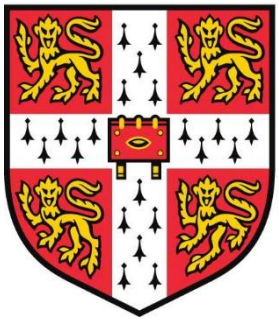

Epidemiological studies of the aetiological associations between nutritional biomarkers and cardiometabolic risk factors in Cameroon

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To my parents Marie-Madeleine Mba and Samuel Mba for believing in me and supporting me in every possible way

Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the preface and specified in the text.

I further state that it is not substantially the same as any work that has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the preface and specified in the text.

It does not exceed the prescribed word limit of 60,000.

Summary

Epidemiological studies of the aetiological associations between nutritional biomarkers and cardiometabolic risk factors in Cameroon

Camille Mba

Suboptimal diets are among the leading factors fuelling the global rise in the prevalence of type 2 diabetes and other metabolic disorders. Most epidemiological studies of the associations between diet and nutritional factors and metabolic outcomes have relied on self-report instruments. Nutritional biomarkers offer a complementary objective approach but have not been widely applied in African settings to test associations between diet and metabolic outcomes. This thesis aimed to examine the relationship between diet and nutritional factors assessed using a wide range of objectively measured nutritional biomarkers and metabolic outcomes in a population-based study in adults in rural and urban Cameroon (n= 651).

I spent the first part of my PhD in the laboratory measuring circulating vitamin D, folate, holotranscobalamin, carotenoids and tocopherol using mass spectrometry techniques. Subsequently, I undertook analyses to describe the patterns and identify factors affecting these nutritional biomarkers reflecting dietary intakes and plasma zinc, which was measured in an external laboratory. Most of the biomarkers showed distinct patterns by age, sex, level of education, physical activity levels and rural/urban area of residence. I then investigated the independent cross-sectional associations of these biomarkers with metabolic risk factors exploring the possibility of both linear and non-linear associations and adjusting for a wide range of potential confounders. Circulating folate and carotenoids, which are associated with intake of fruits and vegetables, showed an inverse association with the metabolic syndrome score and fasting glucose respectively. Holotranscobalamin, a biomarker that reflects intake of animal-sourced foods, was positively associated with the metabolic syndrome score. Circulating zinc, reflecting intake of protein-rich foods, was inversely associated with several markers of glucose homeostasis (fasting and 2-h glucose and homeostatic model assessment for insulin resistance). Higher 25-hydroxy-vitamin D, a marker of vitamin D status, was associated with lower fasting glucose. Finally, I investigated the potential effect modification by rural/urban area of residence, sex and body mass index on the association between the biomarker and metabolic outcomes.

Overall this PhD showed 1) Rural and urban differences in the distribution of the nutritional biomarkers in Cameroon 2) Significant associations of the studied biomarkers reflecting dietary

intakes with metabolic risk factors. Findings from my PhD advance the understanding of the role of diet and nutritional factors on metabolic health in adults in Cameroon. Diet is modifiable, making it a realistic target for public health intervention to improve metabolic health in this population.

Acknowledgements

I am deeply indebted to my supervisor Prof Nicholas Wareham for his guidance and support throughout my PhD journey. Prof Wareham did not only act as an academic supervisor but as a real mentor who encouraged me, gave constructive feedback on my work and continues to help in my personal and professional development. He appreciated every little progress I made especially during the difficult times of the COVID-19 pandemic, which was very encouraging.

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I am grateful to the Cambridge Trust and Islamic development bank who funded my PhD project and gave me the opportunity to study in this prestigious institution. You made a dream come true.

I cannot end this without expressing my deepest appreciation for my fiancé and siblings who have encouraged me all the way. Thank you for your daily words of encouragement that made my PhD journey more enjoyable. I am truly blessed to have you.

Contributions

I was not involved in the design and data collection of the Cameroon study. I designed the research questions presented in this PhD thesis with guidance from my supervisor, analysed, and interpreted the data. I wrote this thesis, including all the papers from this thesis and incorporated feedback from the co-authors. I conducted the laboratory analyses of the nutritional biomarkers with assistance from laboratory members mentioned in the acknowledgement section, except for plasma zinc. I coordinated the shipping of plasma samples for zinc analyses to Southampton and these were analysed by staff at the Southampton University Hospitals. Vasileios Kaimakis supervised the retrieval of the blood samples from the storage site offsite. Sarah Meadows and Tabasum Tabasum assisted with the laboratory analyses of serum vitamin D and folate and plasma carotenoids and tocopherol. Damon Parkingston assisted with the laboratory analysis of plasma vitamin C. In this PhD thesis, I use the pronoun “we” to indicate the collaborative work.

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List of publications from this thesis

Peer reviewed journals

Mba CM, Koulman A, Forouhi NG, Imamura F, Assah F, Mbanya JC, Wareham NJ. Associations of Serum Folate and Holotranscobalamin with Cardiometabolic Risk Factors in Rural and Urban Cameroon. Nutrients. 202;14(1):178.

Mba CM, Koulman A, Forouhi NG, Imamura F, Assah F, Mbanya JC, Wareham NJ. Association between circulating 25-hydroxyvitamin D and cardiometabolic risk factors in adults in rural and urban settings. (Manuscript in revision at Nutrition & Diabetes)

Mba CM, Jones K, Forouhi NG, Imamura F, Assah F, Mbanya JC, Wareham NJ. The association between plasma zinc concentrations and markers of glucose metabolism in adults in Cameroon. (Under review at Public Health Nutrition)

Mba CM, Koulman A, Forouhi NG, Imamura F, Assah F, Mbanya JC, Wareham NJ. The associations of plasma carotenoids and α-tocopherol concentrations with fasting glucose in adults in Cameroon. (Under review by co-authors)

Book chapter

Mba CM, Wareham NJ. Diabetes Epidemiology (In preparation to be submitted at Endotext)

Conference abstracts

Mba C, Koulman A, Sharp S, Forouhi N, Imamura F, Assah F, et al. Associations of Serum Folate and Holotranscobalamin with Cardiometabolic Risk Factors in Rural and Urban Cameroon. Current Developments in Nutrition. 2021;5(Supplement_2):1058. https://doi.org/10.1093/cdn/nzab053_051

Mba CM, Koulman A, Assah F.k, Mbanya JC, Wareham NJ. Association between circulating 25-hydroxyvitamin D and cardiometabolic risk factors in rural and urban Cameroon. Diabetologia. 63, 1–485 (2020). Abstract 286. https://doi.org/10.1007/s00125-020-05221-5

Conference presentations

American Society for Nutrition (June 2020). Associations of Serum Folate and Holotranscobalamin with Cardiometabolic Risk Factors in Rural and Urban Cameroon. Poster presentation.

56th European Association for the Study of Diabetes Annual meeting (September 2020). Association between circulating 25-hydroxyvitamin D and cardiometabolic risk factors in rural and urban Cameroon. Poster presentation.

List of abbreviations

BMI	Body mass index
CVD	Cardiovascular diseases
FFQ	Food frequency questionnaire
GBD	Global Burden of Disease
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
HoloTC	Holotranscobalamin
HOMA-IR	Homeostatic model assessment for insulin resistance
IDF	International Diabetes Federation
LDL	Low density lipoprotein
LPA	Light intensity physical activity
MVPA	Moderate-to-vigorous intensity physical activity
NCD	Non Communicable Disease
PAEE	Physical activity energy expenditure
SSA	Sub-Saharan Africa
RCT	Randomised controlled trial
RNI	Reference nutrient intake
WHO	World Health Organisation
25(OH)D	25-hydroxyvitamin D

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Chapter 1 : Introduction and background

Publication

Parts of this chapter are for publication as a book chapter in Endotext. South Dartmouth (MA): MDText.com

Mba CM, Wareham NJ. Diabetes Epidemiology.

Summary

Diabetes mellitus is a major cause of cardiovascular disease, end-stage kidney disease, vision loss and non-traumatic amputations placing a huge strain on health care systems. The rising prevalence of diabetes globally is of major public health concern. Recent estimates show a global prevalence of 537 million adults with diabetes in 2021, of which 80% live in low and middle-income countries. This number is predicted to rise to 783 million by 2045, with 94% of the increase occurring in low and middle-income countries. Africa is the region with the highest death rate in people of economically productive age and highest predicted rate of increase in diabetes. The rise in the incidence of diabetes has been strongly linked to the rise in obesity, and shifts in dietary and physical activity patterns towards unhealthy patterns. Dietary and nutritional factors are amongst the leading modifiable risk factors for diabetes. Much of the evidence on the association between diet and diabetes have relied on the subjective traditional dietary assessment methods, which have a number of limitations. Nutritional biomarkers offer a complimentary and objective approach to assess dietary intake without the errors commonly associated with the traditional dietary assessment methods but have not been widely applied to test diet-metabolic disease associations in African settings. The overall aim of this PhD was to examine the associations between a wide range of objectively measured nutritional biomarkers and cardiometabolic risk factors in adults in rural and urban areas of Cameroon

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1.1 Epidemiology of type 2 diabetes

Geographic distribution

Globally, an estimated 537 million adults had diabetes in 2021, with over 80% living in low and middle-income countries. Almost half of the people with diabetes globally are undiagnosed, with the highest proportion of undiagnosed in the African region (54%) (1). Type 2 diabetes is the most prevalent of all diabetes types and accounts for ~ 85% of all diabetes cases. There are marked differences in the geographic distribution of type 2 diabetes, with the age-adjusted prevalence ranging from 1.1% in Benin to 30.8% in Pakistan. Table 1.1 shows the global and regional age-adjusted prevalence of diabetes. The top five countries with the highest age-adjusted prevalence of diabetes are Pakistan (30.8%), French Polynesia (25.2%), Kuwait (24.9%), New Caledonia (23.4%), and Northern Mariana Islands (23.4%). Only a few studies all in high and middle-income countries, have reported on the incidence of type 2 diabetes. These studies show over a 20-fold variation in the incidence of type 2 diabetes with the age-standardised incidence ranging from 1.1 per 1000 person-years in Russia to 24.3 per 1000 person-years in the US (2).

Table 1.1 : Diabetes prevalence in adults aged 20-79 years in 2021 by regions (IDF 2021) (1)

	Diabetes prevalence	
	Age-adjusted (%)	Age-standardised (%)
World	10.5	9.8
Africa	4.5	5.3
Europe	9.2	7.0
MENA	16.2	16.2
North America and Caribbean	14.0	11.9
South and Central America	9.5	8.2
South East Asia	8.7	10.0
Western Pacific	11.9	9.9

Age-adjusted: Standardised to the national population

Age-standardised: Standardised to the world population

MENA, Middle East and North Africa

Distribution by age, sex and ethnicity

Age is a major determinant of type 2 diabetes prevalence, which increases sharply with age. The gradual demographic shift in the population structure towards an older population especially in the developed countries means that the total diabetes prevalence will continue to rise especially in the elderly populations. In high-income countries, type 2 diabetes prevalence is highest in the > 65-year-old category, while in LMIC countries, the prevalence is highest in those of working age. There is a slight male excess in the prevalence of type 2 diabetes; globally, 17.7 million more men than women had diabetes in 2021 (1). Type 2 diabetes incidence also increases with age and is highest in adults aged ≥ 40 years. The incidence is extremely low in children before puberty but rises slightly at puberty probably due to hormonal changes and insulin resistance associated with puberty.

Diabetes prevalence and incidence vary by ethnic group and socioeconomic status. The prevalence of diabetes is higher in non-white ethnic groups and people of lower socioeconomic status. In the US, the age-adjusted prevalence of type 2 diabetes is higher among American Indians and Black populations than White-Hispanic populations (Figure 1.1) (3). The higher diabetes prevalence in ethnic minorities groups living in high-income countries is independent of socioeconomic status.

The prevalence of diabetes is higher in urban areas than in rural areas worldwide (12.1% vs 8.3% in 2021) (1). As urbanisation increases, leading to shifts in dietary and physical activity patterns toward a more sedentary lifestyle and higher intake of unhealthy diets, the number of people with type 2 diabetes in the urban area is also expected to rise.

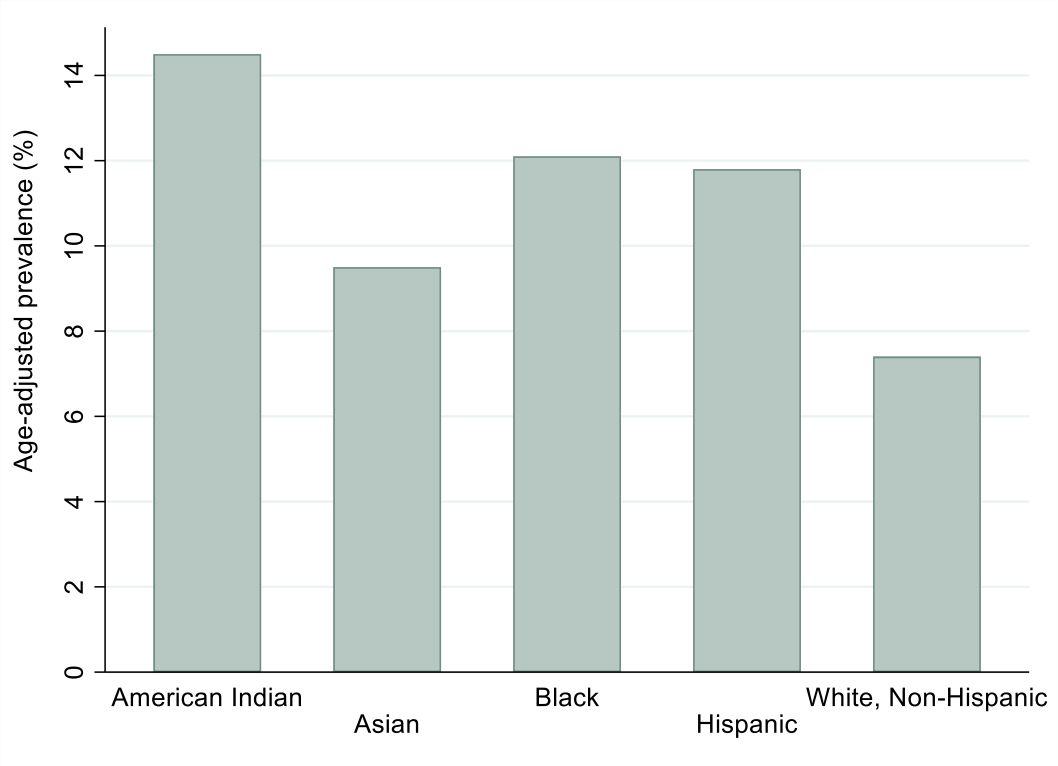

Figure 1.1 : Ethnic variation in diabetes prevalence in adults aged ≥ 18 years in the US in 2018-2019 (CDC 2022 report) (3)

Variation by time

The prevalence of type 2 diabetes has increased globally over the last four decades (4). The World Health Organisation (WHO) estimated there were 108 million adults aged ≥ 18 years with diabetes in 1980, rising to 422 million adults in 2014. In the same period, diabetes prevalence increased from 4.7% to 8.5%. The most dramatic increase occurred in low and middle-income countries. For example, in Africa, the prevalence rose from 3.1% to 7.1% between 1980 and 2014 (Figure 1.2).

The global prevalence of diabetes has continued to rise since 2014 and is projected to reach 12.2% by 2045. The highest predicted increase of 129% is expected to occur in the African region (Figure 1.3). These projections are likely to underestimate the true prevalence, as they do not take into account the changes in some risk factors that affect diabetes incidence like overweight/obesity. Nonetheless, an increase in prevalence does not necessarily reflect an

increase in the incidence rate of diabetes as other factors such as improved survival of people with diabetes may contribute to the rising prevalence.

Although the incidence of type 2 diabetes continues to rise in many countries (with an annual increase ranging from 0.9% to 5.6%) (1), there is evidence that it has started to plateau or even fall in some high-income countries. There are no available data to assess trends in incidence of type 2 diabetes in low-income countries. The number of people with diabetes living in urban areas globally is predicted to increase by 65% between 2021 and 2045, while it appears to have plateaued in rural areas.

