/g dw). These variations may arise from the type of mushrooms used for experiments, the differences in the duration and intensity of sun exposure, and the place of cultivation. Fig. 3 depicts the content of vitamin D₂ across the durations of sun exposure and slice sizes of oyster mushrooms. The figure shows that the contents of vitamin D₂ were increased with the durations of sun exposure.

The mean \pm SD of vitamin D₂ contents produced in 30 min, 1 h and 3 h of sun exposure were 28.5 \pm 8.5, 36.6 \pm 8.7 and 73.7 \pm 12.9 μ g/g dw, respectively. In 1 h of sun exposure, Urbain and Jakobsen (2015) obtained 3.9 μ g/g dw of vitamin D₂ from white button mushroom in Germany. But, Simon et al. (2011) obtained 3.8 μ g/g dw of vitamin D₂ from white button mushroom exposed to the sun for 2.5 h in United States of America. These two results were by far lower than the present findings. Possible explanations for the discrepancies in vitamin D₂ contents are the differences in the latitude and altitude of the locations, cultivars/types, sizes of the mushroom preparations, moisture contents and temperature as well as duration and intensity of sun exposure. Latitude and season affect both the quantity and quality of solar radiation reaching the surface of the earth which in turn have an influence on the synthesis of vitamin D (Webb et al., 1988).

During the first 3 h of sun exposure, vitamin D₂ contents were rapidly increased in all size groups and the maximum content was found in 1 cm³ size of slice group with mean \pm SD of 88.1 \pm 3.1 μ g/g dw. Some fluctuations in the distribution of vitamin D₂ were observed along with the durations of sun exposure. From 3 h to 8 h of sun exposures, the content of vitamin D₂ in 1 cm³ size



Fig. 1. Oyster mushroom before (A) and after (B) sun treatment.

Table 1
Percentage of moisture loss and changes in color and texture of sun treated oyster mushroom.

Size	Duration of sun exposure (hour)	Loss of moisture (%)	Minimum Temp. (°C)	Maximum Temp. (°C)	Color change	Texture change
1 cm ³	0.5	19.3	21	23	Not observable	None
	1.0	31.4	21	27	Very light brown	Very slight shrinking
	3.0	67.0	21	32	Brown	Shrank
	8.0	91.0	21	34	Dark brown	Totally shrank
	16.0	90.2	21	34	Dark brown	Totally shrank
4 cm ³	0.5	11.4	21	23	Not observable	None
	1.0	27.4	21	27	Very light brown	Very slight shrinking
	3.0	59.7	21	32	Light brown	Shrank
	8.0	89.7	21	34	Dark brown	Totally shrank
	16.0	90.9	21	34	Dark brown	Totally shrank
9 cm ³	0.5	12.0	21	23	Not observable	None
	1.0	23.4	21	27	Not observable	Not observable
	3.0	62.2	21	32	Brown	Shrank
	8.0	90.2	21	34	Dark brown	Totally shrank
	16.0	90.7	21	34	Dark brown	Totally shrank
Whole	0.5	0.5	21	23	Not observable	None
	1.0	7.6	21	27	Not observable	None
	3.0	35.0	21	32	Very light brown	Very slight shrinking
	8.0	74.6	21	34	Brown	Shrank
	16.0	90.8	21	34	Dark brown	Totally shrank

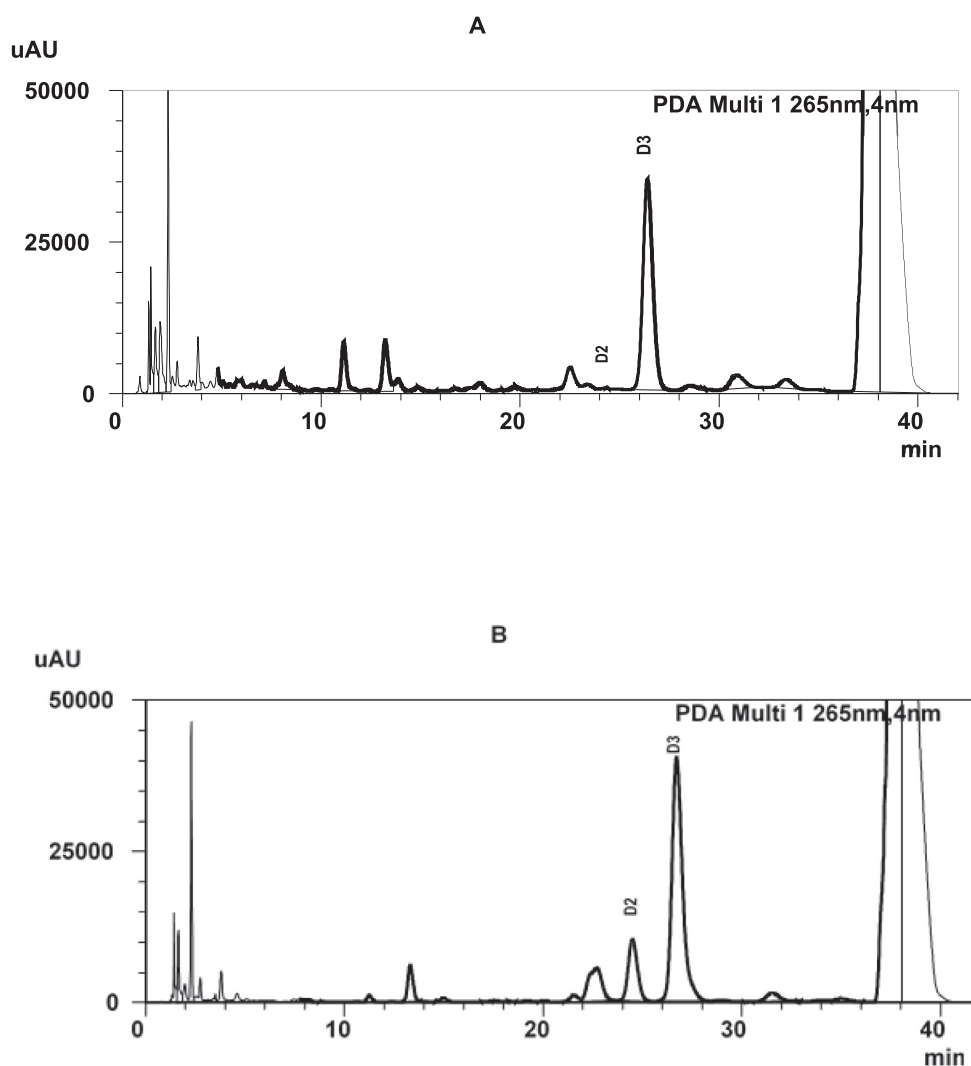


Fig. 2. HPLC chromatograms of vitamin D₂ analysis in non-treated oyster mushroom (A) and sun treated oyster mushroom (B). Vitamin D₃ was used as internal standard.

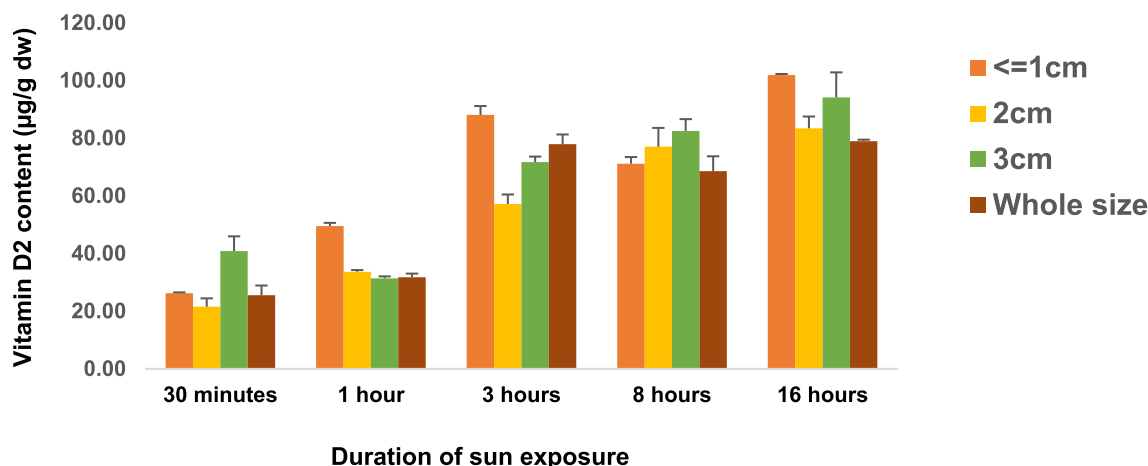


Fig. 3. Contents of vitamin D₂ across different sizes groups and duration of sun exposure.

of slice group was decreased but from 8 h to 16 h of sun exposure its content was increased again. Per the results of the overall pairwise comparisons, 1 cm³ size of slice group had significantly higher content of vitamin D₂ than 4 cm³, 9 cm³ and whole size groups in 1 h, 3 h and 16 h of sun exposures ($p < 0.05$).

There were statistically significant differences in vitamin D₂ contents among different size groups ($p < 0.05$). The contributions of durations of sun exposure, size of slice groups and the interaction of the two were also statistically significant on the content of vitamin D₂ in oyster mushroom ($p < 0.05$). The overall distributions of vitamin D₂ indicated that the smaller the mushroom sliced, the higher the content of vitamin D₂. These results were corroborated with the findings of Urbain and Jakobsen (2015), and Urbain et al. (2016). Nölle et al. (2017) also observed the highest conversion of ergosterol to vitamin D₂ in sliced fruit bodies in 2016.

The effects of durations of sun exposure on the content of vitamin D₂ were also statistically analysed. More than 93% of the variations in vitamin D₂ contents amongst 1 cm³, 4 cm³, 9 cm³ and whole size groups were accounted for by the duration of sun exposure. As compared to durations of sun exposure, the size of slices in the group had smaller effects on the content of vitamin D₂. After 8 h of sun exposure, there was no statistically significant difference in the contents of vitamin D₂ across all size groups ($p > 0.05$). However, in 16 h of sun exposure, the difference in the contents of vitamin D₂ became significant. This showed the variabilities in the contents of vitamin D₂ in extended periods of sun exposure. One of the factors that account for such variabilities is the intensity of UVA irradiation. The longer wavelength UVA present in sunlight could potentially photodegrade vitamin D (Chen et al., 2010).

Vitamin D₂ may also be subjected to the action of tissue mono-oxygenases that reduce the overall conversion of ergosterol to vitamin D₂ (Jasinghe and Perera, 2005). However, in the presence of ergosterol, the conversion processes might eventually resume and elevate the concentration of vitamin D₂ as the case of 16 h of sun exposure in our findings. The efficacy of ergosterol to vitamin D₂ conversion was positively dependent on the irradiation intensity. The content of vitamin D₂ dramatically increases when the irradiation intensity increases (Wu and Ahn, 2014).

3.3. Vitamin D₂ as the result of UVB irradiation

An experiment on artificial UVB irradiation was conducted for the sake of comparisons. The bar graphs clearly depicted the differences of the contents of vitamin D₂ across the size of slice groups (Fig. 4). The maximum content of vitamin D₂ was

814.1 ± 21.9 µg/g dw which was measured in 1 cm³ size of slice group. This amount was more than 9 folds higher than the one produced with 3 h of sun exposure in the same size group. In agreement with this, Urbain and Jakobsen (2015) noted that the content of vitamin D₂ produced with artificial UVB irradiation was more than 10 folds higher than the one produced with solar radiation during the process of drying.

Wu and Ahn (2014) obtained 239 ± 4.5 µg/g dw of vitamin D₂ content in oyster mushroom with operational conditions of 28.2 °C, 94.28 min and 1.14 W/m². This value was lower than the present finding in the whole size oyster mushroom (316.0 ± 5.1 µg/g dw). Urbain et al. (2016) reported the highest vitamin D₂ content after the largest UVB dose of 2.01 J/cm² and consisted of 101.5 ± 4.3 µg/g dw. However, this finding was nearly 8 times lower than our finding in 1 cm³ size of slice group (814.1 ± 21.9 µg/g dw). This difference could be emanated from the difference in moisture content, types and sizes of mushroom slices as well as the doses of UVB irradiation.