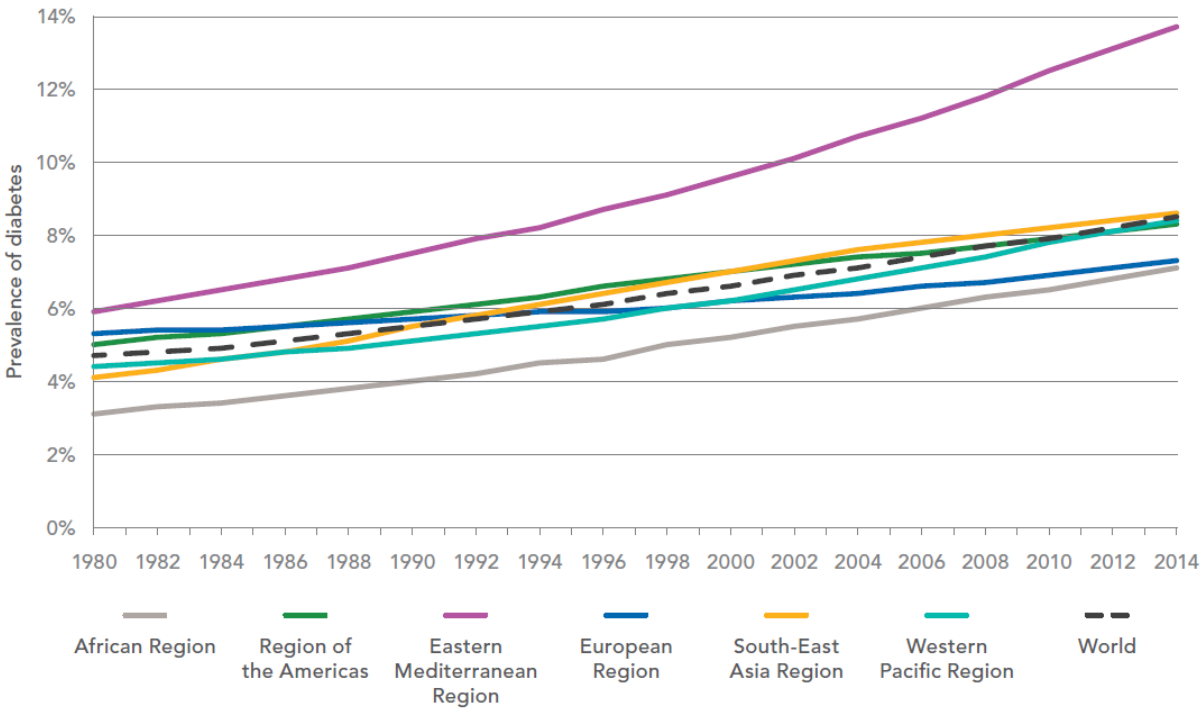

Figure 1.2: Trends in the prevalence of diabetes by region (WHO report, 2016) (4)

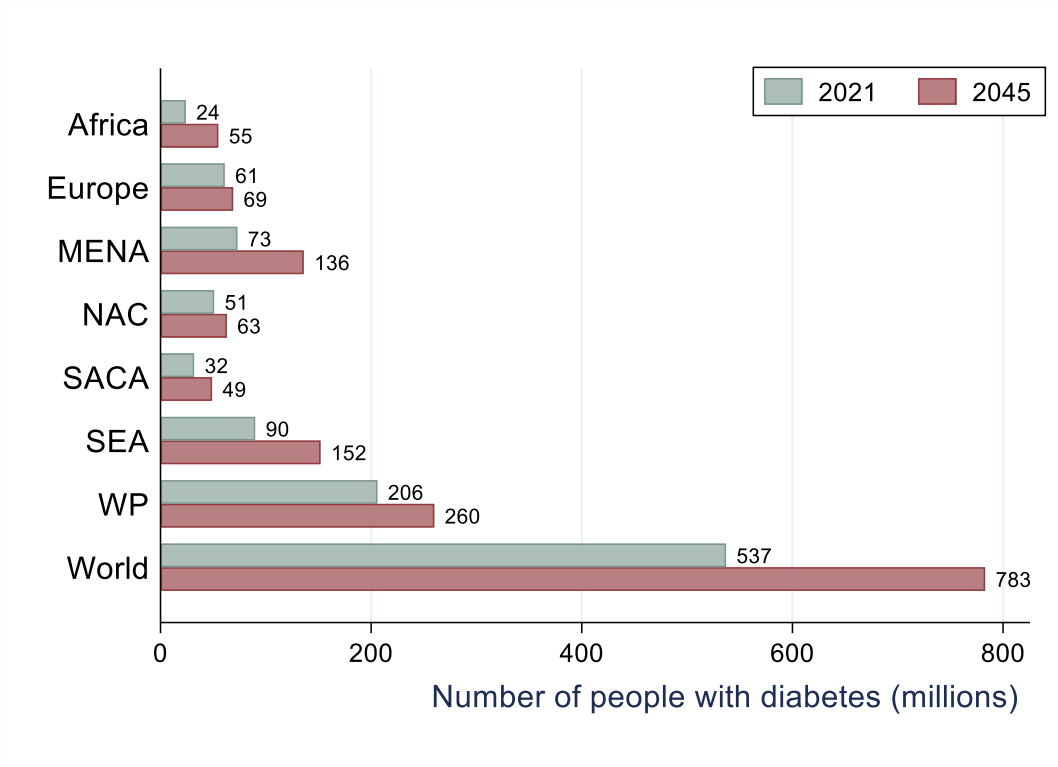

Figure 1.3 : Number of people with diabetes aged 20-79 years globally and per IDF region in 2021 and projected estimates for 2045 (1).

MENA, Middle East and North Africa; NAC, North America and Caribbean; SACA, South and Central America; SEA, South East Asia; WP, Western Pacific

1.2 Risk factors

Type 2 diabetes is a multifactorial disease that results from a complex interaction between genetic predisposition, early growth and programming and health-related behaviours throughout life.

Genetic predisposition

Twin studies show a high concordance in the development of glucose intolerance and type 2 diabetes with estimates ranging between 36-83% for monozygotic twins and 16-40% for dizygotic twins (5). Genetic studies to date have identified over 240 genomic regions and 400 genetic variants associated with type 2 diabetes (6). However, the contribution of individual genetic variants is small. Even when the genetic factors are combined to create a genetic risk score, this is not effective in predicting future diabetes cases over simple risk prediction scores.

Current knowledge of genetic risk factors for type 2 diabetes has been driven predominantly by studies in European descents and there is a need for large trans-ethnic genetic studies.

Overweight and obesity

Obesity is a major independent and modifiable risk factor for type 2 diabetes with strong evidence from large cohort and intervention studies. Higher body mass index (BMI) is associated with higher type 2 diabetes risk (Figure 1.4). The relative risk of type 2 diabetes increases progressively from a BMI of 22 Kg/m^2 independently of family history, age, sex, smoking habits, and physical activity. The rise in incidence and prevalence of diabetes mirrors the rapid rise in obesity worldwide. Regardless of the genetic predisposition, obesity increases diabetes risk by more than six times compared to those with normal weight (7). Sustained weight loss of 15 Kg or more may induce sustained type 2 diabetes remission for up to 2 years in adults with overweight and obesity (8).

The ethnic disparities in diabetes distribution could also be partly explained by the ethnic differences in obesity prevalence. Obesity is more common in Black than White populations in the US, with age-adjusted prevalence of obesity of 49.6% in non-Hispanic Black adults, 44.8% in Hispanic Whites, 42.2% in non-Hispanic Whites, and 17.4% in non-Hispanic Asians (3). However, the effect of adiposity on diabetes risk may vary across populations. For example, Asians develop diabetes at lower BMI than other ethnic groups.

Ethnic minority groups living in high-income countries have a higher prevalence of type 2 diabetes independently of socioeconomic status. In the UK for example, type 2 diabetes is 4 to 6 times more prevalent in South Asians and African-Caribbean populations than in the White European population (9). This is in support of the thrifty phenotype hypothesis which proposes that undernutrition during pregnancy and early childhood produces permanent changes in glucose-insulin metabolism which results in a phenotype that would be advantageous during food famine but deleterious during food abundance by promoting overweight/obesity (10). The slight excess diabetes risk in men compared with women may be due to the higher central adipose tissue distribution in men, despite women having a higher BMI.

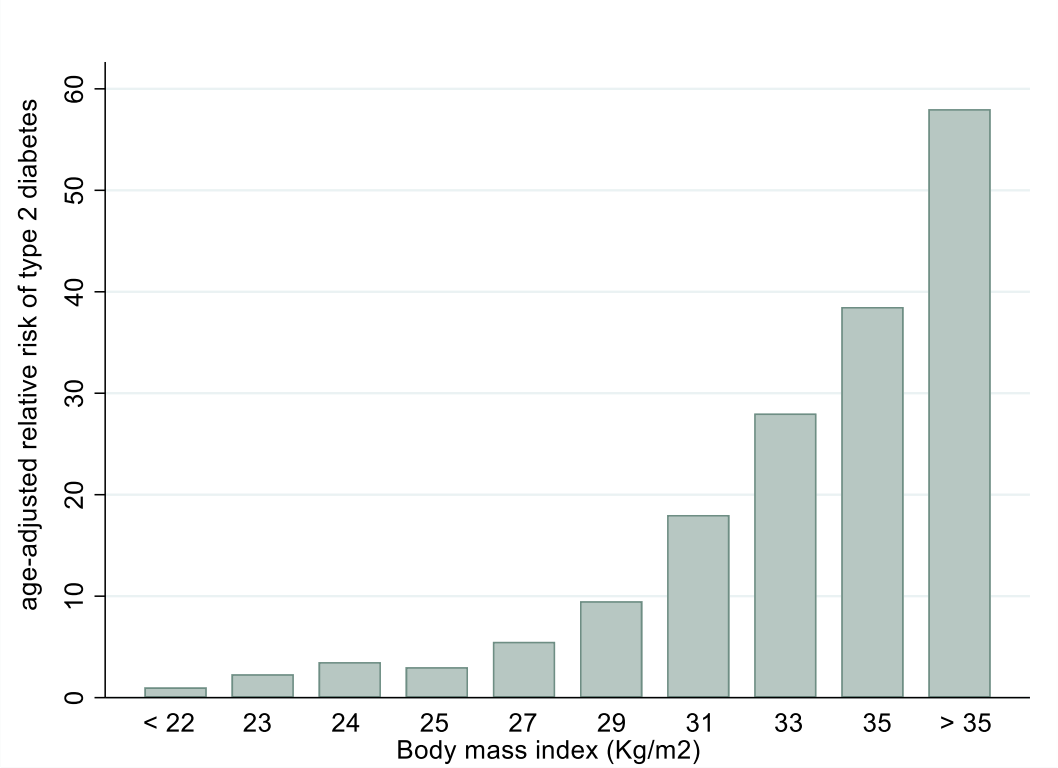

Figure 1.4 : Association between body mass index and type 2 diabetes amongst women aged 30-35 years in the Nurses' Health Study (adapted from Colditz et al ((11))

Physical inactivity and unhealthy diet

Physical inactivity and unhealthy diets are amongst the leading modifiable risk factors for type 2 diabetes. Increasing physical activity and improving dietary habits can reduce type 2 diabetes risk. The greatest relative benefits of physical activity on type 2 diabetes risk are observed in physically inactive individuals who replace their sedentary time with physical activity of any type, intensity, and duration. Compared with physically inactive individuals, achieving the recommended 150 minutes of moderate physical activity per week reduces type 2 diabetes risk by 26% (12).

Dietary risk factors account for ~ 20% of the attributable risk of all non-communicable diseases (13). Low-fat diet and low carbohydrate diet interventions are effective for weight loss. Long-term sustainability is linked to the extent of dietary adherence. A high intake of fruits and vegetables, whole grains, legumes, nuts, and fermented dairy products lowers diabetes risk while a high intake of processed and unprocessed meat, sugar-sweetened beverages, and refined grains, raises diabetes risk (13). In recent years, the importance of the overall patterns

of food consumption has gained interest as food combinations may have synergistic or antagonistic effects. Dietary patterns such as the Mediterranean diet and dietary approaches to stop hypertension (DASH) have shown robust evidence in type 2 diabetes prevention. A meta-analysis showed that adherence to a Mediterranean diet or DASH reduced diabetes risk by 13% and 19% respectively when comparing the highest vs the lowest quartile of intake (14).

1.3 Diabetes clusters

The classification of diabetes into the two main types that constitute ~ 90% of all diabetes relies on the age at diagnosis and the presence or absence of markers of β-cell auto-immunity (GADA, glutamic acid decarboxylase autoantibody; ICA, islet cell autoantibody; IA-2A, insulin antigen-2 autoantibodies; IAA, insulin autoantibodies). Patients with type 1 diabetes have absolute insulin deficiency and require insulin for their survival. However, type 2 diabetes is a multifaceted disease and management is more complex. Early optimal metabolic control is crucial for the prevention of diabetes complications because target tissues remember poor metabolic control even decades later (metabolic memory).

In recognition of the heterogeneous group of patients often classified as having type 2 diabetes, a sub-classification that could provide a step towards precision medicine and improve clinical outcomes is needed. Some authors have proposed using cluster analysis to sub-classify adult-onset diabetes into five groups with distinct underlying disease mechanisms, phenotypes and risk of complications (15). For example, patients with severe insulin deficiency are more likely to develop retinopathy and neuropathy and those with severe insulin resistance are more likely to develop nephropathy and fatty liver disease. Other authors have suggested the use of simple quantitative measures to predict clinical outcomes (for example, baseline kidney function to risk diabetes nephropathy, or age at diagnosis to predict changes in HbA1c) (16).

1.4 Burden of diabetes

Deaths attributable to diabetes and disability-adjusted life years (DALY) attributable to diabetes have risen globally over the past 2 decades. Globally, between 2007 and 2017, deaths from type 1 and type 2 diabetes increased by 15.1% and 43% respectively. An estimated 1.37 million deaths were attributable to diabetes in 2017 (17) . Similarly, DALY rates for diabetes increased by 24.4% globally between 1990 and 2019, placing diabetes amongst the top ten leading causes of all-age DALY in 2019 (18).

1.5 Diabetes and Cardiovascular diseases in sub-Saharan Africa

The co-existence of infectious diseases and non-communicable diseases (NCDs) in sub-Saharan Africa (SSA) imposes a huge strain on the already fragile health care systems. Although infectious diseases are still the leading causes of morbidity and mortality in the region, the disease burden in SSA is gradually shifting from infectious diseases to NCDs. Many of the countries in Africa that are projected to have the greatest increase in the burden of NCDs are least prepared for the challenge. It is projected that morbidity and mortality from NCDs will surpass those from infectious diseases in SSA by 2030 (19).

In 2021, diabetes affected 24 million adults and was responsible for 416,000 deaths in Africa. Over 70% of deaths from diabetes in the region occur in those who are in an economically productive age group, which has substantial implications at the individual, household and societal levels. The number of people living with diabetes is expected to increase by 129% by 2045, which is the highest predicted increase in all the regions (1). The burden of hypertension is also high in the region, with a prevalence of 30% and up to 70% in some countries (20), (21). One third of people with hypertension and over half of those with diabetes are unaware of their disease status (1), (20). As in the rest of the world, the prevalence of diabetes and hypertension is higher in urban areas than rural areas, but the prevalence of both conditions is gradually rising in the rural communities (22), (21), (23). The number of people living with hypertension is projected to reach 216.8 million by 2030 (24). Cardiovascular diseases (CVDs) are the leading causes of deaths from NCDs accounting for 13% of all deaths and 37% of all NCDs deaths (25). Ischaemic heart disease and stroke are the leading contributors to CVDs mortality in the region. In 2009, over a million deaths were attributed to CVDs in SSA (26).

The rapid urbanisation in many SSA characterised by decreased levels of physical activity and higher consumption of unhealthy foods and obesity have fueled the rise in diabetes and CVDs in the region. This is in line with the thrifty phenotype hypothesis mentioned above, where poor nutrition during pregnancy and early childhood, combined with shifts in dietary habits during later childhood and adulthood resulting from urbanisation in SSA might explain in part the rising prevalence of type 2 diabetes. In Cameroon, lower physical activity levels have been reported in people living in urban areas compared with those living in rural areas which may partly account for the rural-urban difference in the prevalence of diabetes and hypertension (23), (27), (28). The prevalence of diabetes in Cameroon is currently estimated at 5.5% and hypertension at ~30% (1), (29). Understanding the role of modifiable risk factors such as diet

in diabetes and related metabolic disorders is important for the design of contextually relevant and effective public health interventions to quell the rapid rise in these disorders in SSA.

1.6 Diet and diabetes

Dietary and nutritional factors are amongst the leading contributors to morbidity and mortality worldwide. The 2019 Global Burden of Disease study reported that suboptimal diet was amongst the top 3 risk factors for deaths globally, accounting for 13.5% and 14.6% of all deaths in females and males respectively (13). The leading causes of diet-related deaths were CVDs, cancer deaths and type 2 diabetes. Moreover, dietary risk was the fifth and sixth leading risk factor globally for attributable DALY in males and females respectively (13).

Evidence from epidemiological studies suggests that a healthy diet can prevent or slow down the progression of diabetes (30), (31), (32). However, these studies have mostly been conducted in North America and Europe and data from Africa and Asia is lacking. Many countries in SSA have undergone a rapid nutrition transition over the past 3 decades. This has been associated with stark shifts in dietary patterns towards the consumption of foods that are energy-dense, high in refined grains, saturated fats and salt (33). In 2015, dietary risk factors accounted for 9.7% of all deaths and 23.1% of NCDs deaths in Ethiopia (34). Specifically, a diet low in fruits, vegetables, low grains and nuts and high in salt were the leading risk factors for NCDs deaths. The nutrition transition in SSA has been associated with the triple burden of malnutrition; co-existence of undernutrition, overweight/obesity and micronutrient deficiencies.

Despite the substantial contribution of diet to the burden of NCDs, the relationship between diet and diabetes and other metabolic disorders has been insufficiently studied in SSA (35), (36), (37), (38). Studies examining the association between diet and diabetes in SSA are limited in part because of the lack of locally adapted and validated dietary assessment methods and food composition tables to assess dietary intake. Lack of knowledge of what constitutes a healthy diet has been highlighted as a barrier to healthy dietary behaviours in Cameroon (39). A study in rural and urban Cameroon reported that the habitual diet in rural areas estimated using a food frequency questionnaire (FFQ) was associated with higher intake of energy, fat, carotenoids, vitamin C, D and E, iron and zinc than in urban areas (40). In another study in Benin where dietary data was collected using a 24-h recall, intake of legumes, fish, and fruits was higher in rural participants compared with urban participants, while meat consumption was higher amongst the urban participants (35). In this study, the micronutrient adequacy score was

positively associated with HDL cholesterol and inversely associated with the total cholesterol/HDL cholesterol ratio. A few other studies examined the association between nutrient patterns derived from FFQs and measures of adiposity and showed a positive association between an animal-driven nutrient pattern and BMI (36), (37).

Most previous studies have relied on the use of traditional self-reported methods to assess dietary intake, which is subject to recall bias and social desirability bias leading to over or underestimation of dietary intakes. This is compounded by the lack of locally adapted food composition databases in many African countries (41). Established biomarkers offer a complementary approach to assess dietary intake or nutritional status without the errors commonly associated with the traditional dietary assessment methods but they have not been widely applied to test diet-diabetes associations in African settings. The clarification of the associations of dietary and nutritional factors with diabetes in populations in Africa is important to inform the design of contextually adapted public health dietary interventions.

1.7 Dietary assessment methods

Diet is complex to measure and as a result, a variety of methods have been developed in an attempt to accurately measure dietary intake. Diet has been traditionally measured using subjective dietary assessment methods. Major limitations of the subjective dietary assessment methods include issues of poor recall and the lack of detailed information from food composition databases to estimate nutrient intakes. Objective dietary assessment methods do not rely on the participant's responses and therefore account for some of the limitations of the traditional dietary assessment methods (42).

1.7.1 Subjective dietary assessment methods

Different dietary assessment instruments have been developed to assess dietary intake and can be broadly grouped into the following categories:

- Estimated food diaries
- Weighed food diaries
- 24-hour recall
- Food frequency questionnaire (FFQ)
- Dietary checklist
- Dietary history.

The common feature of subjective dietary assessment methods is that they rely on individuals or (proxy-reporters) reporting their diets during a specified period. Subjective dietary assessment methods broadly aim to provide information on the types of foods consumed and portion sizes. Data on the type and quantity of diet consumed can be linked to a food composition database to estimate nutrient intake.

Food diaries, 24-hour recalls and FFQs have been most commonly used in nutritional epidemiology research. Food diaries are less reliant on memory as they involve individuals recording their intake of food or beverages at the time of consumption. This method may impose a high participant burden depending on the number of days of recording and the detailed dietary intake data collected. Participants may also change their diet during the recording period (Hawthorne effect). 24-hour recalls are used to retrospectively list dietary intakes in the last 24 hours. 24-hour recalls can be used to estimate population mean intakes or even habitual intakes and variations of dietary intake (if multiple recalls are available). The FFQ is the most frequently used dietary assessment instrument in large-scale epidemiological studies since it is less expensive and easy to administer compared with the other methods. FFQ provides estimates of usual intakes and is mainly used to rank individuals within a population (42).

Different self-report dietary assessment instruments have different strengths and limitations. Although subjective methods are non-invasive, relatively cheap, easy and flexible to implement, their major limitation is that they rely on the participants' responses. As a result, they are prone to recall bias and social desirability bias leading to underestimation or overestimation of dietary intake (43). Moreover, participants tend to change their eating patterns when taking part in a study and there is a need for repeat measures of dietary intake to take into account the day-to-day variation (44). These problems can be further compounded by coding and data entry errors and inadequate food composition databases (41).

1.7.2 Objective dietary assessment methods

Objective dietary assessment methods do not rely on the participants' responses, thereby minimising errors related to recall and social desirability bias or inadequate food composition databases. Objective methods are often used to validate subjective dietary assessment methods or can be used to complement the traditional dietary assessment methods.

The 3 main types of objective dietary assessment methods are: direct observations, duplicate diets and nutritional biomarkers.

Direct observation

Direct observations involve trained staff recording peoples' diets at the time of consumption. With direct observations, researchers unobtrusively observe individuals during a meal and independently record their behaviours including the type and quantity of diet received, consumed or spilled throughout a defined period (for example during school lunch). This could involve the use of video recording (45).

Direct observations are often used to validate other dietary assessment tools or evaluate interventions, given that they provide objective measures of actual intakes. Direct observations do not provide information on habitual dietary intakes unless multiple observations are conducted. With direct observations, imprecise recordings of the observers may introduce errors and interobserver reliability should be assessed. Direct observations are costly and require extensive training of the observer (46). Moreover, individuals may alter their behaviours when they know they are observed.

Duplicate diet

The duplicate diet method involves weighing and retaining a duplicate portion of the diet during a defined period (usually 24-hours) for chemical analyses. This is usually done concurrently with a weighed food record to check on the completeness of the duplicate diet. The duplicate diet method is considered the gold standard to assess actual nutrient intake at the individual level, particularly mineral intakes. It can also be used to validate nutritional biomarkers. The duplicate diet method is expensive to execute, requires sophisticated laboratories and imposes a high participant burden (47).

Nutritional biomarkers

Nutritional biomarkers are more commonly used in nutritional epidemiology than direct observations and duplicate diets. A nutritional biomarker is any biological indicator of dietary intake or nutrient status in relation to the metabolism of dietary constituents (48). Biomarkers may sometimes be used to complement traditional dietary assessment instruments to provide a better estimate of dietary exposure. Broadly, nutritional biomarkers have three main uses: validation of dietary instruments, indicators of dietary intake and indicators of nutritional status (49).

Depending on the biological specimen in which the biomarker is measured, nutritional biomarkers may reflect dietary exposure/nutritional status over a short, medium or long-term

period. For example, urine reflects dietary exposure over a few hours to days, serum or plasma reflects exposure over a few days to months, erythrocytes reflect exposure over 3-4 months (lifespan of the red blood cells) and others such as hair, nails and adipose tissues reflect long-term intake (months to years) (50).

Classification of nutritional biomarkers

Two main classifications of nutritional biomarkers are commonly used. The first classification distinguishes between biomarkers of dietary exposure and biomarkers of nutritional status as follows (49):

- Biomarkers of dietary exposure: These are biomarkers that reflect intake of foods. Biomarkers are particularly useful when the nutrients and food composition of the same food vary considerably depending on where the food was grown, processing and cooking methods. In some cases, several measures of food components may better reflect intake than a single indicator.
- Biomarkers of nutritional status: These biomarkers do not only reflect intake but possibly the metabolism of the nutrient. Some of these biomarkers may not reflect the status of a single nutrient but possibly the interaction of several nutrients.

The second classification groups the different biomarkers based on biokinetics and intended use as follows (51):

- Recovery biomarkers: Recovery biomarkers constitute the most important group of biomarkers and are mainly used for the validation of other biomarkers or dietary instruments. Recovery biomarkers provide estimates of absolute dietary intake and are based on the notion of metabolic balance between dietary intake and excretion over a given period. Assessing recovery biomarkers is expensive and complex, and thus not suitable for large-scale epidemiological studies. Few recovery biomarkers have been identified to date. Examples include doubly labelled water to assess total energy intake, urinary nitrogen for protein intake and urinary potassium for potassium intake.
- Predictive biomarkers: Just like recovery biomarkers, predictive biomarkers are sensitive, time-dependent and show a dose-response relationship with dietary intake except that their overall recovery is lower. Therefore, they do not completely reflect dietary intake but predict it to some extent. An example is 24-hour urinary fructose, which is a predictive biomarker for sugar intake.

- Concentration biomarkers: Concentration biomarkers correlate with dietary intakes, although the correlation coefficients are usually lower than those of recovery and predictive biomarkers. They are mainly used to rank individuals in relation to usual intakes and do not provide estimates of absolute intakes because their concentrations may be affected by some factors (listed below). Examples of concentration biomarkers are plasma carotenoids.
- Replacement biomarkers: Replacement biomarkers are similar to concentration biomarkers and are often used for compounds that have incomplete or inadequate information in food composition databases. For example plasma aflatoxins. Replacement biomarkers and concentration biomarkers are the most commonly used nutritional biomarkers in nutritional epidemiology to test diet-diseases associations.

Advantages and limitations of nutritional biomarkers

The major advantage of nutritional biomarkers is that they are objective measures and are independent of errors and biases associated with the traditional dietary assessment methods. However, the assessment of dietary intake or nutritional status using nutritional biomarkers is not without challenges and measurement errors (48), (49), (51). Some of the advantages and limitations of nutritional biomarkers are summarised in Table 1.2.

Table 1.2 : Advantages and limitations of nutritional biomarkers

Advantages of biomarkers	Limitations of biomarkers
- Minimises bias related to reactivity bias, social desirability bias or recall bias - Less time consuming than dietary instruments - Several biomarkers can be measured from the same biological specimen - Do not require the use of food composition databases	- Collection and analysis of samples are costly - Measurement of some biomarkers requires sophisticated laboratories - Some biological specimens such as the adipose tissue are difficult to obtain and not applicable in large-scale studies - Lack of or poor quality control in samples collection, processing and storage can affect the biomarker measurements

To correctly interpret biomarkers concentrations in relation to dietary intake, it is important to consider the following factors that affect the bioavailability of biomarkers:

- Genetic hormonal, metabolic, gut-microbiota and homeostatic variations between individuals and the interplay between them.
- Health-related behavioural factors such as physical activity, smoking and alcohol intake
- Disease state and medication use
- The interaction between different biomarkers

As a result, the concentrations of some biomarkers (such as concentration and replacement biomarkers) cannot be translated into the participant's absolute dietary intake. At this point, biomarkers of intake are limited to research applications. In addition, it is sometimes difficult to identify the specific dietary component associated with the biomarker.

1.8 PhD project aims

There is a dearth of information on the use of nutritional biomarkers to assess dietary intake or nutritional status in populations in Africa and little is known of the associations between dietary and nutritional factors and diabetes and other metabolic disorders. To address this gap in the literature, in this PhD project, I aimed to examine the associations between a wide range of objectively measured nutritional biomarkers and diabetes in adults in rural and urban settings of Cameroon. The specific aims of this PhD are presented in Figure 1.5.

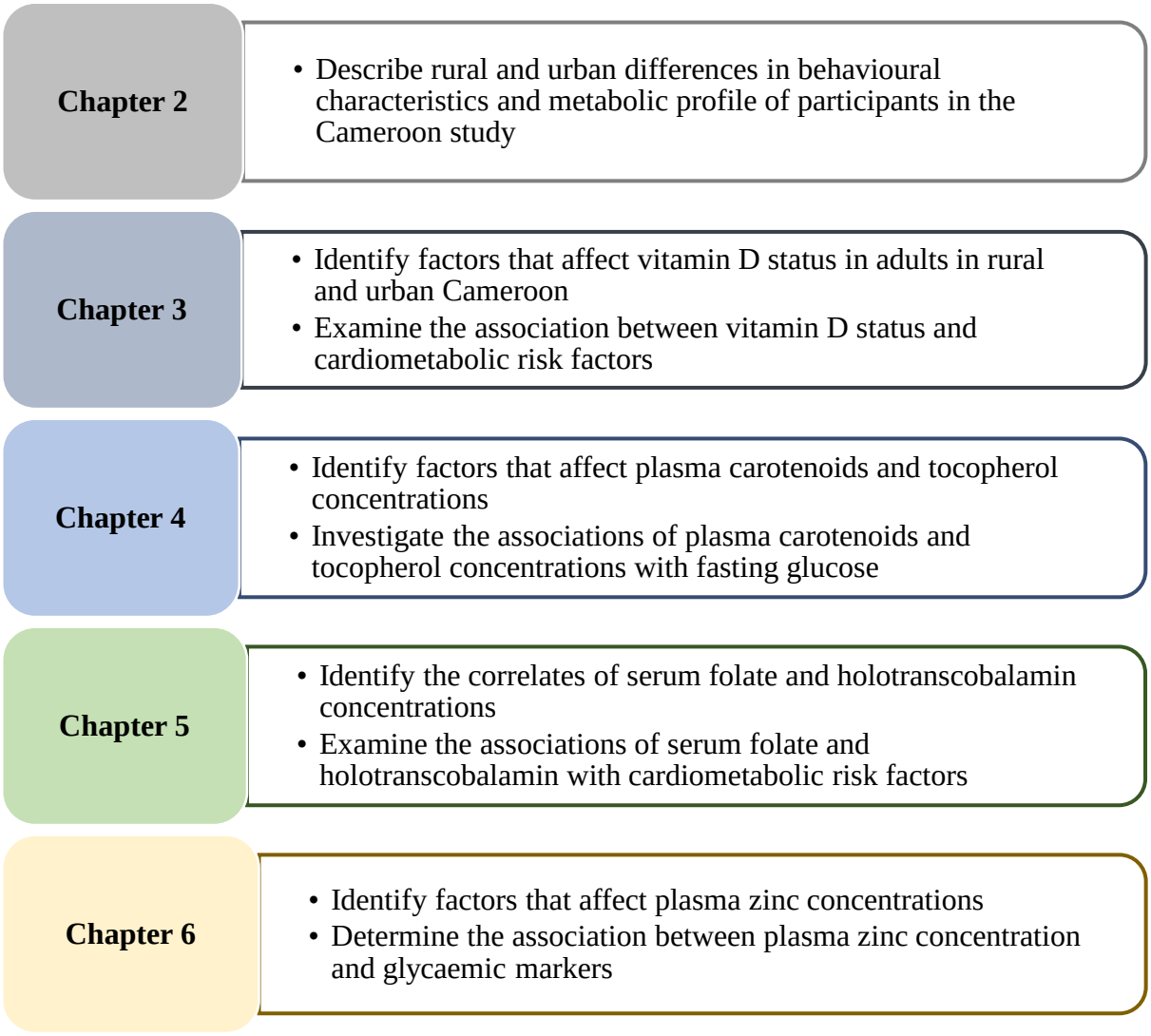

Figure 1.5 : PhD aims according to the different chapters

Chapter 2 : Overview of the Cameroon study and characteristics of participants

Summary

Aim: I aimed to describe the common methods used in the Cameroon study and present the characteristics of the participants.

Methods: The Cameroon study is a population-based cross-sectional study of 596 adults aged 25-55 years without a history of diabetes or CVD. Participants were recruited from two (high plateau and bimodal forest) of the five ecologic zones in Cameroon. In each ecologic zone, a rural and urban setting was selected. Data on socio-demographic characteristics and health-related behaviours including intake of fruits and vegetables were collected using an adapted version of the WHO STEPS instrument. Free-living physical activity energy expenditure (PAEE) was objectively measured using individually-calibrated heart rate and movement sensing. Metabolic markers including fasting glucose and insulin and 2-h post-load glucose levels were measured.

Results: Of the 596 adults who accepted to take part in this study, 275 participants lived in rural areas and 321 in urban areas. The mean ± SD age of participants was 38.3 ± 8.6 years (63.5% were women). 33.2 % of participants living in urban areas were obese compared with 11.4% of participants in rural areas (p-value < 0.001). Obesity was five times more prevalent in women than men. The means of systolic and diastolic blood pressure were higher in urban residents (126.2 ± 22.4 mmHg and 79±14.0 mmHg respectively), than in rural residents (118.3 ± 17.6 mmHg and 73.5 ± 12.0 mmHg respectively), p-value for both comparisons < 0.001. Participants living in rural areas reported a higher frequency of intake of fruits (3(1-6) times/week) and vegetables (5(2-9) times/week) than urban residents (fruits: 2(1-4) times/week, vegetables: 4(2-6) times/week), p-value for both comparisons< 0.01. Women reported a higher frequency of intake of fruits (3(1-6) times/week) and vegetables (4(1-8) times/week) than men (fruit: 2(1-4) times/week and vegetables: 3(2-6) times/week), p-value < 0.001 for both comparisons. Men and rural residents accumulated higher levels of PAEE than their counterparts did (men: 58.2 ± 25.1 KJ/Kg/day vs women: 45.89 ± 20.6 KJ/Kg/day; rural residents: 59.3 ± 23.5 KJ/Kg/day vs urban residents: 43.2 ± 20.2 KJ/Kg/day, both p-value < 0.001). Glycaemic markers and triglycerides concentrations did not differ between rural and urban residents or between men and women.