With the same operational condition, Wu and Ahn (2014) found 498.1 µg/g dw of vitamin D₂ content in lyophilized powder. However, this finding was higher than our finding in lyophilized powder (314.5 ± 8.2 µg/g dw). With equal dose of UVB irradiation, the content of vitamin D₂ in lyophilized sample was 2.6 times lower than the one in 1 cm³ size of slice group. One of the reasons for such a difference could be moisture content. Lyophilized powder contained very little moisture content as compared to 1 cm³ size of slice group. This in turn ascertained the essentiality of the moisture content which is important for the dilution of ergosterol in the photochemical reaction and its conversion to vitamin D₂. Perera et al. (2003) reported that the conversion of ergosterol to vitamin D₂ was affected by the moisture content of the mushrooms.

Further comparison was made on lyophilized powder. One group of lyophilized powder was exposed to the sun for 20 min and compared with the one irradiated with artificial UVB. The content of vitamin D₂ produced in the former was about 31 times lower than in the latter. These results clearly showed how the difference in the doses of UVB irradiation influences the production of vitamin D₂ in oyster mushroom.

3.4. Contribution of sun treated oyster mushroom

In general, vitamin D₂ is effectively produced in oyster mushroom under sun treatment. Vitamin D₂ has similarity with vitamin D₃ in physiological responses such as regulation of calcium and phosphate homeostasis and, cell proliferation and differentiation (Jones et al., 1998; Jurutka et al., 2001). Although there is a slight

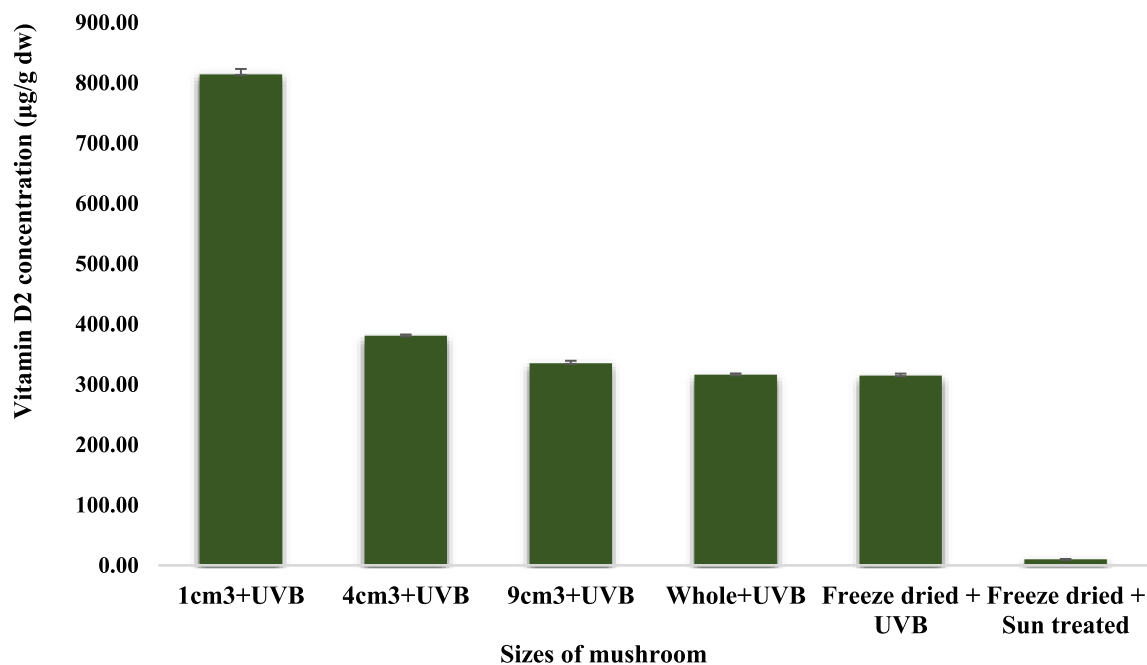


Fig. 4. Vitamin D₂ concentration in oyster mushroom treated with UVB irradiation up to a dose of 1.5 J/cm².

difference in the chemical structure at the side chains, recent reports confirmed that vitamin D₂ is as effective as vitamin D₃ in maintaining the contents of 25-hydroxy vitamin D in the blood (Biancuzzo et al., 2010; Urbain et al., 2011; Keegan et al., 2013). Hence, its importance in the fight against vitamin D deficiency is worth noting particularly in tropical and subtropical areas.

The analyses done based on the dietary reference intakes (DRI) revealed that sun treated oyster mushroom contained a large amount of vitamin D₂. For instance, a group of 9 cm³ size slices of oyster mushroom which was treated with sun for 30 min produced vitamin D₂ to the levels equivalent to 40.9 ± 5.1 µg/g dw. This was comparable to the content of vitamin D₃ in cod liver oil (40.3 µg per 1 tablespoon) (Bueno and Czepielewski, 2008).

A regular 100 g serving of such mushrooms could provide vitamin D₂ more than 20 times of the current recommended dietary allowance (RDA) of 15 µg (600 IU) for all ages between 1 and 70 years as indicated in Table 2 (IOM, 2010; Simon, et al. 2011; EFSA, 2016). In other words, 5 g fresh weight of such mushrooms are sufficient to satisfy the RDA of vitamin D.

Table 2
Contribution of 100 g edible portion of oyster mushroom based on dietary reference intakes.

Duration of sun exposure	Size of slices	Vitamin D (AI ^a = 15 µg)	
		Mean (µg/100 g fresh weight)	Contribution (%)
30 min of sun exposure	1 cm ³	197.8	1318.8
	4 cm ³	162.5	1083.5
	9 cm ³	308.1	2054
1 h sun exposure	1 cm ³	373.5	2490.2
	4 cm ³	253.8	1692
	9 cm ³	236.6	1577.2
3 h sun exposure	1 cm ³	664.1	4427.1
	4 cm ³	431.2	2874.7
	9 cm ³	540.9	3605.8

AI – Adequate Intake.

^a Based on European Food Safety Authority (EFSA), 2016 recommendation.

3.5. Limitations

In the present study, no measurements were taken on the UVB doses of different durations of sun exposure. In addition, data on the contents of other UV sensitive substances in the oyster mushrooms like vitamin C or β-carotenes were not included in this study. However, food composition tables show that oyster mushrooms do not provide notable amount of such substances.

4. Conclusion

In conclusion, sun-treated oyster mushroom is an excellent resource of vitamin D₂ which is comparable to the content of vitamin D₃ in cod liver oil. Increasing the surface areas of sun exposure maximizes the synthesis of vitamin D₂ in oyster mushroom. Our findings also indicated that duration of sun exposure, sizes of mushroom slices and their moisture content at the time of sun exposure are the factors that determine the synthesis of vitamin D₂. Exposing slices of oyster mushroom to the sun light for a brief period (≤30 min) provide the amount that satisfies the current RDA of vitamin D₂ without any visible change in color and texture. Thus, sun treatment of oyster mushroom is an effective and economically cheap strategy in the fight against vitamin D deficiency particularly in tropical and subtropical areas where there is a year-round sun shine. In the future, further researches are warranted to precisely understand the clinical importance of vitamin D₂ and the changes in the chemical compositions of oyster mushrooms as imparted by sun-treatment.

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Author contributions

T.S.K and H.K.B, study concept; T.S.K., experimental design, samples analyses; data analyses and interpretations, and drafted the manuscript; N.N., C.L., D.N., and H.K.B., critical review and approval of the final version of the manuscript.

Conflict of interest

The authors declare no competing financial interest.

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CHAPTER 3.5. VITAMIN D₂ FROM SUN-EXPOSED OYSTER MUSHROOM: ITS IMPACT ON THE TREATMENT OUTCOMES OF TUBERCULOSIS

3.5. Vitamin D₂ from sun-exposed oyster mushroom: Its impact on the treatment outcomes of tuberculosis

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Submitted paper

VITAMIN D₂ FROM SUN-EXPOSED OYSTER MUSHROOM: ITS IMPACT ON THE TREATMENT OUTCOMES OF TUBERCULOSIS

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Abstract

Background: Despite the availability of effective antimicrobials, tuberculosis (TB) remains as a public health threat globally. There is a need for simple and inexpensive strategies to improve the treatment outcomes of TB. Due to its immunomodulatory and antimicrobial properties, vitamin D could be one of the potential candidates.

Objective: To deal with the potential use of vitamin D₂ derived from sun-exposed oyster mushroom as adjunctive therapy to standard anti-TB treatment.

Methods: Randomized controlled trial was conducted on 64 pulmonary TB patients (32 assigned to intervention and 32 assigned to control) in North Shewa, Ethiopia. Intervention group was provided with a sandwich bread prepared from sun-exposed oyster mushroom containing 146 µg (5840 IU) vitamin D₂ continuously from Monday to Friday during the first 16 weeks of anti-TB treatment. Blood and sputum specimens were taken at the beginning and end of the study. The primary outcomes were changes in vitamin D status, clinical improvements (assessed by TB score and Karnofsky performance status scale) and immunologic responses. Sputum smear and culture conversion were evaluated as secondary outcomes. P<0.05 was considered as statistically significant.

Results: Vitamin D₂ intervention brought significant difference of 8.1 ± 6.2 ng/mL (95% CI: 5.9 to 10.3 ng/mL) in the serum 25-hydroxy vitamin D (25(OH)D) level and corrected vitamin D deficiency in more than 35% of TB patients. After intervention, 96.9% vs 21.5% of TB patients found in TB score Severity Class (SC)-I and had mean ± SD of Karnofsky performance status scale of 80.3 ± 6.9% vs 64.7 ± 5.7% in the intervention group vs control group, respectively. Interferon (IFN)-γ and cathelicidin LL-37 levels were showed significant improvement solely in the intervention group. But, the changes in the levels of Interleukin (IL)-4 and IL-10 as well as sputum smear culture conversion were not significant in both groups.

Conclusion: Vitamin D₂ derived from sun-exposed oyster mushroom was effective in improving vitamin D status, clinical outcomes and immune responses. And hence, it could serve as potential, safe, easily available and cost-effective adjunctive therapy for TB.

Key words: Vitamin D₂, sun-exposure, oyster mushroom, TB, adjunctive treatment

Abbreviations: 25(OH)D, 25-Hydroxy Vitamin D; AFB, Acid Fast Bacilli; ALERT, All Africa Leprosy Rehabilitation and Training; AHRI, Armauer Hansen Research Institute; BMI, Body Mass Index; CRP, C-

Reactive Protein; DOTS, Directly Observed Treatment Short Course; ELISA, Enzyme Linked Immunosorbent Assay; FFQ, Food Frequency Questionnaire; HPLC, High Performance Liquid Chromatography; IFN- γ , Interferon- γ ; IL, Interleukin; IQR, Inter Quartile Range; MUAC, Mid Upper Arm Circumference; T regs, Regulatory T cells; sVDD, Severe Vitamin D Deficiency; SC-I, Severity Class; VDS, Vitamin D Sufficiency; TB, Tuberculosis; UVB, Ultra Violet B; VDD, Vitamin D Deficiency; VDR, vitamin D Receptors.

Introduction

Despite the availability of effective antimicrobials, tuberculosis (TB) remains as a public health threat globally. The treatment requires multiple drugs with longer durations that often have mild to severe side effects [1]. Thus, there is a need for simple and inexpensive strategies to improve treatment outcomes. Vitamin D, a fat soluble secosteroid, has immunomodulatory and antimicrobial properties which make it as a candidate adjunctive immunotherapy in TB [2]. In pre-antibiotic era, vitamin D rich resources such as sun-exposure and cod liver oil together with TB sanatorium were commonly used to treat patients infected with TB [3].

Vitamin D moderates cell growth and differentiation, and regulates gene transcription by binding to vitamin D receptors (VDR) [4]. T cells, monocytes and macrophages are known to express VDR [5,6]. The interaction of T-cells with infected macrophages depends on the interplay of cytokines released and, is crucial for eliciting protective immunity against TB [7].

Vitamin D deficiency (VDD) is common in active TB [8]. Susceptibility to TB and risk of progression from infection to disease, tends to occur more often in patients with low 25-hydroxy vitamin D (25(OH)D) levels [9]. Several studies have been conducted on the role of vitamin D supplementation in modifying the treatment progress of TB, however, the results are still a matter of debate. Experimental data have shown that Th₁ cells through production of Interferon (IFN)- γ are crucial for the release of cathelicidine by macrophages, bacterial killing, and containment of *M. tuberculosis* in granulomas [10]. On contrary, other groups have shown that vitamin D inhibits the generation of Th₁ responses and the production of IFN- γ by promoting the generation of regulatory T cells (T regs) [11].

To look into such paradoxical effects of vitamin D on TB and determine whether provision of vitamin D has roles on clinical recovery, immune response and vitamin D status, we designed the current study that deals with the impact of vitamin D₂ (Ergocalceferol) derived from sun-exposed oyster mushroom on the treatment outcomes of TB. Our recent study revealed that

sun-exposed oyster mushroom has excess amount of vitamin D₂ [12]. The bioavailability of vitamin D₂ from Ultra Violet B (UVB) light treated mushroom is almost comparable with that of the supplement [13]. To the best of our knowledge, this is the first study in dealing with the use of vitamin D₂ derived from sun-exposed oyster mushroom as an adjunctive therapy to standard anti-TB treatment.

Materials and methods

Study site and design

This randomized two-arms interventional trial was conducted between December 2014 and June 2015 at Debre Birhan Referral Hospital and 5 different health centres in North Shewa Zone of Amhara Regional State, Central Ethiopia.

Patients

Patients were recruited from TB clinics of all health facilities. Newly diagnosed, smear positive, active pulmonary TB patients who showed commitment to participate for the duration of the study were included. Smear positive pulmonary TB, as per the diagnostic algorithm, is defined by at least two initial sputum smear examinations positive for acid fast bacilli (AFB), or one initial smear examination positive for AFB and culture positive, or one initial smear examination positive for AFB and radiographic abnormalities consistent with active TB as determined by a clinician [14,15]. On the other hand, patients with clinical history of pregnancy, breastfeeding, extra pulmonary TB, multi drug resistant TB, HIV infection, diabetes mellitus, liver cirrhosis, renal failure and other chronic diseases, and who were taking any corticosteroids, immunosuppressive drugs, thiazide diuretics or vitamin D supplementation were excluded from the study.

Randomization and follow up

Eligible patients were randomly assigned into two equal groups. Group I (vitamin D₂ intervention group) consisted of those patients who were provided with sandwich bread which was prepared from sun-exposed oyster mushroom (containing 146 µg or 5840 IU vitamin D₂) for five days in the week for the first 16 weeks of 6 months anti-TB treatment. Group II (the control group) included patients who received the first line anti-TB chemotherapy only. As per the national treatment guidelines of Ethiopia, the standard first line anti-TB treatment consists of a combination of isoniazid, rifampicin, pyrazinamide and ethambutol for 2 months (intensive

phase treatment) followed by isoniazid and rifampicin for 4 months (continuous phase treatment) [16]. Adherence to the DOTS (Directly Observed Treatment Short Course) programme and consumption of mushroom sandwich bread were supervised by the clinical nurses. Every week, patients were checked for any kind of complaints and undergone physical examination. Follow-up of each patient continued for 4 months.

Data collection

Sociodemographic data were collected using pre-tested and structured questionnaire which is translated to Amharic (local language). Medical records were also used to collect clinical characteristics. Vitamin D intake was assessed using a modified food frequency questionnaire (FFQ). The questionnaire included the availability of vitamin D rich foods, monthly income, money spent for foods, duration of sun-exposure, working hours in the sun, clothing style and use of sun protection. The types of all market and traditional foods were completely listed and, self-administered supplementary vitamin D intake was also considered in the assessment of dietary intake.

Sun-exposure of oyster mushroom

Fresh Oyster mushrooms (*Pleurotus ostreatus*) were procured from BioEnguday Production and Sale Micro-enterprise (Debre Birhan, Ethiopia) with the moisture content of 92.5% as determined by oven drying method. To facilitate the production of vitamin D₂, mushrooms were chopped down to the volume of 9cm³ (3cm x 3cm x 1cm) and exposed to the sun for 3 hours. Immediately after sun-exposure, mushrooms were put into plastic bag and kept at - 20°C. The detailed process of sun-exposure was described in our previous work [12].

Sandwich bread preparation and vitamin D₂ analysis

Sandwich breads were prepared every day morning using ingredients of sun-exposed oyster mushroom, wheat bread, olive oil, onion and salt. We used 27g of sun-exposed oyster mushroom (containing 146 µg of VD₂) to prepare one sandwich bread. Samples were taken, packed into an insulated plastic bags together with dry ice and transported to University of Hohenheim (Stuttgart, Germany) where further analysis of vitamin D₂ was done. The extraction of vitamin D₂ was performed based on the method indicated in Keflie et al. [12]. A system of High Performance Liquid Chromatography (HPLC) (Shimadzu technologies) equipped with a DGU-20A3R degassing Unit, two LC-20AT pumps, a SIL-20ACHT auto

sampler and a CBM-20A communication bus module (Shimadzu GmbH, Duisburg, Germany) as well as Reprosil 80 ODS-2 analytical column, 4.6 × 250 mm, 3 µm particle size (Dr. Maisch GmbH, Ammerbuch, Germany) was used to measure vitamin D₂ at Institute of Biological Chemistry and Nutrition. Our pre-test experiment on the effect of cooking temperature indicated that vitamin D₂ was almost stable during the process of cooking (Data not shown).

Samples collection

Blood and sputum samples were taken at the beginning (Day 0) and end of the study (4th month). After overnight fasting, 10 mL of venous blood was withdrawn from antecubital fossa vein into a non-heparinized vacutainer tube between 8:00 and 10:00 am at all health facilities. Blood samples were allowed to clot for about 1 h in the dark and subjected to centrifugation at 2504xg for 10 min at room temperature. Samples with visible haemolysis were discarded. The sera were separated immediately into aliquots of sterile Eppendorf tubes by means of sterile pasteur pipettes and stored at -20 °C. Later, the sera were transported in the ice-box to Armauer Hansen Research Institute (AHRI) in Addis Abeba, Ethiopia, where they were stored at -80 °C until the time of analyses. We used the sera for the analyses of 25(OH)D, cytokines, cathelicidin (LL-37) and C-Reactive Protein (CRP). Similarly, overnight sputum specimens were collected in sterile plastic cap for AFB smear examination and mycobacterial culture. Sputum specimens were stored at -20 °C and later transported in the ice-box to AHRI.

Measurements

Assays of 25(OH)D, cytokines, LL-37 and CRP were performed in duplicate at AHRI using Enzyme Linked Immunosorbent Assay (ELISA) technique.

25 Hydroxy (OH) Vitamin D

Serum 25(OH)D (vitamin D₂ plus D₃) levels were assayed using ELISA kit purchased from Enzo Life Sciences, GmbH (Lörrach, Germany). The assay was done as per the manufacturer's instructions. The kit had 1.98 ng/mL detection limit with 0.5 to 1010 ng/mL assay range. Measurements were categorized as severe vitamin D deficiency (sVDD) (≤ 10 ng/mL), deficiency (VDD) (≤ 20 ng/mL), insufficiency (VDI) (≤ 30 ng/mL) and sufficiency (adequate level) (VDS) (> 30 ng/mL) [17, 18].

Cytokines, Cathelicidin (LL-37) and C-Reactive Protein (CRP)

Levels of IFN- γ , Interleukin-4 (IL-4), IL-10, LL-37 and CRP in the serum were measured using ELISA kits. The kits for IFN- γ and IL-4 were purchased from Sigma-Aldrich (Saint Louis, MO63103 USA) with detection limits of 15 pg/mL and 5 pg/mL, respectively. The kit for IL-10 was purchased from Enzo Life Sciences, GmbH (Lörrach, Germany). This kit had detection limit of <7.81 pg/mL. Likewise, the kits of LL-37 and CRP were purchased from Hycult GmbH (Beutelsbach, Germany) and their detection limits were 0.1 ng/mL and <5 ng/mL, respectively. Mean value of CRP ≥ 10 $\mu\text{g/mL}$ was considered as positive indicator of infection or inflammation. The assays were carried out as per the manufacturer's instructions.

Mycobacteriology

Duplicated sputum specimens were collected from each patient. AFB smears were prepared using Ziehl-Neelsen staining technique as previously described by Keflie and Ameni [15] at all health facilities. AFB smears were denoted as +1, +2, +3 and +4 whenever 1-9 AFB in 100 high-powered fields, 1-9 AFB in 10 high-powered fields, 1-9 AFB in 1 high powered field or > 9 AFB in 100 high powered fields were observed, respectively [8]. Sputum specimens were decontaminated with 4% sterile NaOH solutions and culture was performed at AHRI with the use of Löwenstein-Jensen solid media as previously reported by Keflie et al. [15]. Bacterial cultures were monitored for 6 to 8 weeks until colonies were detected.

Anthropometry

Anthropometric measurements such as body weight, height, and mid upper arm circumference (MUAC) were obtained using standardized procedures. All patients were weighed while wearing light clothes using an electronic platform weighing scale to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm by means of a seca stadiometer. Body mass index (BMI) was calculated as body weight (kg) divided by height (m) squared (kg/m^2). BMI values of 18.5, 17.0, and 16.0 kg/m^2 were used as the cut-off values below which patients were classified as having mild, moderate, or severe malnutrition, respectively [19]. MUAC was measured half way between the olecranon and acromion processes of the left arm using a flexible non-stretch measuring tape to the nearest 0.1 cm while the arm is hanging relaxed, without compressing the tissues. MUAC less than 23 cm for male and 22 cm for female is used to define undernutrition as per FANTA III [20].

Outcomes

The primary outcomes were changes in vitamin D status, clinical improvements and immunologic responses. Clinical outcomes were assessed using TB score and Karnofsky performance status scale. TB score measures change in the clinical status of TB patients and its components include self-reported symptoms (cough, dyspnea, night sweat, chest pain, haemoptysis), clinical signs (tachycardia, pallor, fever, auscultatory findings), BMI (Low BMI: $\leq 18.5 \text{ kg/m}^2$, $<16 \text{ kg/m}^2$) and MUAC ($<23 \text{ cm}$ for male or $<22 \text{ cm}$ for female, $<20 \text{ cm}$). Each variable contributed 1 point and the total score varies from 0 to 13 points. The score was grouped as mild (Severity Classes (SC)-I: 0-5 points), moderate (SC-II: 6-7 points) or severe (SC-III: 8 points and more). Low TB score correlates with favourable outcomes, cure, and completed treatment [21]. Likewise, Karnofsky performance status scale correlates purely to physical ability and covers 11 points, each scored as a percentage from normal health to death (100 to 0%) [22]. Sputum smear conversion and culture negativity were evaluated as secondary outcome.

Adverse effects

Patients were interviewed for the occurrence of adverse events related to hypercalcemia such as nausea, vomiting, excessive thirst, anorexia, symptoms of kidney stones, and confusion. In addition, the occurrence of itching, arthralgia, jaundice, headache, malaise, dyspepsia and others were asked.

Ethics

This study was undertaken in accordance with Helsinki declaration and approved by Ethics Review Committee of AHRI-ALERT (All Africa Leprosy Rehabilitation and Training Centre) as part of the project entitled ‘‘ Effect of micronutrients on the treatment outcomes of TB’’ (Project Reg. No. P057/14). All concerned health bureau of North Shewa Zone of Amhara Regional State gave us supports by facilitating the processes of the study. After explaining the aim and purpose of the study, written informed consent was obtained from each of the participating patients.

Statistical analysis

Sample size estimation was done based on the previous findings of TB Score reduction after 2 months of anti-TB treatment with the mean \pm SD of 3.2 ± 2.3 [21]. With the assumption of

36% more reduction in the primary clinical TB score after vitamin D₂ intervention at 5% level of significance, 80% power and 20% dropout rate, the sample size was calculated to be 75 for each group with total of 150 patients. The study was analysed using IBM SPSS version 23 statistical program and data were summarized as mean± SD or median with IQR (Inter Quartile Range) for continuous variables and frequencies with percentages for categorical variables. Means were compared using a two-tailed paired t-test or Wilcoxon signed rank sum test depending on the results of Shapiro-Wilk test for normality of distribution. The differences in the proportions were analysed using Chi-square (X²) or Fisher's exact test (when more than 20% of the cells have expected count less than 5). Kruskal-Wallis test was applied to assess the changes in the serum 25(OH)D levels across different categorical variables. Pearson's and Spearman correlation tests were used to identify the associations between parametric and non-parametric variables, respectively. Variables with p-value less than 0.25 in bivariate analysis were entered into multiple linear regression model in order to evaluate the relationship between the changes in the serum 25(OH)D levels and the clinical outcomes by adjusting independent factors. A p-value less than 0.05 was considered statistically significant.