Conclusion: In this population-based study including participants from rural and urban settings of Cameroon, the distribution of some health-related behavioural characteristics and cardiometabolic risk factors varied markedly by sex and rural/urban area of residence.

2.1 Common methods

2.1.1 Study design and site

Study design

The Cameroon study is a population-based cross-sectional study including participants recruited from rural and urban areas of Cameroon. Data was collected between October 2005 and December 2006. I was not involved in the design and data collection of the Cameroon study. The initial aims of the study were to describe physical activity levels and identify the sociodemographic correlates of physical activity and examine the associations of physical activity with metabolic health in rural and urban Cameroon.

Geographic scope

Cameroon is a country located in central Africa and lies between longitude 8° and 16° east and latitude 2° and 13° north. It is bordered by Nigeria in the west, the Central African Republic in the east, Chad in the north-east, Equatorial Guinea, Gabon and Congo-Brazzaville in the south. Cameroon covers a surface area of 475,442 km². It is made of 10 administrative regions and five ecologic zones (Figure 2.1).

Cameroon is sometimes referred to as Africa in miniature because of its geographic, climatic and cultural diversity that characterises the region. Cameroon is home to about 250 ethnic groups speaking about 260 local languages. Cameroon is a bilingual country with English and French as the official languages. The estimated population of Cameroon in 2022 is 27.57 million, with an annual population growth of 2.75% (52). Currently 58.7% of the population live in urban areas with an annual urbanisation rate of 3.43%. Over 3/4 of the population is under 24 years, and only 3.11% is ≥ 65 years (Figure 2.2). The sex ratio is 0.99 male/female (53).

Data for this PhD project was collected in two of the five ecologic zones of Cameroon (high plateau and bimodal forest zones) (Figure 2.3 and 2.4). In each zone, a rural and an urban site were selected.

High plateau zone:

- Rural site: Bafut health district, North West region
- Urban site: Nkwen health district, Bamenda, North West region

Bimodal forest zone:

- Rural: Mbankomo health district, Centre region
- Urban site: Biyem-Assi health district, Yaoundé, Centre region

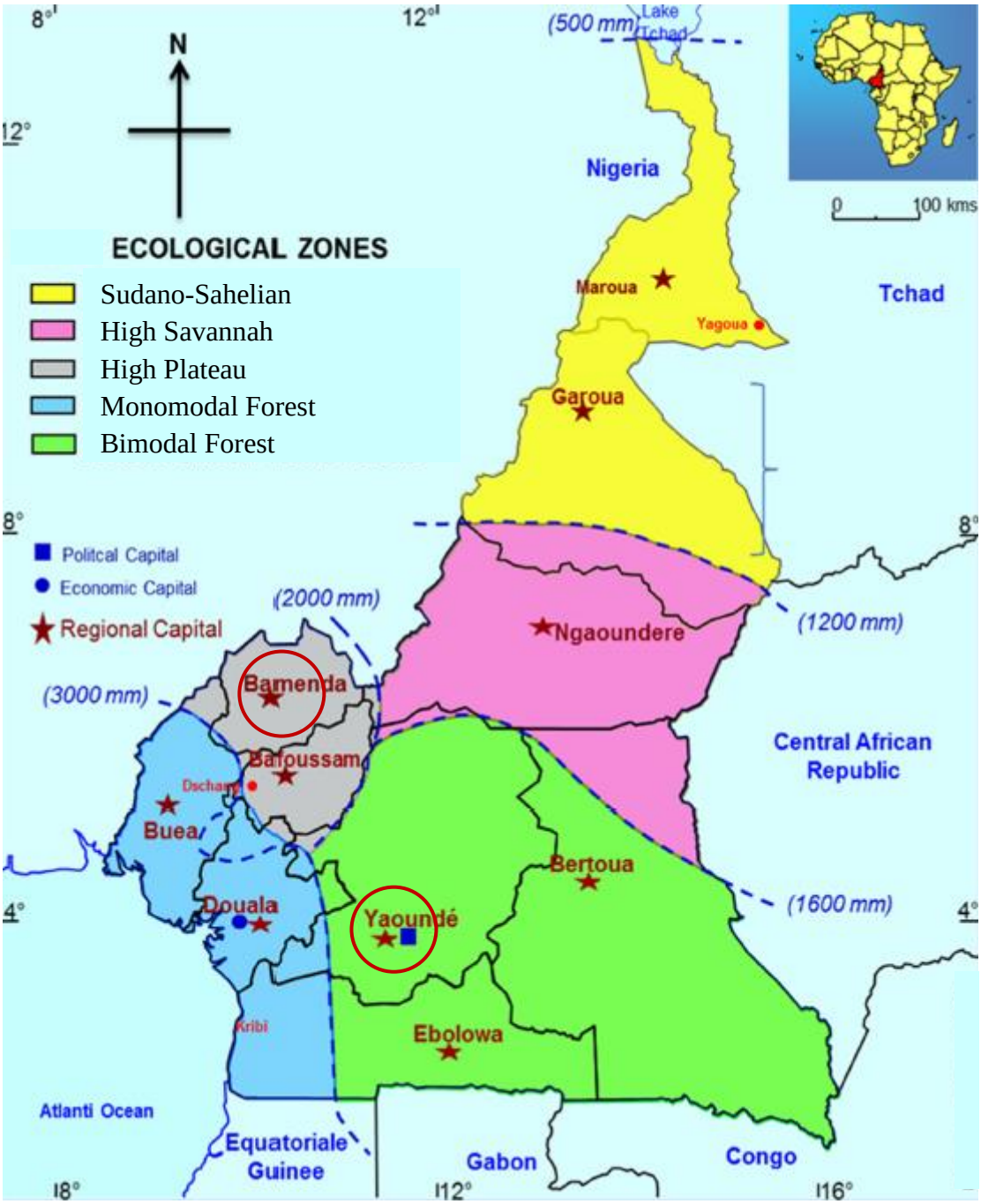

Figure 2.1: Map of Cameroon showing the five ecologic zones (adapted from IRAD report 2007)

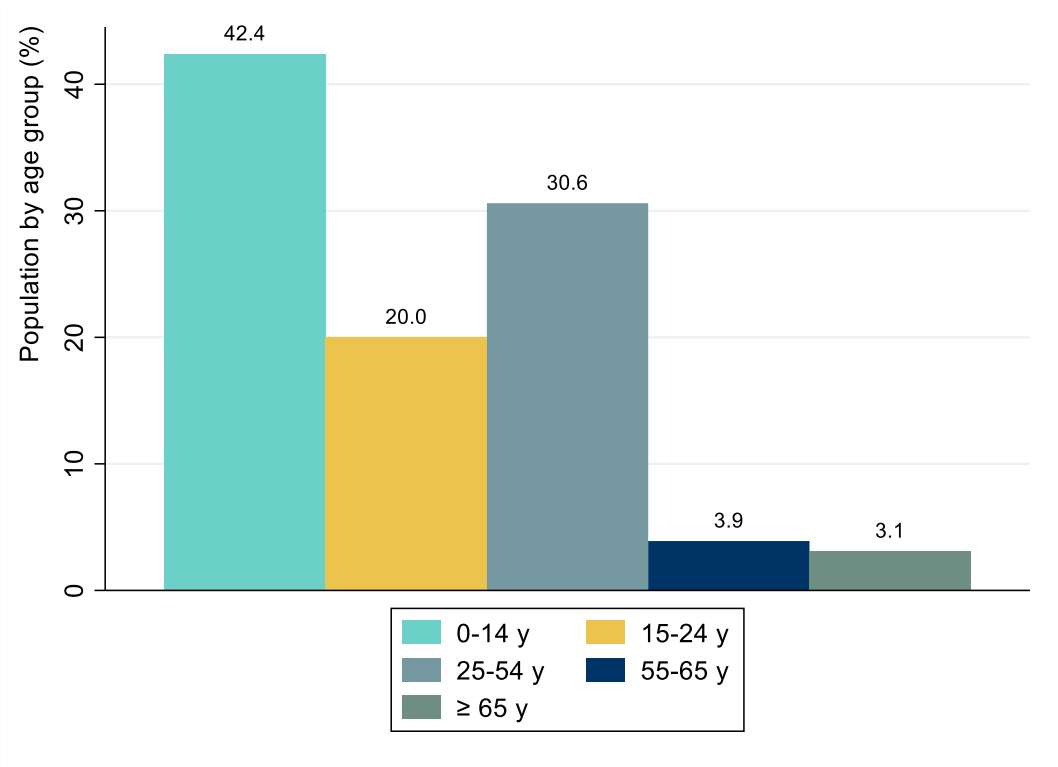

Figure 2.2 : Age structure of the population of Cameroon in 2022 (53)

Rural: Bafut

Urban: Nkwen

Figure 2.3 : Health districts in the North West region, Cameroon, (Source: DHIS, Ministry of Public health, Cameroon)

Figure 2.4 : Health districts of Yaoundé, Centre region (Source: DHIS, Ministry of Public health, Cameroon)

The urban sites for the data collection were Biyem-Assi and Nkwen located in Yaoundé and Bamenda respectively, which are 2 of the largest cities in Cameroon. Yaoundé is the capital of the Centre region and the administrative capital of Cameroon with a population currently estimated at 3.5 million. Bamenda is the capital of the North West region and has ~ 573,000 inhabitants (53). A high proportion of inhabitants in the urban areas (Yaoundé and Bamenda) are employed in the civil service and salaried occupations or are businesspersons. The rural sites were Bafut in the North West region and Mbankomo in the Centre region. Mbankomo is located ~ 20 km from Yaoundé, covers a surface area of about 3500 km², and currently has an estimated population of 12,325. Bafut is situated 20 km from Bamenda, and stretches on a surface of roughly 340 km², with slightly over 100,000 inhabitants. At the time of data collection in 2005-2006, most of the inhabitants of Mbankomo and Bafut (rural areas) made a living from farming.

2.1.2 Study population and sampling

Inclusion criteria:

- Adults aged 25 to 55 years

Exclusion criteria:

- History of diabetes or CVD

Sampling

Due to the absence of complete population registers, a study-specific sampling frame was established which consisted in the enumeration of all eligible adults in the households in delimited areas of the study site. A total of 3854 eligible participants were registered in the delimited areas (urban: n = 1616, mean age = 35.4 ± 8.3 years, rural: n= 2238, mean age= 35.3 ± 7.7 years). Because of the absence of fixed house addresses or telephone lines, it was difficult to retrace the participants and so the study-established sampling frame could not be used to conduct a random sampling. Instead, door-to-door recruitment of volunteers from the sampling frame was undertaken. Volunteers were provided with verbal and written information about the study and invited to the testing facility in their local hospital specifically set up for the study. Recruitment of volunteers and data collection were carried out by 4 fieldworkers in teams of 2. The teams alternated every week between Bamenda and Bafut or Yaoundé and Mbankomo.

2.1.3 Ethical approval

Ethical approval for this study was obtained from the Cameroon National Ethics Committee, Committee (Ethical approval number Approval: FWA IRB00001954; approval date: 26th December 2005) and all participants provided written informed consent.

Volunteers who had any condition that was a contra-indication to the exercise testing and pregnant women were excluded from the study. A questionnaire adapted from the WHO Rose angina questionnaire (54) was used to screen for pre-existing CVD that would not allow participants to take part in the exercise. In total, 651 eligible participants (rural: n = 303, mean age 38.5 ± 8.3 years; urban: n = 348, mean age 37.9 ± 9.1 years) agreed to take part in this study. Of these, blood samples for measurement of the nutritional biomarkers were available for 596 participants and constituted the analytical sample for this PhD project (Figure 2.5).

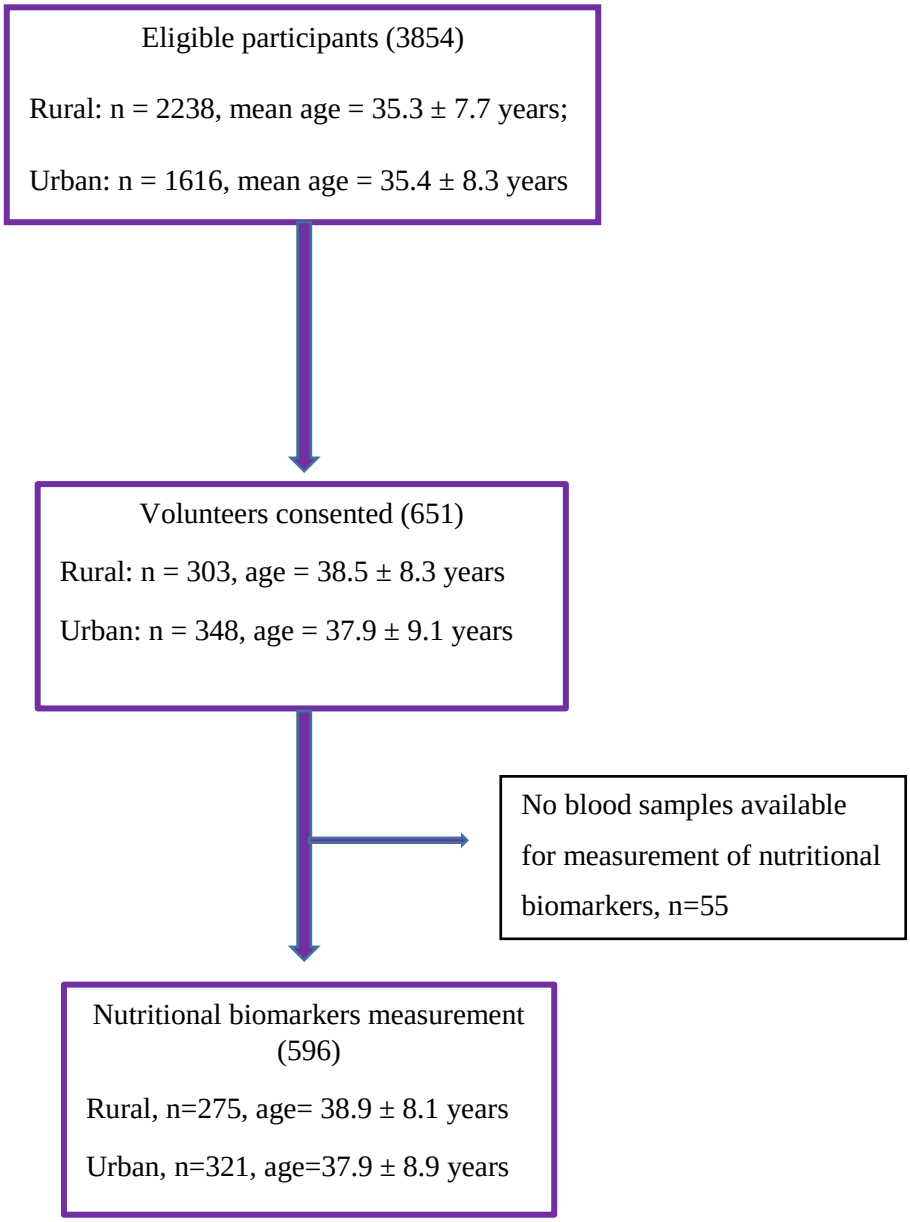

Figure 2.5 : Flowchart of the inclusion process of participants in the Cameroon study

2.1.4 Data collection

Socio-demographic and anthropometric measurements

Data were collected over 15 months between 2005-2006. Using an adapted version of the WHO STEPwise approach to Surveillance (STEPS) questionnaire (55), trained interviewers collected self-reported data on socio-demographic (age, sex, level of education, residential site) and behavioural characteristics (alcohol intake, smoking, physical activity, fruit and vegetable intake). Based on responses to the questions "have you ever smoked any tobacco product/consumed a drink that contains alcohol?" and "do you currently smoke any tobacco product/did you consume a drink that contains alcohol within the past 12 months?" smoking status and alcohol intake were categorised as never, past or current. Participants were asked four questions relating to the frequency of fruit and vegetable intake: i) the number of days in a typical week when they eat fruit or ii) vegetables and iii) the number of times in a typical week they eat fruits or iv) vegetables. Using the date of blood draw, I derived the season of data collection as long dry (December-March), light rain (April-May), short dry (June-July), and heavy rain (August-November).