Results

Description of recruitment

A total of 155 TB patients were assessed for eligibility. Of these, 35 patients failed to meet the inclusion criteria and 20 patients refused to provide their consents. One hundred patients were recruited and assigned into vitamin D₂ intervention group and control group. Due to various reasons, 36 patients were lost and the remaining 64 patients (32 patients in vitamin D₂ group and 32 patients in control group) completed the study. The flow of participants is illustrated in detail in **Figure 1**.

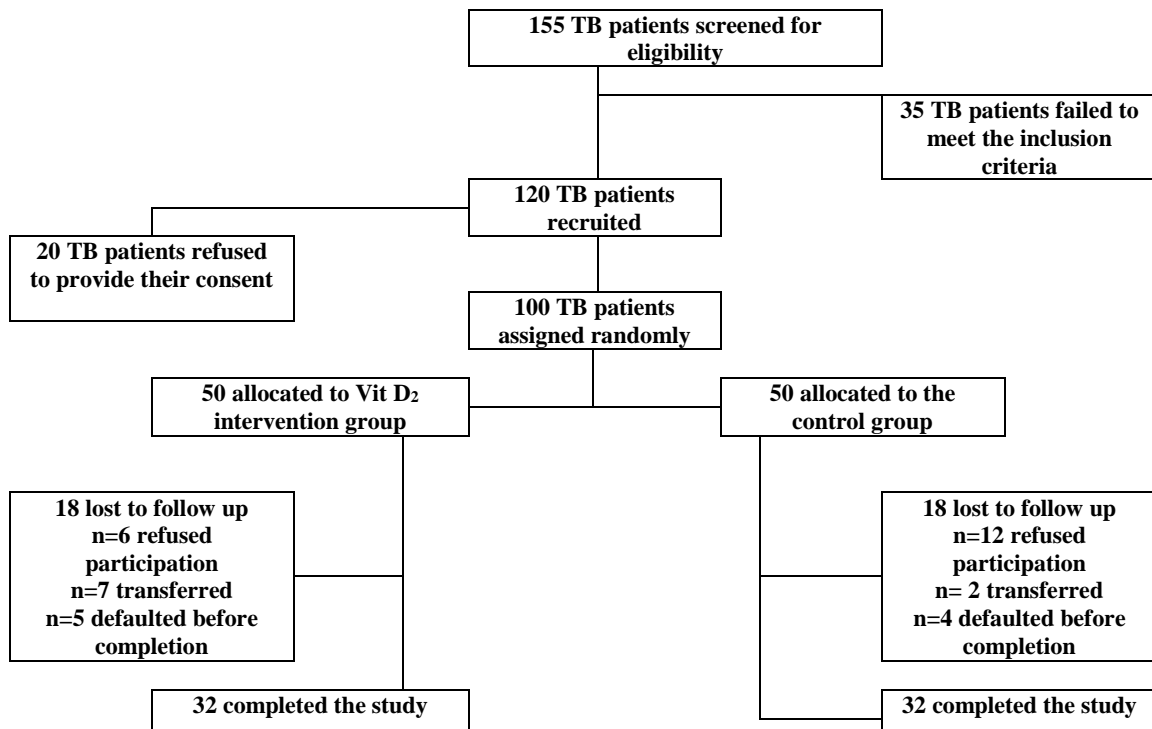


Figure 1: Study recruitment, inclusion and completion profile of TB patients

Basic Characteristics

Basic characteristics were compared between vitamin D₂ intervention group and the control group. As it is indicated in **Table 1**, the two groups had almost similar baseline characteristics. The median age with IQR was 28 (14) years in the intervention group and 26 (8.5) years in the control group.

Table 1: Basic Characteristics of TB patients assigned in vit D₂ intervention group and control group

Variables		Vit D ₂ Intervention	Control	P-value
Age (years) median (IQR)	Age	28 (14)	26 (8.5)	0.90
Sex n (%)	Male	18 (56.3)	16 (50.0)	0.62
Location n (%)	Urban	21 (65.6)	20 (62.5)	0.79
Educational Status n (%)	Illiterate	2 (6.3)	3 (9.4)	0.43
	Primary Education	11 (34.4)	15 (46.9)	
	Secondary Education	14 (43.7)	13 (40.6)	
	Tertiary Education	3 (9.4)	1 (3.1)	
	Religious Education	2 (6.2)	0 (0.0)	
Occupation n (%)	Student	7 (21.9)	6 (18.7)	0.79
	Farmer	6 (18.7)	6 (18.7)	
	Working for the government	6 (18.8)	10 (31.3)	
	Working for NGO*	9 (28.1)	6 (18.8)	
	House Wife	4 (12.5)	4 (12.5)	
Marital Status n (%)	Single	17 (53.1)	11(34.4)	0.13
	Married	15 (46.9)	21 (65.6)	
Family Size n (%)	1 to 4	12 (37.5)	5 (15.6)	0.12
	5 to 8	15 (46.9)	22 (68.8)	
	> 8	5 (15.6)	5 (15.6)	
Monthly Income n (%)	≤ 1000 ETB*	3 (9.4)	10 (31.3)	0.11
	1001 to 3000 ETB*	20 (62.5)	12 (37.5)	
	3001 to 5000 ETB*	6 (18.7)	6 (18.7)	
	> 5000 ETB*	3 (9.4)	4 (12.5)	
Money spent for food n (%)	30 to 40% of monthly income	16 (50.0)	7 (21.9)	0.072
	40.1 to 50% of monthly income	7 (21.9)	14 (43.7)	
	50.1 to 60% of monthly income	5 (15.6)	4 (12.5)	
	> 60% of monthly income	4 (12.5)	7 (21.9)	
Working in the Sun for 1hour/day n (%)	No	12 (37.5)	18 (56.3)	0.13
	Yes	20 (62.5)	14 (43.7)	
Clothing style (covering the body) n (%)	No	5 (15.6)	10 (31.3)	0.14
	Yes	27 (84.4)	22 (68.7)	
Use of sun protection n (%)	No	7 (21.9)	10 (31.3)	0.39
	Yes	25 (78.1)	22 (68.7)	
Vitamin D rich food consumption (E.g. Oily Fish) n (%)	No	24 (75.0)	25 (78.1)	0.77
	Yes	8 (25.0)	7 (21.9)	
AFB Smear Examination n (%)	AFB Smear ≥ +3	24 (75.0)	31 (96.9)	0.012
BMI mean (SD)	BMI	17.5 (2.4)	18.7 (3.8)	0.13
MUAC median (IQR)	MUAC	21 (5)	20 (3)	0.93

*NGO-Non-Governmental Organization; 1USD ≅ 27.96 ETB (Ethiopian Birr)

Sun-exposed oyster mushroom

The content of vitamin D₂ in fresh oyster mushroom was almost nil. However, large amount of vitamin D₂ was produced after sun-exposure and we obtained concentrations of 540.9 µg/100 g fresh weight.

Vitamin D

More than 65% of the study participants spent 30 to 50% of their monthly income on food. But, less than 26% of them tried to include vitamin D rich foods like oily fish in their diet. The correlation between the serum 25(OH)D level and vitamin D rich food intake, working in the sun for 1 hour per a day, use of sun protection and clothing style was not significant. Although more than 40% of TB patients were working in the sun for about 1 hour per day, their skin did not directly expose to the sun as most of them (>68%) cover their body by clothes and using sun protection (**Table 1**).

The mean \pm SD of serum 25(OH)D level at baseline was 29.1 ± 12.3 ng/mL versus 30.6 ± 16.8 ng/mL in the intervention group and control group, respectively. In the intervention group, the proportions of VDD and VDI at baseline were nearly 42% and 58%. Whereas, these values in the control group were about 41% and 59%, respectively (**Table 2**).

Table 2: Serum 25(OH) vitamin D level in VD₂ intervention group and the control group of TB patients

25(OH)Vitamin D Level	VD ₂ Intervention Group			Control Group		
	Before n (%)	After n (%)	P - value	Before n (%)	After n (%)	P - value
sVDD‡ (≤10ng/mL)	-	-	0.0012	1 (3.4)	1 (3.4)	0.73
VDD§ (≤20ng/mL)	13 (41.9)	2 (6.4)		12 (41.4)	7(24.1)	
VDI† (≤30ng/mL)	18 (58.1)	8 (25.8)		17 (58.6)	15 (51.7)	
Sufficient VD (>30ng/mL)	13 (41.9)	23 (74.2)		12 (41.4)	14 (48.3)	

‡sVDD - Severe Vitamin D Deficiency; §VDD - Vitamin D Deficiency, †VDI - Vitamin D Insufficiency, and Sufficient VD

After 4 months of VD₂ intervention, the mean \pm SD of the serum 25(OH)D level was higher in the intervention group than the control group (37.2 ± 10.9 vs 34.4 ± 17.6). The intervention induced 27.8% increase in the mean of serum 25(OH)D level in the intervention group (for the difference: mean \pm SD of 8.1 ± 6.2 ng/mL; 95% CI of 5.9 to 10.3 ng/mL, $p < 0.001$) (**Figure 2**). In addition, the proportions of VDD and VDI were significantly reduced in the intervention group by 35.5% and 32.3%, respectively. Although not statistically significant, the

corresponding values for the control group were 17.3% and 6.9% (**Table 2**). The changes in the serum 25(OH)D level were not different across sex and age categories.

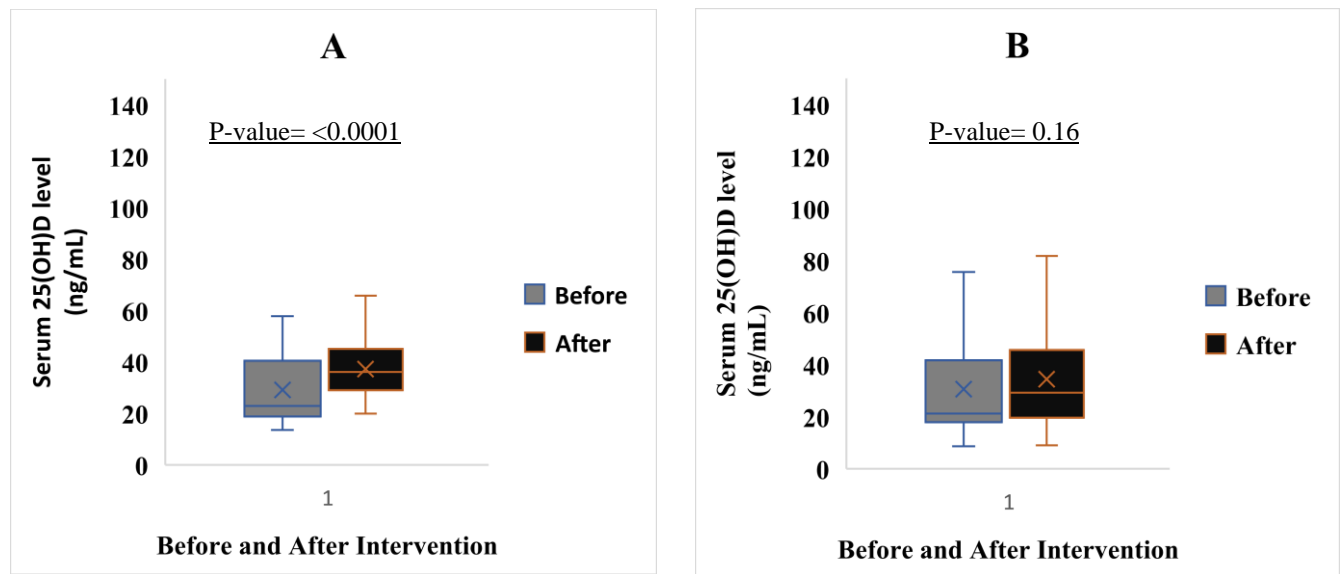


Figure 2: The changes of 25(OH)D level in the serum of TB patients before and after intervention. Significant change was observed in intervention group (A) but not in the control group (B).