Waist circumference was measured to the nearest 0.1 cm using a non-stretch fiberglass tape at the level of the midpoint between the lower costal margin and the anterior superior iliac crests of participants wearing light clothing. Central obesity was defined as waist circumference ≥ 80 cm in women or ≥ 94 cm in men. Bodyweight and composition were measured using electronic scales and bio-impedance (Tanita TBF-531 scales; Tanita UK, Uxbridge, Middlesex, U.K.), respectively. Height was measured in individuals without shoes and belts using a standard rigid stadiometer and body mass index (BMI in kg/m^2) computed as the body weight (Kg) divided by the square of height (m^2). Where relevant the following cut-offs were used to categorise BMI: less than 18.5, underweight; 18.5-24.9 Kg/m^2, normal weight; 25-29.9 Kg/m^2, overweight and ≥ 30 Kg/m^2, obese. Blood pressure was measured on the dominant arm of the participants after at least 5 minutes of rest using an automated blood pressure measuring device (OMRON M4-I). Three measurements of blood pressure were taken at 1-minute intervals and the blood pressure value was computed as the average of the three recordings.

Both self-reported and objectively measured physical activity data were collected from all the participants. Self-reported activities at work, recreational activities and travel were recorded using the global physical activity questionnaire (GPAQ) and estimates of energy expenditure in each domain in metabolic equivalents of task (METs)-min/week and physical activity energy

expenditure (GPAQ PAEE) derived (56). Objectively measured physical activity energy expenditure (PAEE) was measured using a combined heart rate and movement sensor (Actiheart; Cambridge Neurotechnology, Cambridge, U.K.) over seven continuous days. Participants were advised not to remove the monitors except for activities such as swimming or bathing. Individual heart rate responses during a step test were used for the individual calibration of heart rate data. This method has been previously validated against the criterion of doubly labelled water in this population *(r*=0.40) (57). PAEE was scaled for body weight and expressed as KJ/Kg/day. Categories were created based on time spent in minutes per day at different intensities of physical activity: < 1.5 METs, sedentary behaviour; 1.5–3 METs, light physical activity (LPA); > 3 METs, moderate to vigorous physical activity (MVPA). Throughout this thesis, PAEE is used to refer to objectively measured physical activity energy expenditure.

Biochemical measurements

All participants provided blood samples in the morning between 7:30 and 9:30 AM after an overnight fast of at least 8 hours. The fasting blood samples collected were centrifuged at ~ 1400 g (gravitational force) and plasma and serum aliquots were stored at -80°C. The aliquots were transported on dry ice by air to Cambridge, United Kingdom and stored at -80 °C until analysis.

Fasting glucose and 2-h glucose post-ingestion of 75g of glucose dissolved in 250 ml of water were measured on fresh capillary whole blood using a Hemocue B-Glucose Analyzer (HemoCue AB, Ängelholm, Sweden) onsite. Subsequent analyses were done at the National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre (BRC), Core Biochemical Assay Laboratory. Fasting plasma insulin was measured by fluorometric assay on a 1235 AutoDELFIA automatic immunoassay system (kit by Perkin Elmer Life Sciences; Wallac Oy, Turku, Finland). C-reactive protein (CRP), plasma cholesterol and triglycerides were measured using automated assays on the Dade Behring Dimension RxL analyser. CRP was measured using a particle enhanced turbidimetric (PETIA) technique and total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were measured by enzymatic method. Low-density lipoproteins (LDL) cholesterol concentrations were derived by the Friedewald formula (LDL cholesterol = total cholesterol - (triglyceride / 2.2) – HDL cholesterol), when triglyceride levels were < 4.5 mmol/L.

2.1.5 Calculation of homeostatic model assessment for insulin resistance and metabolic syndrome score

The homeostatic model assessment for insulin resistance (HOMA-IR) was used to assess insulin resistance. Estimates of insulin resistance derived from HOMA-IR are strongly correlated with estimates from the hyperinsulinaemic clamp (r=0.88), which is considered the gold standard for assessing insulin sensitivity (58). I calculated HOMA-IR using the formula = ([FPI x FBG]/22.5)), where FPI is fasting plasma insulin (mU/L) and FBG is fasting blood glucose (mmol/L) (58). The metabolic syndrome which is a cluster of cardiometabolic risk factors including central obesity, high blood pressure, blood glucose, and triglycerides and low HDL cholesterol has been reported to highly predict diabetes (59) and CVDs (60). I computed a continuous metabolic syndrome score based on the five risk factors (waist circumference, fasting blood glucose, blood pressure, triglycerides and HDL cholesterol) in the definition of the National Cholesterol Education Program Adult Treatment Program III (NCEP ATP-III). The purpose of using a continuous score instead of a binary definition was to maximise statistical power. Dichotomisation leads to loss of power and secondly underestimates the extent of variation between the groups (61). Moreover, there is increasing evidence supporting the use of a continuous metabolic syndrome score in epidemiological research (62), (63). The metabolic syndrome score was calculated by summing sex-specific standardised continuous values of central obesity (waist circumference), glycaemia (fasting blood glucose), mid blood pressure [(systolic blood pressure + diastolic blood pressure)/2] (64) and blood lipids (triglycerides and HDL cholesterol with the latter coded in an opposite direction to the other factors; i.e. inverted HDL). I standardised each of the five factors by subtracting the sample mean from individual values and dividing it by the standard deviation (SD) of the sample mean. Triglyceride was log-transformed to meet the normality assumption. The five components of the metabolic syndrome were equally weighted in the calculation. A higher score is indicative of a less favourable metabolic syndrome profile.

2.2 Statistical analyses

Statistical analyses were performed using Stata 15 (StataCorp, College Station, TX). All analyses were stratified by rural-urban area of residence. Descriptive statistics are presented as means and standard deviations (SD) for normally distributed continuous variables (or median and [25^{th} - 75^{th} percentile] for non-normally distributed variables) or numbers and percentages for categorical variables. I tested differences between rural and urban dwellers using a t-test for normally distributed continuous variables (or Mann Whitney test for non-normally

distributed variables) and a chi-squared test (or fisher exact test when the number of observation in a cell was < 5) for categorical variables.

2.3 Results

In total, blood samples were available for 596 participants and are included in this study. The characteristics of participants with missing blood samples compared with those included in this study are presented in Table 2.1. The characteristics of participants in the Cameroon study stratified by sex and rural/urban residential site are presented in Tables 2.1 and 2.2. Of the 596 participants with blood samples available for measurement of nutritional biomarkers (275 rural and 321 urban), 63.3% were women. Mean age ± SD was 38.3 ± 8.6 years. There was no difference in age between rural (38.8 ± 8.2 years) and urban residents (37.9 ± 8.9 years). Participants living in rural areas had spent fewer years in education than those in urban areas.

Regarding health-related behaviours, there were rural-urban differences in self-reported intake of fruits and vegetables and physical activity levels. The median (25^{th}-75^{th} percentile) number of times participants self-reported consuming fruits in a typical week was 2(1-5) times/week and the comparable figure for vegetable intake was 4(2-7) times/week. Participants living in rural areas reported a higher frequency of intake of fruits (3(1-6) times/week) than urban participants (2(1-4) times/week), p-value=0.008. The frequency of self-reported vegetable intake was higher in rural participants (5(2-9) times/week) than in urban participants 4(2-6) times/week), p-value < 0.0001. Women reported a higher frequency of consumption of fruit (3(1-6) times/week) and vegetables (4(1-8) times/week) than men (fruit: 2(1-4) times/week and vegetables: 3(2-6) times/week), p-value < 0.001 for both comparisons. Mean ± SD PAEE was higher in rural residents (59.3±23.5 KJ/Kg/day) than in urban residents (43.2 ± 20.2 KJ/Kg/day), p-value < 0.0001. This is depicted in the rural to urban left shift in the distribution of PAEE (Figure 2.6). Men accumulated a higher mean ± SD of PAEE (58.2 ± 25.12 KJ/Kg/day) than women (45.89 ± 20.6 KJ/Kg/day), p-value < 0.001. There was no difference in alcohol intake and smoking status between rural and urban residents.

The means of individual cardiometabolic risk factors including waist circumference, BMI, blood pressure and HDL cholesterol were higher in urban residents compared to rural residents (Table 2.2). The proportion of participants with obesity (BMI ≥ 30 Kg/m^2) was 23.3% in the overall sample, higher in those living in urban areas (33.2%) compared with those living in rural areas (11.4%), p-value < 0.001. Figure 2.7 shows the rural-to-urban right shift in the distribution of BMI. There were more women with obesity than men (32.5% in women vs 6.5%

in men), (p-value < 0.001). Mean ± SD systolic blood pressure and diastolic blood pressure were 118.3 ± 17.6 mmHg and 73.5 ± 12 mmHg respectively for rural residents compared with 126.2 ± 22.4 mmHg and 79 ± 14.0 for urban residents (p-value for both comparisons < 0.001). Mean systolic blood pressure was higher in men 125.8 ± 20.1 mmHg, compared with women 120.8 ± 20.9 mmHg (p-value = 0.005). There was no difference in diastolic blood pressure between men and women. The prevalence of hypertension in the overall sample was 17.2%, higher in urban residents (23.3) than in rural residents (9.9%). Glycaemic markers and triglycerides concentrations did not differ between men and women or rural and urban residents. As shown in Figure 2.8 there was a rural-to-urban right shift in the distribution of the continuous metabolic syndrome score.

Table 2.1: Characteristics of participants with and without missing blood samples for measurement of nutritional biomarkers

Characteristics	Excluded (n=55)	Included (n=596)	p-value
Age	36.9 ± 9.5	38.3 ± 8.6	0.26
Sex			
Women n(%)	35(63.6)	377(63.3)	0.54
Education (years)	10.1 ± 5.7	10.5 ± 5.2	0.54
Smoking status n (%)			
Never	43(78.2)	464(77.9)	
Former	11(20.0)	79(13.3)	0.08
Current	1(1.82)	53(8.9)	
Alcohol intake n (%)			
Never	11(20.0)	65(10.9)	
Former	8(14.5)	58(9.7)	0.06
Current	36(65.4)	473(79.4)	
PAEE (KJ/Kg/day)	46.6 ± 22.2	50.5 ± 23.2	0.06
BMI (Kg/m2)	25.1 ± 5.3	26.1 ± 5.2	0.19
Systolic blood pressure (mmHg)	118.7 ± 15.6	122 ± 20.8	0.17
Diastolic blood pressure (mmHg)	73.5 ± 12.2	76.4 ± 13.4	0.12
Fasting glucose (mmol/L)	4.95 ± 1.0	4.78 ± 1.34	0.34
2-h blood glucose (mmol/L)	6.06 ± 1.35	6.29 ±1.89	0.37
HDL cholesterol (mmol/L)	1.03 ±0.23	1.22 ±0.33	0.13
Metabolic syndrome score	-0.28 ± 1.87	-0.18 ± 1.64	0.41

Results are presented as arithmetic mean or n (%). p-values are from a t-test for continuous variables and from a chi squared test for categorical variables (or Fisher exact test if the number of observations was less than 5 in a cell).

Excluded: participants with missing blood samples for measurement of nutritional biomarkers

Included: Participants with blood samples for measurement of nutritional biomarkers

BMI, body mass index; PAEE; objectively measured physical activity energy expenditure, HDL, high density lipoprotein.

Table 2.2 : Socio-demographic and health-related behavioural characteristics of the study population stratified by sex and urban/rural residence (Cameroon study: n=596)

Characteristics	Women (n=377)			Men (n=219)			P-value for sex-site interaction
	Rural (n=172)	Urban (n=205)	p-value	Rural (n=103)	Urban (n=116)	p-value	
Age (years)	39.8±8.1	38.6±8.8	0.168	37.3±8.3	36.3±8.9	0.368	0.88
Education (years)	8.0±4.1	11.8±5.2	<0.001	9.0±4.5	13.2±5.2	<0.001	0.45
Education level, n (%)							
< Primary education	51(29.7)	24(11.7)		23(22.6)	5(4.3)		
Primary education	92(53.5)	79(38.5)		59(57.8)	34(29.3)		
Secondary school	25(14.5)	68(33.2)	< 0.001	16(15.7)	41(35.4)	< 0.001	0.224
University	4(2.3)	34(16.6)		4(3.9)	36(31.0)		
Alcohol intake, n (%)							
Never	24(14.0)	24(11.7)		11(10.7)	06(5.2)		
Past	16(9.3)	29(14.2)	0.485	03(2.9)	10(8.6)	0.104	0.34
Current	132(76.7)	152(74.1)		89(86.4)	100(86.2)		
Smoking status, n (%):							
Never	161(93.6)	188(91.7)		60(58.2)	55(47.4)		
Past smoker	10(5.8)	16(7.8)	0.820	21(20.4)	32(27.6)	0.357	0.748
Current smoker	1(0.6)	1(0.5)		22(21.4)	29(25.0)		
Season, n(%)							
Long dry	60(34.9)	70(34.1)		44(42.7)	39(33.6)		
Short rainy	17(9.9)	26(12.7)	0.08	11(10.7)	19(16.4)	0.32	0.65
Short dry	67(38.9)	90(43.9)		37(35.9)	50(43.1)		
Heavy rain	28(16.3)	19(9.3)		11(10.7)	8(6.9)		
Fruit (times/week)	3(2-6)	2(1-5)	0.06	2(1-6)	2(1-3)	0.03	0.94
Vegetable (times/week)	6(3-9)	4(2-6)	<0.0001	4(2-6)	3(2-6)	0.153	0.031
Fruit and vegetable intake, n(%)							
< 3 times/ week	7(4.3)	15(8.2)		15(15)	16(14.8)		
3-6 times/ week	41(25.2)	74(40.2)	0.001	30(30)	53(49.1)	0.01	0.035
≥ 7 times per week	115(70.6)	95(51.6)		55(55)	39(36.1)		
PAEE (KJ/Kg/day)	54.3±20.9	38.3±16.6	<0.0001	65.9±26.2	52.1±22.6	0.0001	0.452
Sedentary time (min/day)	925.1±143.7	1016.5±137.3	<0.0001	878.1±154.1	961.0±150.9	0.0003	0.56
LPA time (min/day)	379.0±95.8	333.1±104.1	0.0001	415.7±97.9	366.5±112.4	0.002	0.996
MVPA time (min/day)	116.2(69.6-201.6)	75.3(47.5-113.2)	<0.0001	133.1(62.5-202.1)	94.4(54.6-149.9)	0.025	0.34
GPAQ PAEE (KJ/Kg/day)	82.5(6.8-178.3)	8.1(3.4-53.8)	<0.0001	35.9(3.2-140.1)	18.4(3.5-69.8)	0.22	0.009
GPAQ work (MET-min/week)	7280(0-16320)	0(0-4800)	<0.0001	2120(0-12600)	0(0-5760)	0.032	0.013
GPAQ leisure (MET-min/week)	0(0-0)	0(0-0)		0(0-0)	0(0-0)		0.65
GPAQ travel (MET-min/week)	1680(560-3360)	720(300-1680)	<0.0001	840(390-4920	840(280-2520)	0.328	0.067

Results are presented as arithmetic mean [or median (25^{th}-75^{th} percentile) for non-normally distributed variables] or n (%). p-values are from a t-test for normally distributed continuous variables (or Mann Whitney test for non-normally distributed variables) and from a chi squared test for categorical variables (or Fisher exact test if the number of observations was less than 5 in a cell). PAEE, physical activity energy expenditure; LPA, light physical activity; MVPA, moderate to vigorous physical activity

Table 2.3 : Metabolic characteristics of the study population stratified by sex and urban/rural residence (Cameroon study: n=596)

Characteristics	Women (n=377)			Men (n=219)			P-value for sex-site interaction
	Rural (n=172)	Urban (n=205)	p-value	Rural (n=103)	Urban (n=116)	p-value	
BMI (kg/m^2)	24.9±4.9	29.2±5.5	<0.0001	23.09±3.23	25.3±4.0	<0.0001	0.006
BMI (kg/m^2), n(%)							
<25	104(60.5)	52(25.4)		78(75.7)	68(58.6)		
25-29.9	40(23.3)	57(27.8)	<0.001	22(21.4)	36(31.0)	0.023	0.055
≥ 30	28(16.3)	96(46.8)		03(2.9)	12(10.4)		
Waist circumference (cm)	85.2±11.5	94.3±12.9	<0.0001	82.9±7.9	89.2±11.3	<0.0001	0.148
Central obesity, n(%)	114 (65.9)	168(84.4)	<0.0001	9(9.0)	31(27.2)	0.001	0.558
Waist to hip ratio	0.84±0.08	0.85±0.07	0.88	0.86±0.05	0.87±0.06	0.11	0.229
Body fat (%)	30.2±8.3	37.4±7.6	<0.0001	15.8±5.5	20.1±7.0	<0.0001	0.011
Systolic blood pressure (mmHg)	117.1±18.8	123.8±21.9	0.002	120.5±15.1	130.4±22.9	0.0003	0.455
Diastolic Blood Pressure (mmHg)	74.2±12.0	79.6±13.7	<0.0001	72.3±11.9	78.0±14.6	0.002	0.968
Hypertension n(%)	19(11.0)	45(22.6)	0.003	10(10.0)	27(23.7)	0.008	0.01
Fasting blood glucose (mmol/L)	4.81±1.39	4.86±1.08	0.682	4.72±1.49	4.63±1.55	0.676	0.498
2-hour blood glucose (mmol/L)	6.23±1.74	6.61±1.93	0.053	5.94±1.55	6.02±1.88	0.737	0.281
Fasting insulin (pmol/L)	22.3(14.2-39.1)	24.7(13.8-37.7)	0.747	14.8(6.5-29.3)	17.2(8.1-28.9)	0.514	0.415
HOMA-IR	0.76(0.46-1.44)	0.87(0.48-1.37)	0.48	0.48(0.20-0.87)	0.59(0.23-1.05)	0.46	0.133
Total cholesterol (mmol/L)	3.84±0.95	4.02±0.98	0.08	3.60±0.90	3.80±0.90	0.318	0.794
LDL cholesterol (mmol/L)	2.26±0.83	2.36±0.85	0.23	2.07±0.79	2.15±0.85	0.363	0.832
HDL cholesterol (mmol/L)	1.18±0.33	1.27±0.32	0.017	1.19±0.35	1.23±0.31	0.362	0.469
Triglycerides (mmol/L)	0.78(0.63-1.01)	0.71(0.57-0.90)	0.026	0.70(0.58-0.94)	0.73(0.56-0.99)	0.876	0.283
CRP (mg/L)	5.26(2.61-8.41)	4.58(2.63-8.31)	0.61	5.69(2.75-10.16)	4.17(2.05-8.24)	0.048	0.532
Metabolic syndrome score	-0.32±2.25	0.35±2.67	0.01	-0.56±2.30	0.37±2.82	0.01	0.688

Results are presented as arithmetic mean [or median (25th-75th percentile) for non-normally distributed variables] or n (%). p-values are from a t-test for normally distributed continuous variables (or Mann Whitney test for non-normally distributed variables) and from a chi squared test for categorical variables. BMI, body mass index; CRP, C-reactive protein. Central obesity was defined as waist circumference ≥ 94 cm in men and 80 cm in women. Hypertension was defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg.

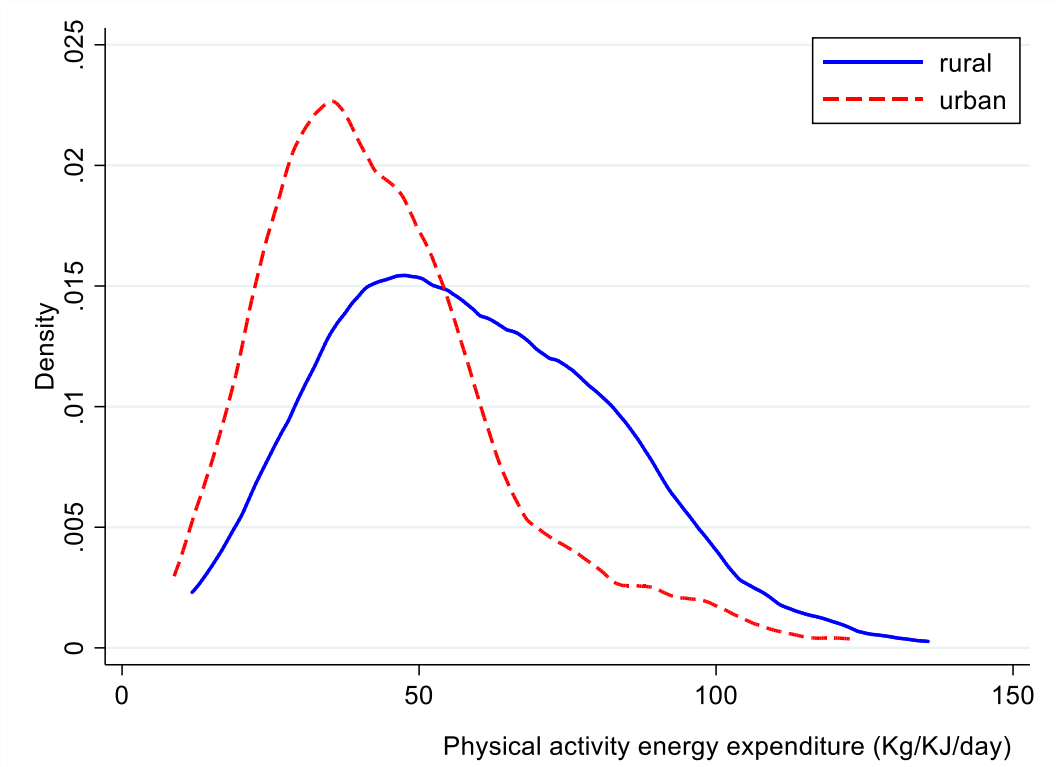

Figure 2.6 : Distribution of physical activity energy expenditure in adults in rural and urban Cameroon

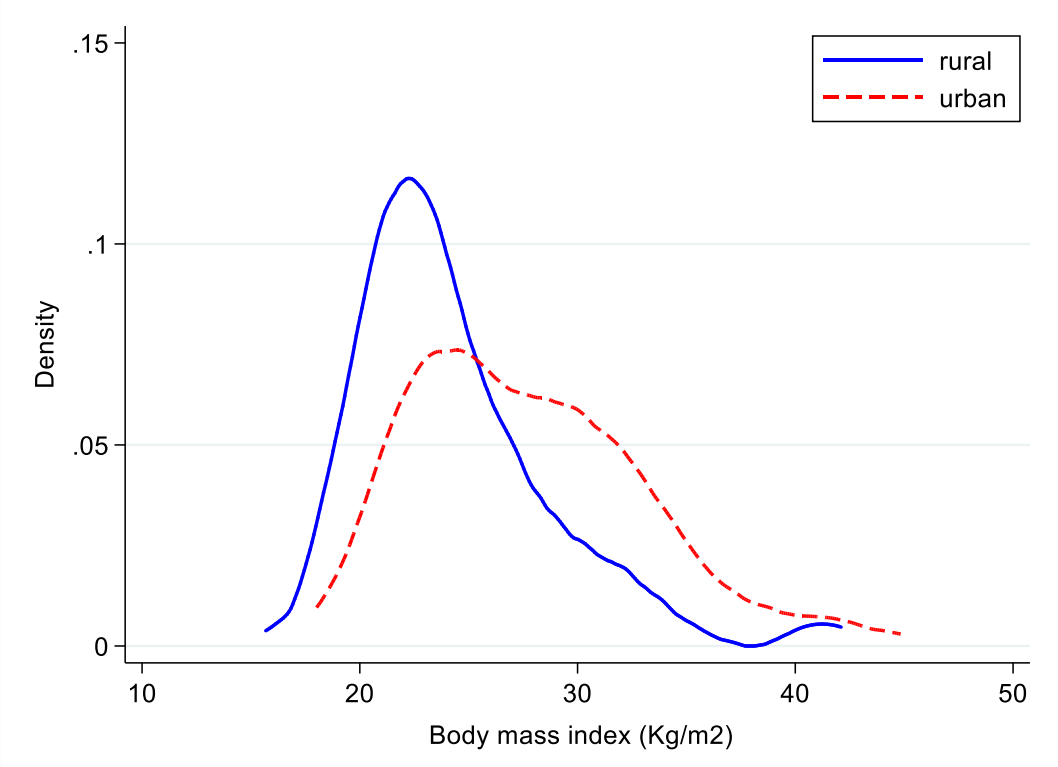

Figure 2.7 : Distribution of body mass index in adults in rural and urban Cameroon

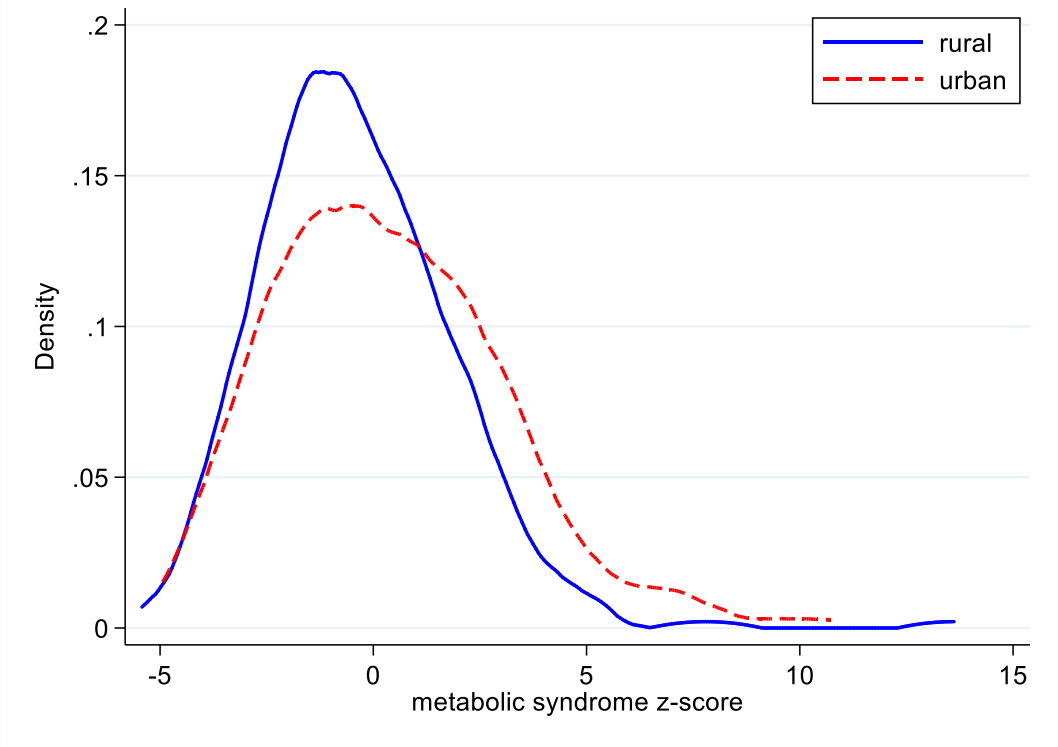

Figure 2.8 : Distribution of metabolic syndrome score in adults in rural and urban Cameroon

2.4 Discussion

In this chapter, I presented the descriptive characteristics of participants in the Cameroon study, which is a population-based study including participants from both rural and urban areas. Participants living in the rural areas reported a higher frequency of intake of fruits and vegetables, were more physically active and had lower measures of adiposity, blood pressure and metabolic syndrome score than those living in urban areas. Overall, these results indicate that participants living in rural areas had a better metabolic profile than those living in urban areas. The rural-urban difference observed in the metabolic profile may partly be attributed to the lower physical activity levels and unhealthy diets in urban settings compared with rural settings.

In this study, the distribution of measures of adiposity, blood pressure and metabolic syndrome score varied by rural-urban area of residence. Consistent with previous studies in SSA, I observed that the mean blood pressure and metabolic syndrome score was higher amongst urban residents than rural residents (21), (65), (66). The proportion of participants with obesity living in urban areas was over three folds higher compared with those living in the rural areas, which is consistent with previous large surveys in Cameroon (67) (68), (69). The higher prevalence of obesity in urban areas compared with rural areas may be attributed in part to urbanisation which has resulted in changes in the food environment and physical activity behaviours in many SSA countries (70), (71). In a previous study in Cameroon, participants reported perceiving obesity as a sign of affluence (39). The prevalence of obesity has risen in both rural and urban Cameroon over the past 2 decades. Data from the Cameroon Demographic Health Survey shows that the proportion of women with obesity aged 15-49 years has doubled between 2004 and 2018 in both rural and urban settings of Cameroon. The increase in the prevalence of obesity in the rural settings of Cameroon may be attributed to the mechanisation of agriculture, which was traditionally manual.

Differences in dietary habits may contribute to the differences in the metabolic profile between rural and urban residents. Compared with urban residents, rural residents reported a higher frequency of intake of fruits and vegetables. The WHO recommends increasing intake of fruits and vegetables for the prevention of NCDs (72). Higher intake of fruits and vegetables can be used as a proxy for healthy eating (73). Reliance on locally sourced foods in many rural communities is associated with substantial health and economic benefits and supports sustainability within the environment (74). In urban settings, fresh foods are gradually replaced

in the markets by processed foods and fast foods, coupled with long work hours which may limit the time to cook healthy meals at home (71).

Similar to the differences in dietary habits between rural and urban residents, results from this study show that participants from rural areas accumulate higher levels of PAEE than those living in urban areas. This may be due to differences in occupational activities and travel time physical activities between rural and urban residents as shown in this study. In rural areas where a high proportion of the population has a low socioeconomic status, reliance on walking for travel, due to poor infrastructure, lack of access to private vehicles or costs constraints with using taxis or motorcycles. Many people in the rural areas also engage in full-time farming, which is mostly manual as a means of livelihood. In contrast, in urban areas, a high proportion of the urban population have office or service jobs and passive travelling with the use of cars and buses is more common. The rural-urban differences in physical activity levels in this sample and the association with metabolic health have previously been reported (28), (27).

Although the Cameroon study is a population-based study including participants from both rural and urban areas, generalisation of the findings from this study to the target population should be made with caution. This study was conducted in adults aged 25-55 years without known diabetes or CVD and our findings may not be generalisable outside of this age range and beyond the geographical location in which the study was conducted. A simple random sampling could not be conducted due to the absence of complete population registers or fixed house addresses and telephone numbers. The use of a study-established sampling frame may have introduced selection bias and may limit our ability to generalise these findings to our target population if the sample was systematically different from the target population. However, the characteristics of participants in this study such as level of education, BMI and blood pressure were similar to those from previous large population-based surveys in Cameroon (75), (68), (76). For instance, in this study, 45.2% of women and 42.7% of men had completed only primary education, which is comparable to the results from the 2004 Cameroon Demographic Health Survey, where 43.1% of women and 38.6% of men had completed only primary education (75). In a previous survey of ~ 10,000 participants from urban Cameroon, the mean± SD BMI in men was 23.4 ± 2.9 Kg/m^2 and women 28.9 ± 8 Kg/m^2, which is similar to the mean of 24.3 ± 3.8 Kg/m^2 in men and 27.2 ± 5.6 Kg/m^2 in women in this sample (68). Similarly, the prevalence of hypertension in the survey was 24.6% which is comparable to the prevalence of 23% among urban dwellers in this sample (76).

2.5 Conclusion

In this chapter, I have described the characteristics of participants in the Cameroon study; a population-based cross-sectional study in rural and urban settings of Cameroon. Results from this study show that the burden of obesity and other cardiometabolic risk factors is high in this population and that there are striking differences in the health-related behavioural characteristics and metabolic profile between participants living in rural and urban areas of Cameroon.

Chapter 3 : Vitamin D status and cardio-metabolic risk factors

Publication

Mba CM, Koulman A, Forouhi NG, Sharp SJ, Imamura F, Jones K, Meadows SR, Assah F, Mbanya JC, Wareham NJ. Associations between circulating 25-hydroxy vitamin D and cardiometabolic risk factors in rural and urban Cameroon. (Manuscript in revision at Nutrition & Diabetes)

Summary

Background: An inverse association between vitamin D status and cardiometabolic risk has been reported but this relationship may have been affected by residual confounding from adiposity and physical activity due to imprecise measures of these variables. I aimed to investigate the relationship between serum 25-hydroxyvitamin D (25(OH)D) and cardiometabolic risk factors in adults in rural and urban settings, with adjustment for objectively-measured physical activity and adiposity.

Methods: In 586 adults in Cameroon (63.5% women), serum 25(OH)D concentrations were measured by liquid chromatography-tandem mass spectrometry. Markers of glucose homeostasis (fasting blood glucose (BG), 2-h post glucose load BG and HOMA-IR) were assessed and a continuous metabolic syndrome score was computed by summing sex-specific standardised scores of waist circumference, blood pressure, fasting glycaemia, triglycerides and HDL cholesterol with the latter included in the score in an opposite direction to the other factors.

Results: Mean ± SD age was 38.3 ± 8.6 years, and serum 25(OH)D concentration was 51.7 ± 12.5 nmol/L. Mean 25(OH)D was higher in rural (53.4 ± 12.8 nmol/L) than urban residents (50.2 ± 12.1 nmol/L), p-value = 0.002. The prevalence of vitamin D insufficiency (< 50 nmol/L) was 45.7%. There was an inverse association between serum 25(OH)D and the metabolic syndrome score in unadjusted analysis (β= -0.30 (95% CI -0.55 to -0.05)); per 1 SD (12.5 nmol/L) of serum 25(OH)D), which became non-significant after adjusting for age, sex, smoking status, alcohol intake and education level. Serum 25(OH)D concentration was inversely associated with fasting BG (-0.21 (-0.34 to -0.08)), which remained significant after adjustment for age, sex, education, smoking, alcohol intake, season of data collection, BMI and objectively measured physical activity (-0.17 (-0.29 to -0.06)). There was an inverse association of serum 25(OH)D with 2-h BG (-0.20 (-0.34 to -0.05)) and HOMA-IR (-0.12 (-0.19 to -0.04)) in unadjusted analysis, but these associations were attenuated and became non-significant after adjustment for age, sex, smoking status, alcohol intake, education level, residential site, season and BMI.