Clinical outcomes

At baseline, the mean \pm SD of TB score was 6.1 ± 3.2 points for intervention group and 6.9 ± 2.1 points for the control group. About 34% of TB patients in the intervention group and 38% in the control group were found in the TB score SC-III. After intervention, progressive change was observed in TB score of the intervention group (mean \pm SD of 2.6 ± 1.8 ; 95% CI of 1.95 to 3.17; $p < 0.001$) as compared to the control group (mean \pm SD of 6.7 ± 1.8 ; 95% CI of 6.13 to 7.37; $p = 0.211$). The number of TB patients with TB score SC-I was significantly improved by 56.3% in the intervention group, but this improvement was very small in the control group (only 3.1%) (**Table 3**). Holding location, occupation and family size constant, there was an inverse relationship between TB score and the serum 25(OH)D level in the intervention group ($\beta = -0.630$, $p < 0.001$). About 33% of the variability of TB score in the intervention group was accounted for by the change in the serum 25(OH)D level. However, the contribution of the change in 25(OH)D for such variability in the control group was 22%.

TB score had statistically significant inverse correlation with Karnofsky performance status scale ($r = -0.554$, $p < 0.001$), body weight ($r = -0.393$, $p = 0.001$), BMI ($r = -0.498$, $p < 0.001$), and MUAC ($r = -0.515$, $p < 0.001$). The mean \pm SD of Karnofsky performance status scale before and

after intervention were $61.9 \pm 6.9\%$ and $80.3 \pm 6.9\%$ in the intervention group, and $64.7 \pm 5.7\%$ and $66.2 \pm 5.5\%$ in the control group, respectively. The change in the Karnofsky performance status scale was statistically significant in the intervention group ($p < 0.001$), but not in the control group ($p=0.52$) (Table 3). There was strong correlation between BMI and MUAC ($r=0.71$, $p<0.001$). After intervention, the group of intervention had significant improvements in BMI (by 0.91 kg/m^2) and MUAC (by 1.16 cm). However, these improvements were not statistically significant in the control group (BMI by -0.01 kg/m^2 and MUAC by -0.1 cm).

Table 3: Clinical and mycobacteriological findings in TB patients before and after intervention

Variables	Vitamin D ₂ Intervention		P-value	Control		P-value
	Before n (%)	After n (%)		Before n (%)	After n (%)	
TB Score Severity Class (SC)						
SC-I (0 to 5 Points)	13 (40.6)	31 (96.9)	<0.001	8 (25.0)	7 (21.9)	0.88
SC-II (6 to 7 Points)	8 (25.0)	1 (3.1)		12 (37.5)	14 (43.7)	
SC- III (≥ 8 Points)	11 (34.4)	0 (0.0)		12 (37.5)	11 (34.4)	
Karnofsky performance status scale						
50 Points	5 (15.6)	0 (0)	<0.001	1 (3.1)	0 (0.0)	0.52
60 Points	16 (50.0)	0 (0)		15 (46.9)	13 (40.6)	
70 Points	11(34.4)	7 (21.9)		16 (50.0)	18 (56.3)	
≥ 80 Points	0(0.0)	25 (78.1)		0 (0.0)	1 (3.1)	
Acid Fast Bacilli (AFB)†						
Smear +1, n (%)	0 (0)	10 (31.3)	<0.001	0 (0.0)	14 (43.7)	<0.001
Smear +2, n (%)	8 (25.0)	14 (43.7)		1 (3.1)	15 (46.9)	
Smear +3, n (%)	16 (50.0)	5 (15.6)		21 (65.6)	1 (3.1)	
Smear +4, n (%)	8 (25.0)	3 (9.4)		10 (31.3)	2 (6.3)	
Culture Positive n (%)	32 (100)	29 (90.6)	0.076	31 (96.87)	29 (90.6)	0.302

† AFB Smear +1: 1 - 9 AFB Observed/ 100 high powered fields; +2: 1 - 9 AFB Observed/ 10 high powered fields; +3: 1 - 9 AFB Observed/ high powered fields); and +4: > 9 AFB Observed/ 100 high powered fields.

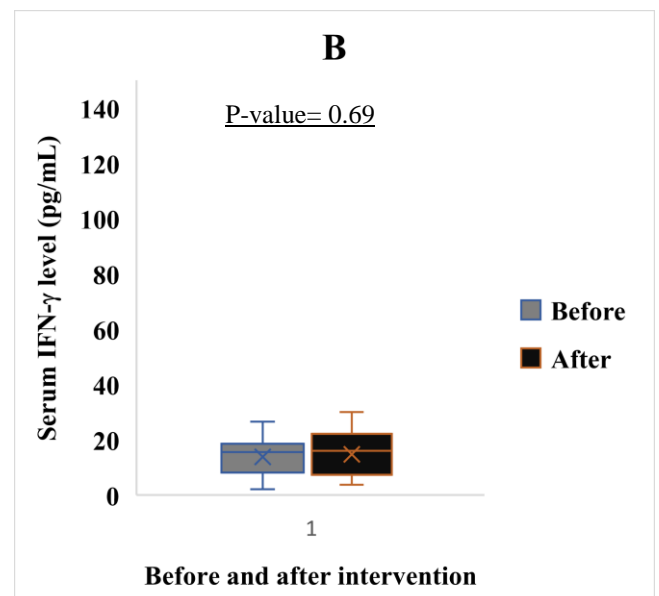
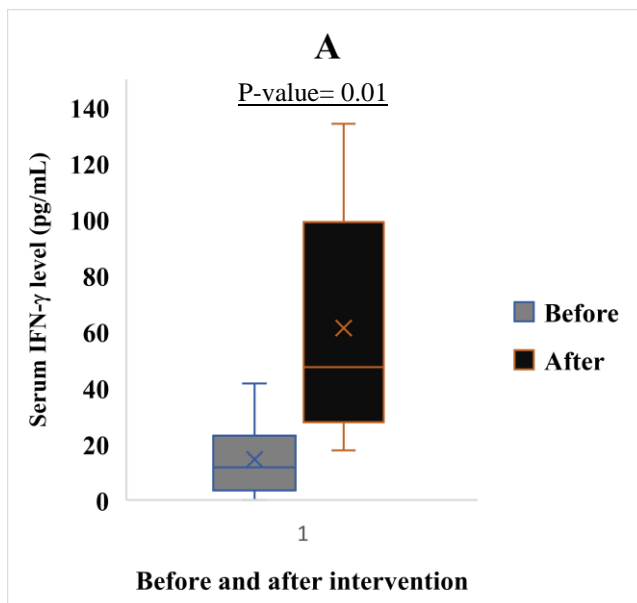
Mycobacteriological outcomes

AFB smear examinations and bacterial culture were performed to analyse the changes in the bacterial load. After intervention, there were significant changes in AFB load in both intervention and control groups ($p<0.001$), in which more than 30% of TB patients had AFB smear examination level +1 (1 to 9 AFB observed/ 100 high powered fields). Despite the changes in the bacterial load, AFB smear negativity could not be achieved and nearly 91% of the patients were culture positive after intervention. In both intervention and control groups, the changes in the culture conversion were not statistically significant (Table 3).

Immunological outcomes and anti-microbial peptides

Figure 3 illustrates the changes in the levels of cytokines and cathelicidin (LL-37) before and after intervention. At baseline, the mean \pm SD of cytokines were 14.9 ± 12.2 vs 13.8 ± 7.0 pg/mL (IFN- γ), 4.6 ± 3.4 vs 9.8 ± 10.4 pg/mL (IL-4), 15.2 ± 5.8 vs 20.3 ± 8.8 pg/mL (IL-10) and that of LL-37 was 192.7 ± 65.2 vs 165.9 ± 92.7 ng/mL in the intervention group versus the control group. After intervention, the corresponding values became 59.5 ± 39.8 vs 14.8 ± 8.7 pg/mL (IFN- γ), 6.5 ± 5.0 vs 9.0 ± 8.2 pg/mL (IL-4), 17.8 ± 5.9 vs 14.6 ± 5.6 pg/mL (IL-10) and 245.3 ± 99.3 vs 174.8 ± 90.1 ng/mL (LL-37). In both groups, there was no significant difference in CRP level.

While comparing the means, we observed significant changes in the levels of IFN- γ and LL-37 ($p < 0.05$) in the intervention group. However, such changes were not observed in the control group. IFN- γ had significant positive correlation with 25(OH)D ($r = 0.426$, $p = 0.017$). Moreover, there were statistically significant relationship between 25(OH)D level and the levels of IFN- γ ($\beta = 0.349$, $p = 0.039$) and LL-37 ($\beta = 0.366$, $p = 0.028$) holding location, occupation and family size constant.



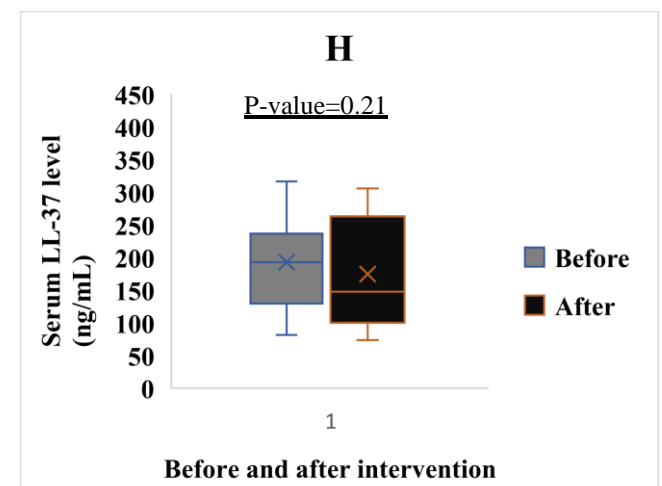
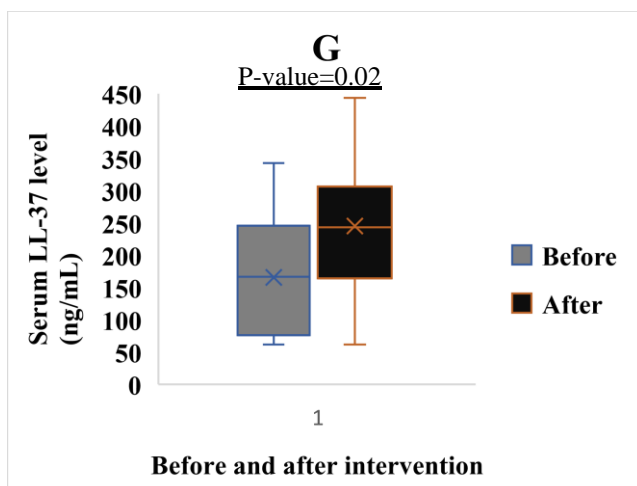
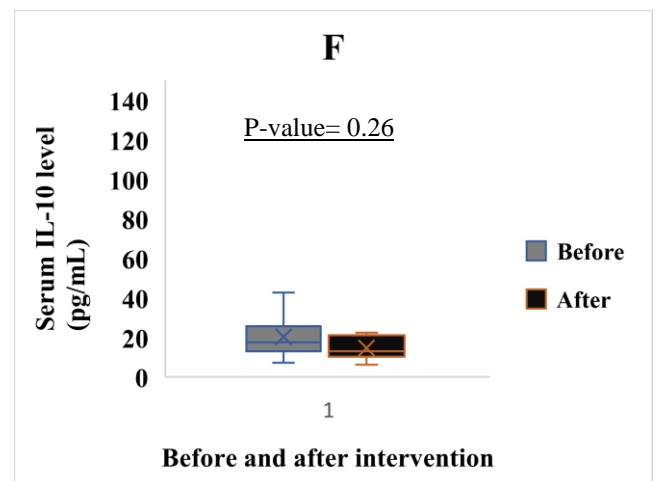
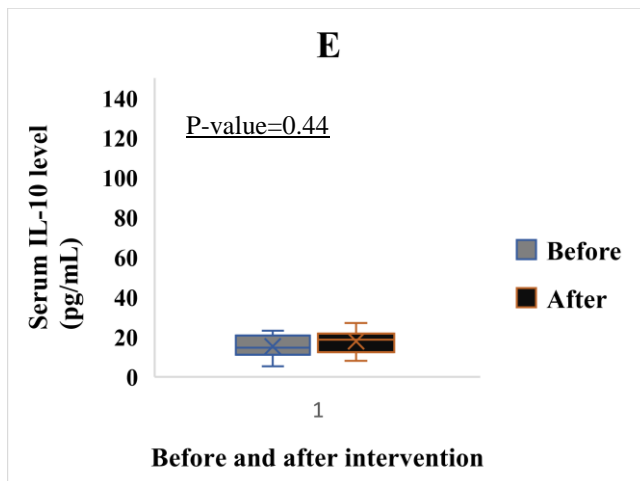
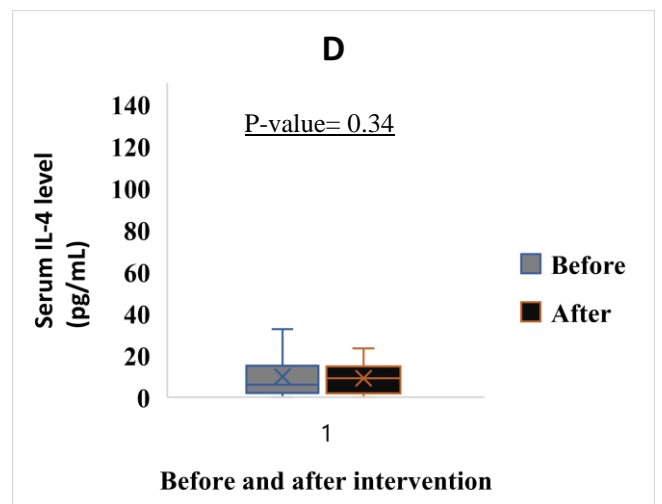
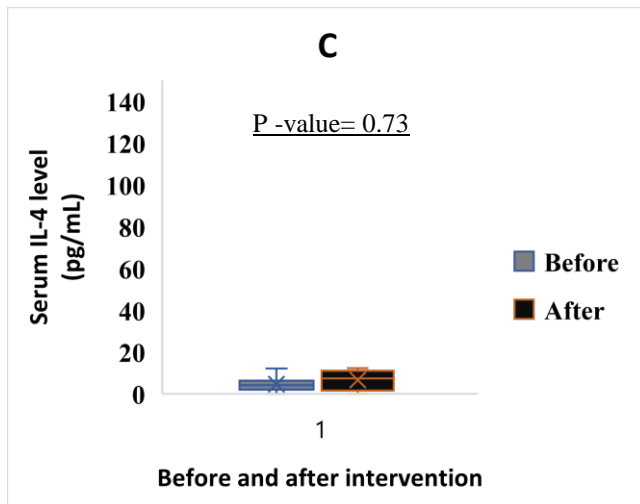