Conclusion: Vitamin D insufficiency was common in this population. This study showed an inverse association between vitamin D status and fasting glucose that was independent of potential confounders including objectively measured physical activity and adiposity suggesting a possible mechanism through insulin secretion.

3.1 Introduction

The burden of cardiometabolic diseases has risen over the last decades in SSA where the highest age-standardized mortality rates occur and therefore is of growing public health concern (77). Vitamin D is suggested to play a role in the prevention of cardiometabolic diseases, but evidence from observational studies of the relationship between vitamin D status and cardiometabolic diseases is inconsistent (78), (79), (80). A meta-analysis of 38 cross-sectional studies and 5 longitudinal studies showed an inverse association between serum 25-hydroxyvitamin D (25(OH)D) concentration and metabolic syndrome in cross-sectional studies but not in longitudinal studies (81). However, there was evidence of high between-study heterogeneity (I^2 > 90%) and few studies included in the review were population-based. Therefore, the association between vitamin D status and metabolic syndrome remains unclear.

Accumulating evidence from observational studies suggests the association between vitamin D and cardiometabolic outcomes may depend on the prevalence of low vitamin D status in the population studied (82), (83). Although most randomised controlled trials (RCTs) suggest no benefit of vitamin D supplementation on cardiometabolic outcomes, these trials were conducted in participants that are in majority replete for vitamin D (84), (85) (Table 3.1). Thus, the context in which an association between vitamin D and cardiometabolic disease is observed may be critical and outstanding questions remain about the role of vitamin D on cardiometabolic outcomes in populations that are inadequate for vitamin D.

Data suggest that vitamin D insufficiency is a public health issue in many SSA countries. A meta-analysis of data from 23 African countries reported a pooled prevalence of low vitamin D status (< 30 nmol/L) of 18.5% (86). This compares with a global prevalence of 6.7% (25(OH)D < 25 nmol/L) (87) and a prevalence of 13.0% (25(OH)D < 30 nmol/L) in Europe (88). Despite the high burden of vitamin D deficiency in SSA, I found only two studies from South Africa in urban populations only that investigated the relationship between vitamin D status and metabolic syndrome and reported no evidence of an association between vitamin D status and the metabolic syndrome or individual metabolic risk factors (89), (90). I found a few other studies in SSA conducted in patients with type 2 diabetes and reported either lower serum 25(OH)D concentration in patients with type 2 diabetes compared with controls (91) or no difference in vitamin D status between those with type 2 diabetes and those without diabetes (92) (Table 3.2).

The inverse association between vitamin D status and cardiometabolic risk factors reported in previous studies might be the result of residual confounding due to imprecise measurements of confounding factors. Amongst the potential confounders in the association of vitamin D status and cardiometabolic risk factors, physical activity and adiposity are some of the most challenging to measure and are highly susceptible to errors. The clarification of the association between vitamin D status and metabolic syndrome requires objective measures of physical activity and more precise measures of adiposity. Most previous observational studies either did not account for physical activity or recorded only self-reported physical activity, which is subject to measurement error and recall bias, thus creating the potential for residual confounding even after adjustment. Physical activity has been reported to be correlated with serum 25(OH)D concentrations, with stronger associations observed for objectively-measured physical activity than self-report (93).

I conducted this study to address the research gap in the literature on the association between vitamin D status and cardiometabolic risk factors with adjustment for objectively-measured physical activity and adiposity in SSA, a region with a high burden of vitamin D insufficiency and cardiometabolic disease (86), (94).

3.2 Study aims

This study aimed to identify the factors that affect vitamin D status and examine the association between serum 25(OH)D concentration and cardiometabolic risk factors with adjustment for objectively measured physical activity and adiposity in rural and urban Cameroon.

Table 3.1 : Evidence from major RCTs of the association between vitamin D supplementation and type 2 diabetes and CVDs.

Study	Characteristics	Endpoints	Results	Comments
Pittas.2019 (US): Vitamin D Supplementation and Prevention of T2D (D2D) (84)	2423 U.S. adults meeting at least two of 3 glycaemic criteria for prediabetes, randomly assigned to 4000IU vitamin D_3/day Double blinded Follow up of 2.5 years	Primary : diabetes Secondary : response to vitamin D_3 supplementation Fasting BG, 2-h BG, HbA1c	HR for vitamin D compared with placebo =0.88(95%CI 0.75-1.04, p=0.12). Post hoc analyses: Subgroup 25(OH)D<30 nmol/L (n=103) HR 0.38 (0.18, 0.80). Pre-specified subgroup analysis: 0.73(0.57-0.92) in non-obese. Not significant in obese	Patients meeting at least 2 of 3 ADA 2010 criteria for prediabetes for inclusion. High baseline vitamin D levels Mean= 69.1 ± 25.5 nmol/L Individuals with obesity may require higher doses of vitamin D
Manson.2019 (US): Vitamin D and Omega-3 Trial (VITAL trial) (85)	2 X 2 factorial design. 25,871 Healthy men> 50 y or women > 55 y Intervention: 2000IU vitamin D_3 and or 1g omega 3 Follow up= 5.3 y	Primary: Invasive cancer and MACE Secondary: All-cause mortality, deaths from cancer	HR CVD= 0.97(0.85-1.12) p=0.69	Supplementation with vitamin D did not reduce risk of CVD nor cancer Participants in majority replete for vitamin D 5106 black participants were included. Participants in both group were allowed to take supplements at RDA levels.
Niroomand.2019 (Iran) (95)	Adults with prediabetes and 25(OH)D < 75 nmol/L Intervention: 50,000IU of vitamin D_3/week 81 in each group Follow up=6 months	Primary endpoint: HOMA-IR Secondary: fasting glucose, 2-h OGTT	Mean of HOMA-IR was significantly lower in the vitamin D group (2.6 vs. 3.1; p-value = 0.04).	Participants had an adequate vitamin D status ~ 50% lost to follow up in both arms

Angellotti.2018 (US): Vitamin D for established type 2 diabetes (DDM2) (96)	127 patients with type 2 diabetes, (HbA1c ≤7.5%) managed with lifestyle only or lifestyle + metformin. Randomly assigned to 4000IU vitamin D_3/ day or placebo Follow up=48 weeks	Primary : change from baseline to week 24 in insulin secretion rate (ISR), estimated from C-peptide from the OGTT Secondary : Changes in HbA1c (week 16, 24, 36, and 48)	Change in ISR=−0.70 ± 5.2 intervention arm vs −1.68 ± 5.33 in control (p=0.89)	No difference in change in ISR or HbA1c between the groups. Baseline HbA1c was 6.6% (which is already good glycaemic control). Small sample size (127) and short follow up (48 weeks). High dose of vitamin D_3 Baseline 25(OH)D was ~ 27 ng/mL which indicates adequacy,
Jorde.2016 (Norway) (97)	Adults > 25 y with prediabetes 255 in each group Intervention: 20 000 IU/week Follow up= 5 y	Primary endpoint: diabetes incidence Secondary: fasting glucose, insulin resistance	Mean baseline 25(OH)D=59.9 ± 21.9 nmol/L. HR: 0.90(0.69–1.18)	Vitamin D supplementation did not prevent progression from prediabetes to diabetes. Similar results in subgroup analyses of vitamin D deficient
Forouhi.2016 (UK) (98)	Adults with prediabetes n=114 in each of 3 groups: vitamin D3; vitamin D2; placebo Intervention: 100,000 IU vitamin D per month for 4 months (~ 3300 IUU/d)	Primary: HbA1c	Mean difference in HbA1c: Vitamin D3 versus placebo 0.19 (95% CI −0.46, 0.83) mmol/mol (p = 0.57)	Short-term supplementation with vitamin D2 or D3 had no effect on HbA1c No hard endpoint Short follow up
Avenell.2009 (UK): Randomised Evaluation of Calcium Or vitamin D (RECORD Trial) (99)	5292 participants, ≥70 years recent history of osteoporotic fracture 2X2 factorial design, 21UK hospitals Randomly assigned to 800IU vitamin D_3, 1000mg Ca, combination, or placebo Follow up=24-62 months	Primary endpoint : new low-energy fracture Post hoc : self-reported new case of diabetes Initiation of diabetic medication	Vitamin D vs placebo Incident diabetes : OR=1.11 (0.77-1.62) Initiation of medications OR = 0.97 (0.62-1.54)	Self-reported endpoints. Only older patients (~77 years), Post hoc analyses

De Boer.2008 (US): Women's Health Initiative) (100)	33,951 healthy post-menopausal women randomly assigned to 400IU vitamin D3 + 1000IU Ca or placebo. Follow up = 7 years	Primary: Bone health related outcomes Post hoc : Incidence of self-reported diabetes, FBG, insulin resistance	61% had baseline 25(OH)D < 50 nmol/L HR= 1.01 (95% CI 0.94–1.10)	Vitamin D + Ca supplementation did not reduce diabetes risk over 7 years of follow up Post hoc analysis. Low dose of vitamin D (400IU/d). Women only. Sample not ethnically diverse Outcome was self-reported diabetes and many cases could have been missed
Pittas.2007: Nurses' Health Study (101)	314 Caucasian adults without diabetes Randomly assigned to 500mg Ca + 700IU vitamin D_3 or placebos / day Follow up= 3 years	Primary endpoint : bone health related outcomes Post hoc : Fasting BG, HOMA-IR	Increase in Fasting BG =0.02 mmol/l in treatment group vs. 0.34 mmol/L in control (p=0.042) Increase in HOMA-IR (0.05 vs. 0.91, P = 0.031).	Lower increase in FBG and HOMA-IR in treatment group vs control Short duration of follow up No hard endpoint

Table 3.2 : Evidence from cross-sectional studies of the association between vitamin D status and metabolic syndrome in SSA

Author, year	Setting	Population characteristics	Exposure (s)	Outcome (s)	Results	Comments
Sotunde, 2016, (90)	Greater J'burg area, South Africa. (PURE-SA-NWP) study)	209 urban black women, > 43 years, mean age=59.6 ± 10.6 y	25(OH)D & parathormone (PTH)	Metabolic syndrome (MetS)	No association between 25(OH)D or PTH and MetS. 25(OH)D negatively associated with body fat %.	Control for adiposity (DEXA), Urban area only, One province, MetS as binary variable. Majority were obese (70%). 25(OH)D measured by immunoassay. Adjusted for self-reported physical activity (PA) and bodyfat %
George, 2013, (89)	South Africa (recruited via the birth to twenty cohort)	374 blacks and 350 Asian Indian. Age 42.1 ± 13.1 y	25(OH)D & PTH	MetS	No association between 25(OH)D and MetS	Greater J'burg area only. Urban area only, 25(OH)D measured by HPLC, Adjustment for BMI, no adjustment for PA
Fondjo. 2018, (91)	Ghana (Kumasi)	192 women with type 2 diabetes, (98 premenopausal and 94 postmenopausal)	vitamin D	Glycaemic indices(Fasting BG, insulin, HbA1c, HOMA-β and HOMA-IR)	Vitamin D deficiency (<50 nmol/L) 60.9%. Negative correlation between 25(OH)D fasting glucose and HbA1c in post-menopausal women only	25(OH)D measured by ELISA,. No adjustment for confounders
Fondjo. 2017, (92)	Ghana (Kumasi) Case control study	118 clinically diagnosed T2D, 100 controls. Data collection: Oct to Dec 2015 Cases=58.81yrs, controls=57.79 yrs	25(OH)D	Insulin resistance measured by (HOMA-β and HOMA-IR)	Vitamin D deficiency (<50 nmol/L): 92.4% among T2DM and 60.2% among controls. No association between vitamin D status and glycaemic markers	25(OH)D measured by ELISA. Adjustment for age, sex and BMI only

Durazo-Arvizu. 2013 (102)	Nigeria (Southwest) and USA (Chicago)	100 Nigerian and 94 African American (AA) women	25(OH)D	Obesity	Mean 25(OH)D in Nigerians=64nmol/L vs 29nmol/L in AA. 76% of Nigerians had levels >50nmol/L, vs 5% of AA. 25(OH)D was inversely associated with BMI	Samples stored at different temperatures in Nigeria (20°C) and US (80°C). 25(OH)D measured by radioreceptor assay

3.3 Background on vitamin D

3.3.1 Sources, metabolism and functions of vitamin D

- **Sources and metabolism of vitamin D**

Vitamin D is a fat-soluble secosteroid and refers to both vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol). Approximately 80% of vitamin D in humans is derived from the cutaneous synthesis in the form of cholecalciferol (vitamin D_3) and ~ 20% comes from diets containing either vitamin D_3 or D_2 (ergocalciferol) (103). Few foods are naturally rich in vitamin D. These are mostly from animal products (egg yolk, meat, liver, kidney and oily fish such as mackerel, salmon and sardines) and contain vitamin D_3. Vitamin D_2 is formed in yeast and fungi by UVB radiation of ergosterol. Rich natural sources of vitamin D_2 include yeast, wild mushroom and some plants. Dietary supplements or fortified foods contain either vitamin D_2 or D_3.

In the presence of sunlight containing UVB radiation, 7-dehydrocholesterol in the epidermis is converted to pre-vitamin D_3, which is thermodynamically unstable (Figure 3.1). Pre-vitamin D_3 is converted to vitamin D_3 in the plasma membrane of epidermal cells. The amount of vitamin D_3 synthesized depends on the exposure of the skin to UVB radiation and the efficiency of the skin to synthesize vitamin D_3. Exposure of the skin to UVB radiation is influenced by factors such as latitude, altitude, air pollution, cloud cover, time of day, season, clothing and sunscreen use.

Vitamin D is fat-soluble and when obtained from the diet, it is incorporated within the enterocytes into chylomicrons and transported through the lymphatic system into the systemic circulation. Dietary vitamin D (D_2 or D_3) and cutaneously produced vitamin D (D_3) are transported in circulation mostly bound to vitamin D binding protein (DBP). Vitamin D has to go through a two-step hydroxylation to be activated. First, in the liver, vitamin D is hydroxylated in the presence of 25-hydroxylase to 25-hydroxyvitamin D (25(OH)D) and then in the kidneys to the active metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) in the presence of 1-alpha hydroxylase (104).