Figure 3: The serum level of different cytokines and antimicrobial peptides before and after intervention. A, C, E and G in the intervention group and B, D, F and H in the control group represent the serum level of IFN- γ , IL-4, IL-10 and Cathelicidin (LL-37), respectively. P-value <0.05 shows significant difference between before and after intervention.

Discussion

Our study demonstrated for the first time the effects of sun-exposed oyster mushroom on the treatment outcomes of TB. A high proportion ($\geq 40\%$) of VDD was found in this study; and it was directly related to factors such as lack of sun exposure and inadequate intake of vitamin D rich diets. These two factors were identified together with others in our previous systematic review as the main predictor variables of vitamin D status among TB patients in Africa [18]. Despite its high content of ergosterol, oyster mushroom contains vitamin D₂ to the level close to nil. However, sun-exposure increases the amount of vitamin D₂ in oyster mushroom [12].

Intervention with sun-exposed oyster mushroom; containing 146 μg (5840 IU) vitamin D₂ daily for 5 days in the week for the first 16 weeks; brought a significant difference of 8.1 ng/mL in the mean 25(OH)D level; and corrected deficiency in more than 35% of TB patients without showing any adverse effects. Comparable to this, in 3 consecutive days of UVB exposure to Asian immigrants in the UK, Yesudian et al. [23] found 9.16 ng/mL increase in the mean 25(OH)D level from a baseline of 11.23 ng/mL. Tukvadze et al. [8] in Georgia also showed that adjunctive high-dose oral vitamin D₃ was safe and led to a substantial increase in plasma 25(OH)D concentrations over 16 weeks. Similarly, Urbain et al. [13] demonstrated that intervention with vitamin D₂-enhanced button mushrooms via UV-B irradiation was effective in improving vitamin D status.

A previous study revealed that oral vitamin D supplementation was associated with significant suppression of the concentrations of circulating inflammatory markers, like CRP [24]. In the present study, however, there was no significant change in the level of CRP. In the same line, Wu et al. [1] confirmed that there was no evidence on the improvement of CRP after the intervention. Our previous work underscored that CRP was not the best option to control the change in the acute phase response of vitamin D and other micronutrient's levels in the serum of TB patients [25].

Intriguingly, our intervention ameliorated the clinical outcomes and immune responses of TB patients, but not sputum culture conversion. Clinical outcomes were assessed by TB score and the Karnofsky performance status scale. Wejse et al. [21] found that TB score declined for 96% of the surviving patients from initiation to end of treatment. At the end of standard chemotherapy at 6 months, most patients had a TB score below 1 [26].

In this study, a progressive change was observed during 4 months of intervention. Most patients (96.9%) found in TB score SC-I, having more than 55% improvement from baseline. During the same duration of treatment, about 22% of patients in the control group was found in TB score SC-I with less than 5% improvement. There was a significant inverse relationship between TB score and serum 25(OH)D level. The variability of TB score in more than one-third of TB patients was attributed to the change in the serum 25(OH)D level. In line with this, a study done in Iran indicated a reduction of TB score in patients who took a single oral dose of 450,000 IU cholecalciferol after 2 and 3 months of treatment [27]. Another study done in Egypt showed better healing after 1000 IUs of oral vitamin D supplementation [28]. More recently, Bekele and his colleagues [26] reported that an additional 25% reduction in the TB score in the intervention group was considered as a significant effect.

TB score was inversely associated with the Karnofsky performance status scale. In this study, there was a significant change in the Karnofsky performance status scale of the intervention group having a mean of 80.3% as compared to 66.2% in the control group. Similarly, there was a significant change in nutritional status as assessed by BMI and MUAC. BMI had a strong relationship with MUAC. We observed the improvements of BMI (by 0.91kg/m²) and MUAC (by 1.16 cm) solely in the intervention group. In agreement with this, Salahuddin et al. [9] showed that 2 doses of 600,000 IU vitamin D administered intramuscularly resulted in a greater weight gain by 1.14 kg and improvement in BMI. Two small randomized studies [28, 29] have also suggested the beneficial effects of vitamin D on weight gain. These implied that the improvement in the level of vitamin D has a contribution to the improvement of the nutritional status of TB patients.

Although there were significant changes in bacterial load as assessed by AFB smear examination, we could not find a whole AFB smear negativity and sputum culture conversion. Comparable to this, several studies done elsewhere [9, 26, 30, 31] demonstrated that vitamin D supplementation did not affect time to sputum smear and culture conversion. However, there were some contrasting reports. Studies done by Nursyam et al. [29] and Coussens et al. [32] indicated that vitamin D supplementation accelerated sputum smear conversion. Martineau et al. [33] also showed that administration of four doses of 2.5 mg vitamin D₃ had a faster effect on sputum smear conversion in patients with the tt genotype of the TaqI VDR polymorphism.

Therefore, the reasons for such inconsistencies could be the presence of the variants of VDR polymorphisms, variability in vitamin D dosages or different phases of baseline serum 25(OH)D level as indicated in Farazi et al. [27].

It's well established that vitamin D intervention can modify the balance between Th₁ and Th₂ responses [34]. We found a significant increment in IFN- γ level, the classical cytokine of Th₁ response in the intervention group. Whereas, non-significant changes were observed in the levels of Th₂ cytokines such as IL-4 and IL-10. These implied that vitamin D intervention skewed the balance of cytokines towards Th₁ response. In contrary, several studies reported that vitamin D intervention inhibits the level of IFN- γ , but enhances the production of IL-4 and IL-10 [7, 10, 35]. However, in agreement with the present study, Salahuddin et al. [9] showed that vitamin D administration brought significant change in IFN- γ production in TB patients with VDD at baseline. Fabri et al. [36] also reported that in vitro supplementation of vitamin D deficient serum with 25(OH)D₃ restored IFN- γ . These recapped that the difference in the effects of vitamin D intervention on Th₁ and Th₂ cytokines was mainly dependent on VDD at baseline.

In the present study, there was a significant change in the level of cathelicidin LL-37. This was supported by several studies done elsewhere [7,8, 10, 37]. We found a direct relationship between LL-37, IFN- γ and 25(OH)D level in the intervention group. This indicated that better status of vitamin D increases the level of IFN- γ mediated cathelicidin LL-37. According to different studies, vitamin D promotes mycobacterial killing [37] or reduced intracellular viability of *M. tuberculosis* [24] in macrophages through the production of cathelicidin LL-37, after activation of macrophages via either toll-like receptor [38] or IFN- γ pathways [36].

Limitation

The first limitation of this study was the inability to separately measure 25(OH)D₂. ELISA kits detect 25(OH)D which is the summation of 25(OH)D₂ and 25(OH)D₃. Secondly, data on the genotypes of VDR polymorphisms were not included as we could not have access to measure the genes of VDR polymorphisms at the time of the study. VDR polymorphisms are, however, expected to influence the effects of vitamin D intervention.

Conclusion

In conclusion, vitamin D₂ intervention from a sandwich bread prepared with sun-exposed oyster mushroom effectively corrected VDD in TB patients taking the standard anti-TB treatment without showing any adverse reaction. The accelerated improvements on the clinical and immunological outcomes although not on sputum smear conversion give us a clue that sun-exposed oyster mushroom could serve as a potential, safe, easily available and cost-effective adjunctive treatment of TB. As this is the first study, further investigations on the interactions of vitamin D₂, sputum smear conversion and immunological responses on the large size and diverse groups of TB patients are warranted.

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Authors contribution

Conceived and designed the experiments: TSK and HKB; performed the experiments, analysed the samples, collected, analysed and interpreted the data, and wrote the manuscript: TSK; and Critically reviewed and approved the manuscript: AS, AZW, AM, MA, CL, DN and HKB.

Conflict of Interest Statement

The authors declared that they have no competing financial interest

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Chapter 4

General discussion and conclusion

CHAPTER 4.1. DISCUSSION

4.1. Discussion

Nutrition plays significant roles in treating TB [1]. Before the advent of anti-TB chemotherapy, a diet rich in calories, proteins, fats, minerals and vitamins was considered in the treatment of TB [2]. The introduction of anti-TB drugs; however, led to reduced emphasis on the importance of nutritional support in the management of TB patients [3]. TB patients with strains susceptible to first line anti-TB drugs are treated only with standardized first line treatment regimen [4].

4.1.1. Dietary and nutritional assessment

In dietary and nutritional assessment, we identified a big nutritional gap in the central part of Ethiopia. The main dietary pattern of the population included cereals (teff, wheat and barley), vegetables (onion, green pepper, tomato and cabbage), legumes (peas, faba bean and lentils), oils (cooking oil) and spices (salt). All these food items are the ingredients of injera, and thick stew made from flour of roasted legumes ('shiro wot'). In agreement with our study, Workicho et al. [5], Kefiyalew and Eshetu [6], Steyn et al [7] and Mekuria et al. [8] reported that cereals, followed by legumes and, root and tubers are the most commonly consumed food groups. These staples dominate the national food basket in Ethiopia, supplying more than 70% of calories [9]. Animal source foods (ASF) were consumed by about one-third of the population. Despite the large livestock population in Ethiopia, consumption of animal products is limited [10] because of decreased access, relatively high price, lack of cooling devices and monotonous food habit [5, 6].

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Healthy food intake is associated with food variety and diet quality of individuals [11]. Food variety score (FVS) and diet diversity score (DDS) are the proxy indicators of the quality of diets in which more than three-fourth of the population in our study had poor FVS (10-19 varieties of foods per week) and DDS (below 4 out of 9 DDS). This implied that most people had poor nutrient adequacy and dietary quality. The median meal frequency per day was 3 with the minimum of 1. Meaning that there were some portion of the population who were eating only one time. Consumption of monotonous diets for longer time lacks essential nutrients and subsequently contributes to the burden of malnutrition and micronutrient deficiencies [12]. In our study, malnutrition was the common problem in the population. Given its commonness in the people with and without TB, it is more likely that malnutrition is paving the way for TB infection and disease advancement. More than half of the population in our study had the risk of micronutrient deficiencies. Deficiency of single or multiple nutrients can reduce an individual's resistance to infection [13].

4.1.2. Vitamin A and zinc deficiencies

The burden of vitamin A and zinc deficiencies in TB patients was very high. Our case-control study revealed that more than half of TB patients had deficiencies of vitamin A and zinc. In line with this, studies done elsewhere reported that TB patients had high proportion of vitamin A (60%) [14] and zinc (47.1%) [15] deficiencies. The low zinc level reported in TB patients was in concert with several studies done in different places [15]. Zinc deficiency impairs the synthesis of vitamin A binding proteins and reduces plasma vitamin A concentration [13].

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Vitamin A deficiency usually occurs in the places where the people consume a monotonous cereal-legume diet [16]. Traditional staple foods, such as cereals, legumes, and tubers, contain zinc, but the presence of phytate, fibre, and lignin reduces its bioavailability. Cow's milk, because of its high concentrations of calcium and casein, and soymilk, because of its phytate content, may further reduce the absorption of zinc from the diet. The tannin content in tea and coffee can potentially inhibit zinc absorption. The soil where the plants are growing has also an influence on the availability of zinc concentration [17].

4.1.3. Protein-Energy malnutrition

While analysing the intake of nutrients, we found that more than three-fourth of TB patients had below half of the energy fulfilment. In patients with active TB, the energy requirement is likely to increase [18] as the result of increasing basal metabolic rate or resting energy expenditure [19]. At the same time, energy intakes are likely to decline as a result of illness-associated anorexia [19]. On the other hand, the protein intake in our study was above the average fulfilment albeit cereal-based diets. Patients with TB used a larger proportion of proteins from oral feeding for oxidation and hence for energy production. Such failure to channel food protein into endogenous protein synthesis is anabolic block which represents one of the mechanisms for wasting in TB patients [19].

Several studies reported that patients with active TB are more likely to be wasted or have a lower body mass index ($BMI = \text{kg/m}^2$) than healthy controls [15]. Our study revealed that about

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one-third of TB patients had BMI less than 18.5 kg/m². This finding was, in fact, less than the reports of Workineh et al. [20] (71.4%), Kant et al. [13] (66%), Zachariah et al. [21] (57%) and Amare et al. [14] (65.4%), but it was higher than the report of Onwubalili [22] (13%). As defined by mid-upper arm circumference (MUAC), close to half of TB patients in our study were undernourished. The possible reasons for such undernourishment could be protein-energy malnutrition, micronutrient deficiencies and impaired metabolism. Undernutrition is associated with increased risk of mortality and poor treatment outcomes. Although nutritional status is improved by effective TB treatment, the most improvements are limited to increase in fat mass with little effect on muscle tissue. The evidence suggests that adequate nutritional intake during TB care and recovery is needed to fully restore nutritional status during and following TB treatment and microbial cure [18].

4.1.4. Vitamin D deficiency

TB has strong relationship with vitamin D [23, 24]. Vitamin D, popularly referred to as sunshine vitamin'' plays significant role in metabolic and immunologic processes [25]. It is mainly obtained endogenously after sun-exposure, and from dietary supplements and food sources [25]. Populations from temperate regions, where exposure to sun is limited, especially during winter season are expected to have vitamin D deficiencies (VDD) unless they rely on dietary intakes [26]. Astoundingly, there were some studies that showed the presence of VDD in the people living in Africa. This caught our attention and posed a question why TB patients in Africa face the challenges of VDD, while sunshine is available in the continent throughout the year.

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Based on this question, we undertook a systematic review and found that above three-fourth of TB patients had VDD and insufficiencies (VDI). Noteworthy to mention, use of sun protection (lack of sun-exposure), inadequate dietary intake, low BMI, skin pigmentation, use of drugs (anti-retroviral or anti-TB), socioeconomic status, season, clothing, comorbidities and age were identified as the main predictor variables that hampered the status of vitamin D. VDD increases the susceptibility to TB infection [27] and encourages the development of advanced TB by causing low macrophage activation and reduced production of antimicrobial factors [3].

4.1.5. Mushroom-derived vitamin D₂

Mushroom is a potential non-animal source of vitamin D [28]. Owing to its nutritional and medicinal properties [29], consumption of mushroom has increased worldwide for the past four decades [28]. Considering its cost, availability and accessibility, we undertook a study on mushroom under subtropical settings. In this study, the impact of sun-exposure on the content of vitamin D₂ in oyster mushroom was assessed. The content of vitamin D₂ was almost nil, but significantly increased after sun-exposure. This was supported by the studies of Phillips and Rasor [30]. The difference in the content of vitamin D₂ before and after sun-exposure is attributed to the presence of sterol, mainly ergosterol in the fruiting body of mushroom [29].

Upon exposure to UVB radiation, pro-vitamin D₂ (ergosterol) was converted to pre-vitamin D₂, which then thermally isomerized to vitamin D₂ [31]. The process of vitamin D₂ synthesis is affected by many factors. Our study indicated that duration of sun-exposure, sizes of

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mushroom slices and their moisture content at the time of sun-exposure determined the synthesis of vitamin D₂. This was corroborated with the work of Jasinghe et al. [32]. Cardwell et al. [28] also described that time of exposure, temperature, and exposure of UVB radiation can influence the production of vitamin D₂. Increasing the surface area of sun-exposure increases the production of vitamin D₂. The maximum content of vitamin D₂ was produced in 1cm³ size of slice group (67.4 ± 28.0 µg/g dry weight (dw)), which was closer to the report of Huang et al. [33] (69.00 µg/g dw).

While increasing the duration of sun-exposure, the content of vitamin D₂ increased; however, there was a change in colour and texture. The change in colour brought discolouration which deterred the consumption of mushroom [28]. Our study showed that the change in the colour and texture could be minimized by shortening the duration of exposure. For instance, exposing a group of 9 cm³ size of slices for 30 minutes produced about 41 µg/g dw of vitamin D₂ without any visible change in colour and texture. This amount of vitamin D₂ was comparable to the amount of vitamin D₃ in cod liver oil (40.3 µg per 1 tablespoon) [34]. Thus, exposing slices of oyster mushroom to the sun for brief period (≤ 30 minutes) produces enough vitamin D that can satisfy the current recommended dietary allowance (RDA) of vitamin D. Our study confirmed that sun-exposed oyster mushroom is the potential alternative source of vitamin D, especially for vegetarians, vegans and for persons who are fasting ASF during religious fasting time.

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4.1.6. Role of mushroom-derived vitamin D₂ on the treatment outcomes of TB

Mushroom-derived vitamin D₂ is bioavailable and relatively stable during storage and cooking [28]. Vitamin D₂ is as effective at increasing and maintaining total serum 25 hydroxy (OH) vitamin D level as vitamin D₃ [29, 35, 36]. In fact, vitamin D₂ and D₃ are structurally very similar. The only exceptions are that vitamin D₂ has double bond between carbon 22 and 23 and a methyl group on carbon 24 [31]. In pre-antibiotic era, vitamin D₃ derived from cod liver oil and direct sun-exposure was once part of regular therapy for TB [15, 24, 37]. Given the implication of VDD and risk for TB, several studies have been performed so far to re-introduce vitamin D₃ as a supplementary therapy to the anti-TB treatment [23]. Dietary source of vitamin D in the form of supplement or fortified foods may represent a new strategy for the TB prevention and shortening of TB treatment in the face of growing drug resistance [24].

To the best of our knowledge, we demonstrated for the first time the role of mushroom-derived vitamin D₂ on treatment outcomes of TB. Intervention with 27 g sun-exposed oyster mushroom in sandwich bread; containing 146 µg (5840 IU) vitamin D₂ daily for 5 days per week for the first 16 weeks; brought an improvement of about 8 ng/mL in the serum 25(OH)D level; and corrected VDD in more than one-third of TB patients without showing any adverse effects. Likewise, Martineau et al. [38] corrected the profound VDD in TB patients by providing a single oral dose of 2.5 mg vitamin D. Hassanein et al. [39] also observed the change in the serum 25(OH)D level in TB patients provided with vitamin D supplementation.

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Our intervention ameliorated the clinical outcomes and immune response of TB patients, but not sputum smear and culture conversion. Clinical outcomes were described in terms of TB score severity class and Karnofsky performance status scale. Different studies done elsewhere reported that the addition of vitamin D supplementation to the standard anti-TB drug regimen leads to the improvement of clinical outcomes in TB patients [24, 37, 40]. More than three-fourth of TB patients were found in the low severity class of TB score (class I) having about 80% Karnofsky performance status scale after intervention. About one-third of the variability of TB score in the intervention group was accounted for by the change in the serum 25(OH)D level. Likewise, Farazi et al. [40] reported that TB patients in the vitamin D supplementation group had lesser TB score than the control group after two to three months. Salahuddin et al. [41] also found that vitamin D supplementation improved TB score. Weight gain is usually an outpatient marker of betterment in TB [40]. The improvements of BMI and MUAC were solely observed in the intervention group. Similarly, Salahuddin et al. [41] and Farazi et al. [40] demonstrated that vitamin D supplementation improved the mean weight gain during anti-TB therapy.

Vitamin D mediates the activation of innate and cell mediated immunity against TB infection [40]. Upon *M. tuberculosis* detection, toll-like receptors (TLR) on the macrophage membrane are activated to induce transcriptional up-regulation of the vitamin D receptor (VDR) and enhance CYP27B1 expression, leading to the increased synthesis of 1, 25(OH)₂D and VDR, two essential components responsible for the VDR-dependent regulation of a variety of genes including the up-regulation of cathelicidin expression [24, 37]. Cathelicidin LL-37 peptide has both antimicrobial and immunoregulatory activities, such as direct killing of

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bacteria via osmotic lysis, but also induction of autophagy, regulation of chemokine production and chemokine receptor expression, modulation of cytokine secretion and chemotactic effects on immune cells [42].

In monocytes and macrophages, IFN- γ can induce CYP27B1-hydroxylase [43] and up-regulates TLR2/1L – induced antimicrobial peptide expression [44]. In our study, there were significant improvements in IFN- γ and cathelicidin LL-37 levels, but not in the levels of interleukin (IL)-4 and IL-10. In agreement with this, Fabri et al. [45] described that the in vitro supplementation of vitamin D – deficient serum with 25(OH)D restored IFN- γ induced antimicrobial peptide expression, autophagy, phagosome-lysosome fusion, and antimicrobial activity. The high IFN- γ level together with the low level of IL-4 and IL-10 implied that our intervention skewed the balance of cytokines towards TH₁ responses. Likewise, Fabri *et al.* [45] confirmed that the capacity of T cell clones to induce monocyte production of cathelicidine LL-37 peptides correlated with the amounts of IFN- γ , but inversely correlated with the amount of IL-4.

Our intervention could not bring a total sputum and culture negativity. In fact, this finding was supported by different studies done elsewhere [41, 46]. On the contrary, there were several studies that reported a total sputum smear and culture conversion in patients provided with vitamin D supplementation [47, 48]. The reasons for such inconsistencies could be the variants of VDR polymorphisms, variability in vitamin D dosages or differing phases of baseline serum vitamin D level [40].

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In conclusion, the cereal-vegetable-legume based dietary pattern has led to poor dietary quality and nutrient adequacy. Micronutrient deficiencies and protein-energy malnutrition are the predominant nutritional problem in TB patients, and hence this calls for giving attention to nutrition in the management of TB. While sun-exposed oyster mushroom is the potential resource of vitamin D₂, the provision of such mushroom effectively corrects VDD and improves the clinical outcomes and immunological responses in TB patients. To get more effects on the treatment outcomes of TB, there is a need to incorporate nutrients enriched with mushroom-derived vitamin D₂ in its management. Well-designed nutrition programme is more helpful to support the first line anti-TB drugs, increase the cure rate and reduce the infectiousness of TB. The control of TB substantially contributes to the control of poverty and food insecurity as they are the causes and consequences of TB. Therefore, nutrition programme needs to be parts and parcels of the national TB control programme in Ethiopia. Further research is warranted to design nutrition formulation for various age and sex groups of TB patients and to determine the interaction of vitamin D₂, VDR polymorphism and TB treatment outcomes.

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Appendixes

A.Affidavit



Annex 2 to the University of Hohenheim doctoral degree regulations for Dr. rer. nat.

Affidavit according to Sec. 7(7) of the University of Hohenheim doctoral degree regulations for Dr. rer. nat.

1. For the dissertation submitted on the topic

Nutrition and tuberculosis in Ethiopia: The role of vitamin D2 derived from sun-exposed oyster mushroom on the treatment outcomes of tuberculosis

I hereby declare that I independently completed the work.

2. I only used the sources and aids documented and only made use of permissible assistance by third parties. In particular, I properly documented any contents which I used - either by directly quoting or paraphrasing - from other works.

3. I did not accept any assistance from a commercial doctoral agency or consulting firm.

4. I am aware of the meaning of this affidavit and the criminal penalties of an incorrect or incomplete affidavit.

I hereby confirm the correctness of the above declaration: I hereby affirm in lieu of oath that I have, to the best of my knowledge, declared nothing but the truth and have not omitted any information.

Place and Date

Signature



Annex 3 to the University of Hohenheim doctoral degree regulations for Dr. rer. nat.

Affidavit, Legal Notice

The University of Hohenheim requires an affidavit declaring that the academic work has been completed independently in order to credibly claim that the doctoral candidate independently completed the academic work.

Because the legislative authorities place particular importance on affidavits, and because affidavits can have serious consequences, the legislative authorities have placed criminal penalties on the issuance of a false affidavit. In the case of willful (that is, with the knowledge of the person issuing the affidavit) issuance of a false affidavit, the criminal penalty includes a term of imprisonment for up to three years or a fine.

A negligent issuance (that is, an issuance although you should have known that the affidavit was false) is punishable by a term of imprisonment for up to one year or a fine.

The respective regulations are documented in Sec. 156 StGB (Criminal Code) (false affidavit) as well as in Sec. 161 StGB (negligent false oath, negligent false affidavit).

Sec. 156 StGB: False Affidavit

Issuing a false affidavit to an authority body responsible for accepting affidavits or perjury under reference to such an affidavit shall be punishable with a term of imprisonment up to three years or with a fine.

Sec. 161 StGB: Negligent False Oath, Negligent False Affidavit:

Subsection 1: If one of the actions described in Secs. 154 and 156 is done negligently, the action shall be punishable by a term of imprisonment of up to one year or a fine.

Subsection 2: Impunity shall apply if the perpetrator corrects the false information in a timely manner. The regulations in Sec. 158 (2) and (3) apply mutatis mutandis.

I have taken note of the information on the affidavit.

(Place and Date)

(Signature)

Appendixes

B. Curriculum vitae

APPENDIX B. CURRICULUM VITAE

Curriculum vitae

Name Tibebeselassie Seyoum Keflie
Birth date 28.01.1979
Nationality Ethiopian
Religion denomination Ethiopian Orthodox Tewahido Christian
Address Institute of Nutritional Sciences
Garbenstr.30, 70599 Stuttgart, Germany
E-mail tibebe.fscuhoh@gmail.com
Tibeselassie.Keflie@uni-hohenheim.de

Education

September 2013 - Present Ph.D. in Nutritional Science (Dr. rer. nat). University of Hohenheim, Stuttgart, Germany (1,0)
Doctoral Study Course Works in Tropical and Infectious Diseases (Tropical medicine) attended at Addis Ababa University, Addis Abeba, Ethiopia
October 2010 to May 2013
September 2005 to April 2007 Master of Science (MSc) in Tropical and Infectious Diseases. Addis Ababa University, Ethiopia

Working Experience

2013 - 2019 Doctoral Fellow at Institute of Biological Chemistry and Nutrition, University of Hohenheim, Stuttgart, Germany
2011 - 2013 President and General Manager of Ethiopian Society of Tropical and Infectious Diseases, Addis Abeba, Ethiopia
2007 – 2011 Assistant Professor of Tropical and Infectious Diseases at Mada Walabu University, Bale-Goba, Ethiopia
Dean of Health Sciences College, Mada Walabu University, Bale-Goba, Ethiopia
Head of Anti-HIV/ AIDS Coordination Bureau of Mada Walabu University, Bale-Goba, Ethiopia

Publications

Tibeselassie Seyoum Keflie, Aregash Samuel, Ashagrie Zewdu Woldegiorgis, Adane Mihret, Markos Abebe, Christine Lambert, Donatus Nohr, Hans Konrad Biesalski. Vitamin D2 from sun-exposed Oyster Mushroom: Its impact on the treatment outcomes of tuberculosis. Submitted manuscript.
Tibeselassie Seyoum Keflie, Aregash Samuel, Christine Lambert, Donatus Nohr and Hans Konrad Biesalski. 2018. Dietary Patterns and Risk of Micronutrient Deficiencies: their Implication for Nutritional Intervention in Ethiopia. J Nutrition Health Food Sci 6(1):1-16 DOI: 10.15226/jnhfs.2018.001120.
Tibeselassie Seyoum Keflie, Aregash Samuel, Ashagrie Zewdu Woldegiorgis, Adane Mihret, Markos Abebe, Hans Konrad Biesalski. 2018. Vitamin A and zinc deficiencies among tuberculosis patients in Ethiopia. J Clin Tuberc Other Mycobact Dis 12 27–33.
Tibeselassie Seyoum Keflie, Nils Nölle, Christine Lambert, Donatus Nohr, Hans Konrad Biesalski. 2015. Vitamin D deficiencies among

tuberculosis patients in Africa: A systematic review. *Nutrition* 31: 1204–1212.

Tibebeselassie Seyoum Keflie, Nils Nölle, Christine Lambert, Donatus Nohr, Hans Konrad Biesalski. 2018. Impact of the natural resource of UVB on the content of vitamin D2 in oyster mushroom (*Pleurotus ostreatus*) under subtropical settings. *Saudi Journal of Biological Sciences*. <https://doi.org/10.1016/j.sjbs.2018.07.014>.

Tibebeselassie Seyoum Keflie, Gobena Ameni. 2014. Microscopic examination and smear negative pulmonary tuberculosis in Ethiopia. *Pan Africa Medical Journal*. Published.

Tibebeselassie Seyoum Keflie. Impact of vitamins A and D, and zinc on immune response and treatment outcomes in patients with pulmonary tuberculosis. 2012. Tuberculosis Research Advisory Council annual proceedings.

Tibebeselassie Seyoum Keflie. Review on impact of interventional supplementation with vitamin A and zinc on Morbidity of Malaria in Children in Rift Valley Areas of Ethiopia. 2011. 13th World Federation of Public Health Association Congress proceedings.

Tibebeselassie Seyoum Keflie. Study on Human Tuberculosis in North West Shewa, Central Ethiopia. 2012. Tuberculosis Research Advisory Council annual proceedings.

Book chapter

Tilahun Bogale, Tibebeselassie Seyoum Keflie. Sero-prevalence of Hepatitis B Surface Antigens (HBsAg) and Its Risk Factor among People Attending Voluntary Counselling and Testing (VCT) Centre and Anti-retroviral Therapy (ART) Clinics of Goba Hospital. 2013. LAP LAMBERT ACADEMIC PUBLISHING, ISBN: 978-3-659-37561-3.

Journal reviewer

Journal of nutrition and metabolism, HINDWAI

BMC Infectious Diseases, SPRINGER NATURE

Journal of Endocrine, Metabolic & Immune Disorders - Drug Target, BENTHAM SCIENCE

Journal of Infectious Disorders - Drug Targets, BETHAM SCIENCE

Journal of Lung Health and Diseases

Grants and awards

2014

Dr. Hermann Eiselen PhD Grant.

September 2013

Food Security Centre PhD Scholarship EXCEED Program supported by DAAD with funds of the Federal Ministry of Economic Cooperation and Development (BMZ), Germany.

April 2012

Travel Grant of Clive West Micronutrient Fund.

Date, Place

Signature

Appendixes

C.Map of study area

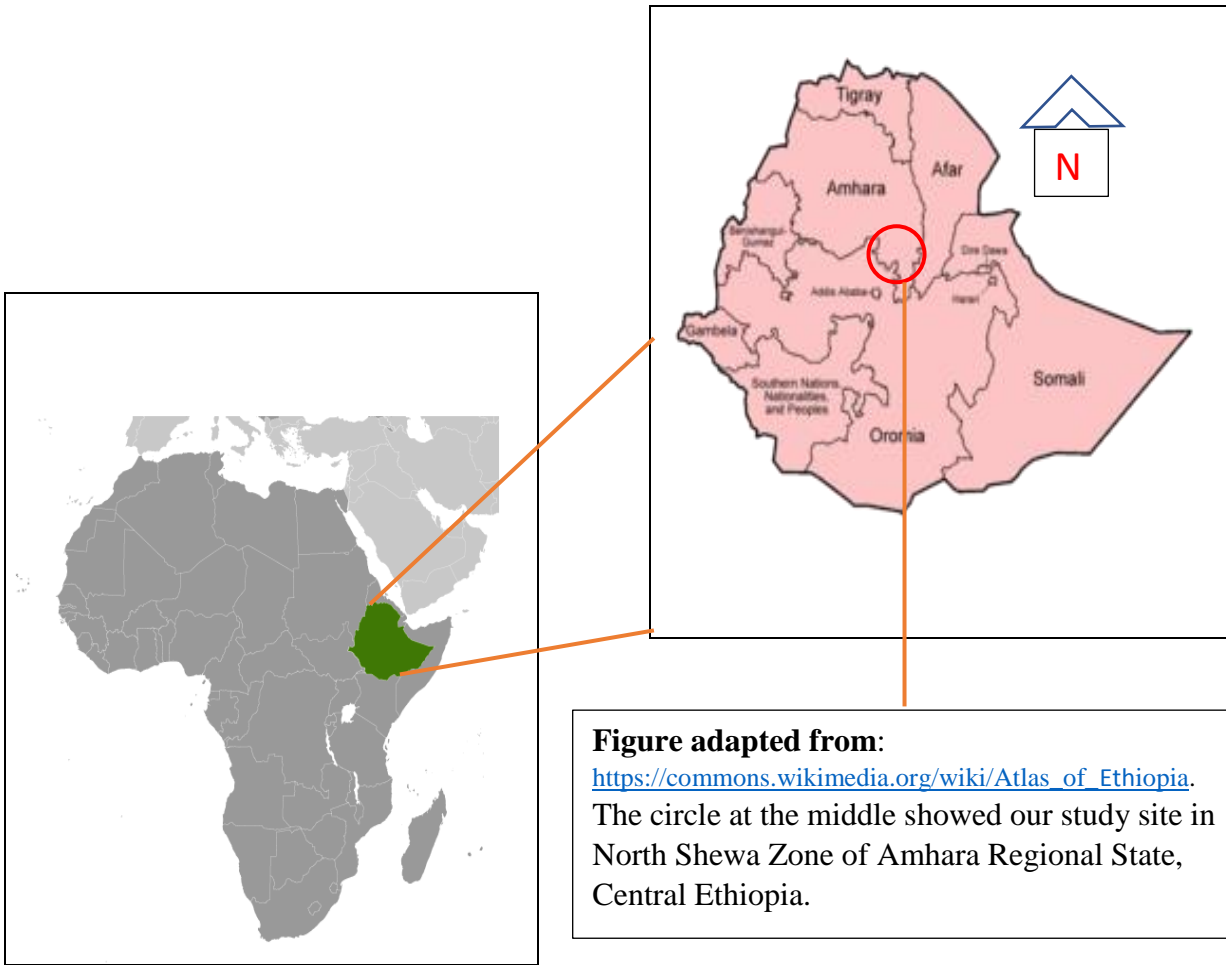


Figure adapted from:
https://commons.wikimedia.org/wiki/Atlas_of_Ethiopia.
The circle at the middle showed our study site in North Shewa Zone of Amhara Regional State, Central Ethiopia.