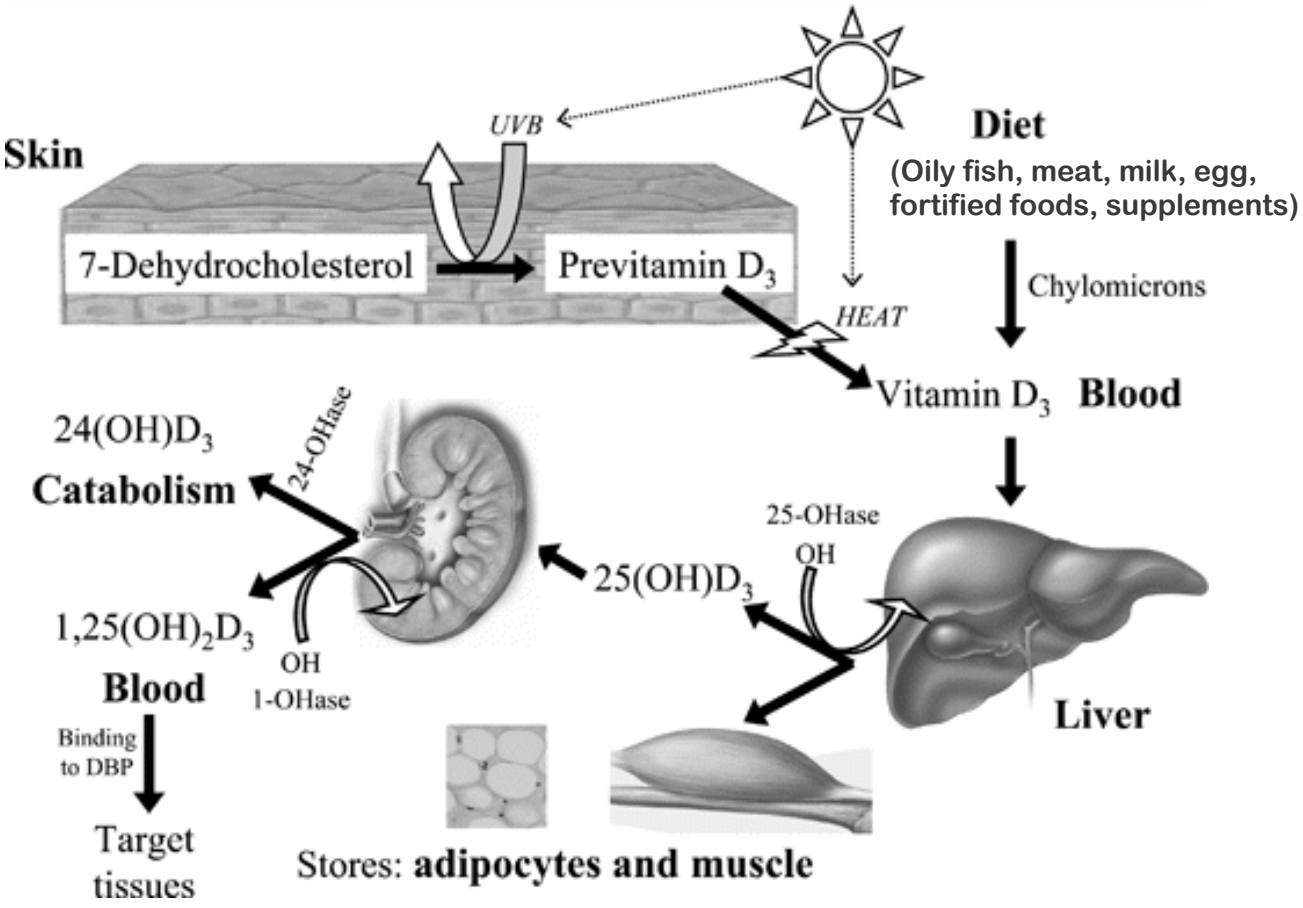

Figure 3.1 : Sources and metabolism of vitamin D (adapted from Palomer et al) *(105)*

- **Functions of vitamin D**:

The active form of vitamin D ($1,25(OH)_2D$) exerts its function by binding to vitamin D receptors mainly in the intestines, bones and kidneys (Figure 3.2). The main function of vitamin D is to maintain calcium and bone homeostasis by increasing intestinal calcium absorption. If calcium needs are not met, vitamin D together with parathyroid hormone stimulates the reabsorption of calcium from the distal convoluted tubules of the kidney and bone resorption of calcium to increase circulating levels of calcium. Vitamin D also stimulates intestinal absorption of phosphate. It is unclear whether there is a direct role of vitamin D in the regulation of the renal reabsorption of phosphorus (106). Over the years, it has become increasingly clear that vitamin D receptors are present on a wide range of organs other than the kidney and the effects of vitamin D may extend beyond the regulation of calcium and phosphate metabolism.

Many organs such as the pancreas, prostate, breast, lung and colon are also capable of synthesizing active vitamin D which has led to the growing interest in vitamin D beyond muscular and bone health (107). Low vitamin D status has been associated with extra renal outcomes including diabetes and CVDs in cross-sectional and prospective studies (108), (109).

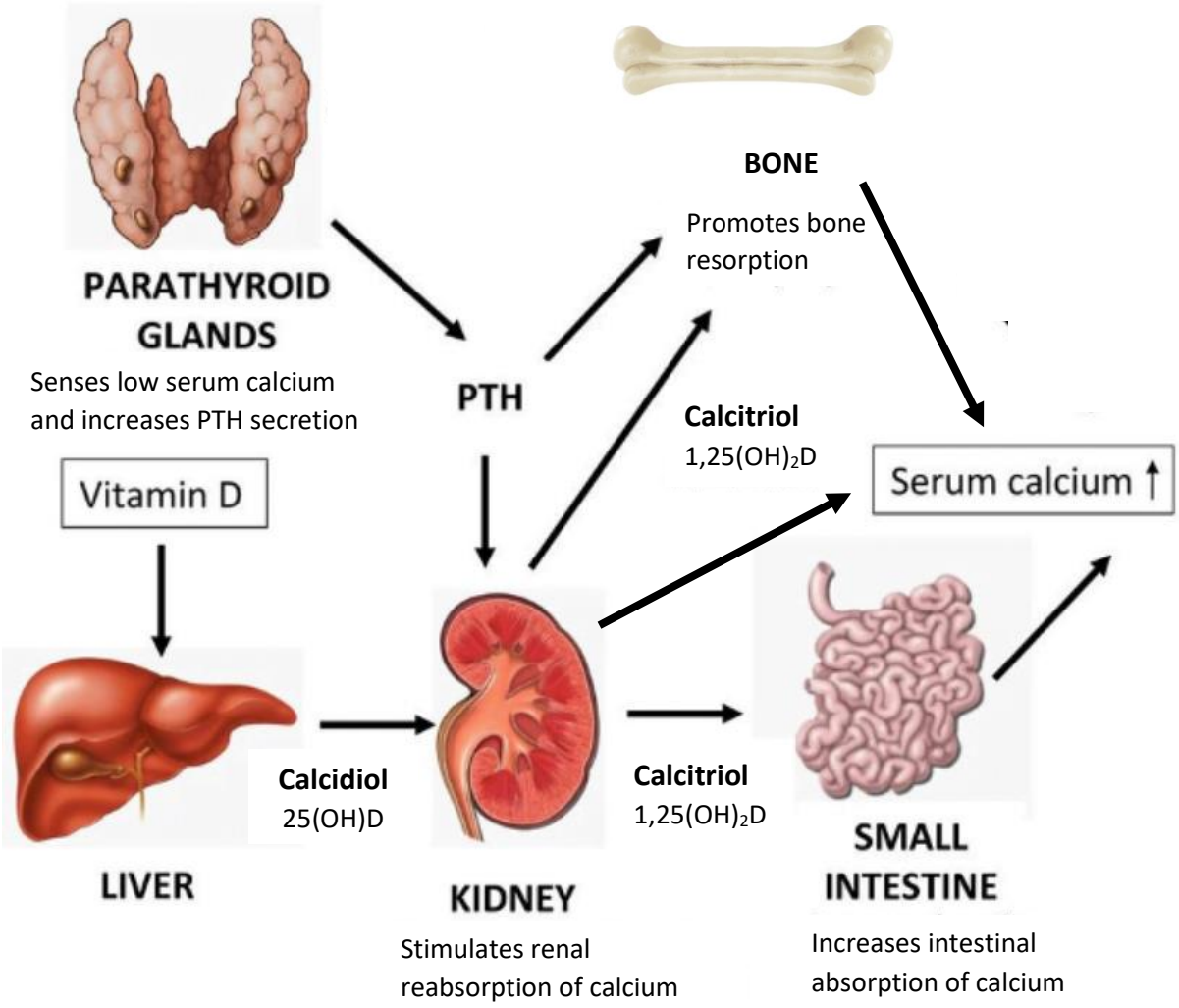

Figure 3.2 : Role of vitamin D in calcium regulation

3.3.2 Biomarker of vitamin D exposure

Blood 25(OH)D concentration is currently considered the best indicator of vitamin D status because of 3 main reasons. Firstly, it reflects both dietary intake and endogenous synthesis of vitamin D. Secondly, it has a long half in the circulation of 2-3 weeks and thirdly, 25(OH)D is not subject to homeostatic control. $1,25(OH)_2D$ on the other hand has a short half-life (< 4 hours) and is under tight homeostatic control. It is stimulated by parathyroid hormone (PTH) and inhibited by foetal growth factor 23 and by $1,25(OH)_2D$ itself (106). However, some points should be considered when using blood 25(OH)D as a marker of vitamin D status. Blood 25(OH)D concentration is affected by factors, such as inflammatory state, personal characteristics (age, sex, BMI and genetic variations, etc) and health-related behavioural characteristics (e.g. physical activity) (110).

Some authors have suggested the use of free 25(OH)D or bioavailable 25(OH)D (free and loosely albumin-bound 25(OH)D)) instead of total 25(OH)D as a marker of vitamin D status. 85-90% of 25(OH)D circulates in the blood bound to the DBP, 10-15% bound to albumin and less than 1% is in the free form. Total 25(OH)D is the sum of three 25(OH)D fractions: DBP-bound, albumin-bound and free fraction. The “free hormone” hypothesis stipulates that only the free fraction or loosely bound fraction of a hormone can enter the cells and exert its biological functions (111). For instance, despite similar concentrations of total 25(OH)D in African Americans and Whites, it has been shown that African Americans have lower concentrations of DBP and higher concentrations of bioavailable 25(OH)D which may have important implications for health outcomes (112).

3.3.3 Physical activity, adiposity and vitamin D status

As mentioned above, vitamin D status is affected by personal and health-related behavioural characteristics. Physical activity and adiposity are some of the most important to consider, given that they are also important determinants of cardiometabolic diseases. There is evidence suggesting that more physically active individuals have higher serum 25(OH)D concentrations (93). It is unclear whether this is the result of higher outdoor activities associated with higher sunshine exposure in more physically active individuals than in less active individuals. In a cross-sectional study of a nationally representative sample of 15,148 adults in the US, self-reported outdoors physical activity was associated with higher serum 25(OH)D concentrations compared with indoor physical activities (113). However, serum 25(OH)D concentrations were

also higher for higher indoor physical activities compared with lower indoor activities. Another study in the US population showed that both self-reported and objectively measured physical activity were positively associated with serum 25(OH)D concentration, but serum 25(OH)D concentration did not differ between self-reported outdoor physical activities and indoor activities (93). In another study in post-menopausal women, the positive association between self-reported physical activity and 25(OH)D concentration was attenuated but remained significant after adjusting for sunshine exposure, waist circumference and season of blood draw (114). These studies suggest that physical activity may be associated with vitamin D status independently of sunshine exposure (115).

The adipose tissue is one of the major sites of storage of vitamin D. Evidence from observational studies suggests that obesity is associated with lower serum 25(OH)D concentration (116), (117). A meta-analysis of 23 studies showed that vitamin D deficiency was 35% and 24% higher in the obese and overweight groups respectively compared with the normal BMI group (118). In a Mendelian randomisation study, higher genetically predicted BMI was associated with lower serum 25(OH)D concentration (119). Some of the reasons for the inverse association between adiposity and vitamin D status is that higher adiposity is associated with a lower release of 25(OH)D in circulation, given that 25(OH)D gets sequestrated in the adipose tissues which reduce vitamin D bioavailability. Another plausible explanation is that people with obesity may spend less time outdoors or have a more concealed dressing style.

3.3.4 Measurement of blood 25(OH)D

Immunoassay-based methods and chromatographic-based methods are the two most common types of assays for measuring blood 25(OH)D. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered the gold standard for the measurement of serum 25(OH)D concentration and allows for the separation of $25(OH)D_2$ and $25(OH)D_3$ from blood samples. Given that Vitamin D_2, mostly comes from supplements or fortified foods (Vitamin D found naturally in most foods is in the form of D_3), LC-MS/MS may have an advantage when evaluating the effect of supplementation or food fortification with vitamin D_2. Immunoassays are cheap, easy to use and rapid but specificity is low due to the high cross-reactivity with $24,25(OH)D_2$ which can result in overestimation of serum 25(OH)D concentration (120). Because of analytical variability between and within methods (especially with immunoassays),

the National Institute of Science and Technology (NIST) has developed protocols for standardising measurement methods of blood 25(OH)D concentration (NIST Standard Reference Material (SRM) 2972).

3.3.5 Classification of vitamin D status and dietary vitamin D recommendations

There is no consensus on the definition of optimal vitamin D status. Many guidelines recommend 25-30 nmol as the threshold for serum 25(OH)D concentration below which there is an increased risk of poor musculoskeletal outcomes (Table 3.3) (121), (122), (123), (124). Although based on limited evidence, the Endocrine Society suggests maintaining blood 25(OH)D concentrations above 75 nmol/L to provide the potential non-skeletal health benefits of vitamin D (125).

Table 3.3 : Suggested cut-offs of serum 25(OH)D in nmol/L to define vitamin D status according to different guidelines

Status	25(OH)D (nmol/L)			
	IOM	ROS/NICE	SACN	ES
Deficiency	<30	<25	<25	<50
Insufficiency	30-50	25-50		50-75
Adequacy	>50	>50		

IOM, Institute of Medicine (121); ROS, Royal Osteoporosis Society (123); NICE, National Institute for Health and Care Excellence (124); SACN, Scientific Advisory Committee on Nutrition (122); ES Endocrine Society (125).

There exists a seasonal variation in blood 25(OH)D concentrations. In the UK, from April to September, UVB radiation in sunlight is sufficient to initiate the synthesis of relevant quantities of vitamin D in white-skinned populations (122). The highest concentrations of 25(OH)D are observed during summer with a peak in September followed by a decline in October till March. Dietary sources of vitamin D become essential, from October to March when sunlight is limited.

In the UK, the reference nutrient intake (RNI) is set at 400 IU/day for everyone aged ≥ 4 years from October to March. For populations at high risk of vitamin D deficiency (e.g. dark skin populations, people with obesity, spend limited time outdoors and elderly populations, the RNI is 400 IU/day all year round (122). The RNI is the amount of nutrient that is sufficient to meet the needs of 97.5% of the population (in terms of bone and muscular health for vitamin D). In the US, the RNI is 400 IU/day for children aged 0-1 year, 600 IU/day for those aged ≥ 1 year and 800 IU/day for elderly populations (> 70 years) (125).

3.3.6 Vitamin D status in Africa

Given the abundant sunshine in many African countries, it is expected that people living in Africa get sufficient ultraviolet B radiation at relevant wavelengths for the synthesis of vitamin D. However, vitamin status does not only depend on the availability of sunshine but on how much sunshine actually reaches the skin which is influenced by religious and cultural practices, the time spent outdoors, and the skin pigmentation. Vitamin D status is also influenced by diet and underlying health conditions, calcium intake and infectious diseases. Africa is heterogeneous in these factors and therefore the prevalence of vitamin D deficiency varies widely within Africa (126).

In the absence of a global consensus to define vitamin D deficiency, various studies have used different cut-offs (< 25 nmol/L or < 30 nmol/L) making it difficult to compare the magnitude of vitamin D deficiency across studies. Despite this, there is evidence suggesting that the prevalence of vitamin D deficiency or insufficiency in some parts of Africa is higher than in the US and Europe where most studies on the association between vitamin D status and metabolic syndrome have been conducted. Using < 30 nmol/L as a cut-off commonly reported, a meta-analysis of vitamin D in Africa showed a prevalence of vitamin D insufficiency (< 30 nmol/L) of 18.5% (86). However, this prevalence is likely to be underestimated as over 90% of the studies included used immunoassay-based techniques. Immunoassays have been shown to yield 25(OH)D concentrations that are ~ 50% higher than liquid-chromatography tandem mass spectrometry (LC-MS/MS) due to the high cross-reactivity with 24,25(OH)2D.

Therefore, recalibration of studies of vitamin D status in Africa to equivalent 25(OH)D measurements from the standardized LC-MS/MS method as has been done in Europe (1) and the US (2) may yield higher estimates of low vitamin D prevalence. For example, recalibrating 25(OH)D data from the Irish National Adult Nutrition Survey or reanalysing all samples using

LC-MS/MS resulted in a prevalence estimate of vitamin D deficiency that was double that of the immunoassay estimate (88).

3.4 Methods

The study design, setting and other common methods and procedures used in this cross-sectional study are reported in Chapter 2.

3.4.1 Measurement of serum 25(OH)D concentrations

- **Pre-analytical step (common step for all the nutritional biomarkers)**

The pre-analytical step consisted in retrieving the plasma and serum samples and providing the right volume and sample matrix for the measurements of the nutritional biomarkers. This was done at the Biorepository of the MRC Epidemiology Unit. I coordinated the retrieving of the samples from Thermo Fisher Scientific, Bishop's Stortford, where the samples were stored at -80°C and transported on dry ice to the Biorepository of the MRC Epidemiology Unit where they were kept in -80°C refrigerators. I generated barcodes with the participant's identification (ID) number, sample matrix and biomarker to be measured using the bartender software, printed and labelled the 1.5L of sarstedt tubes (689 x 6 tubes) with the help of a laboratory assistant. I added freshly prepared metaphosphoric acid (MPA) into the tubes labelled for vitamin C.

I removed the samples and sorted them out on dry ice to avoid repeated freeze-thaw cycles which might affect our analyses. Sorting consisted in rearranging the tubes with samples in ascending order of their IDs in new boxes. In total, 18 boxes of serum and plasma collected in heparin tubes and plasma EDTA each were sorted out. Two tubes each of the same IDs and matrices were thawed and combined because the volume of the samples needed was more than that available from one tube using an automated liquid handling robot (Hamilton star®). However, I still needed to transfer the samples manually because the volume of the samples was insufficient for the robot to perform its task. After merging samples from two tubes, we aliquoted the required volumes into the newly labelled tubes using either the robot or manually when it failed.

Processing of any thawed sample was conducted within 2 hours and quickly refrozen to -80°C. For the samples for folate and vitamin E measurement, this pre-processing stage was conducted in the room with lights switched off and minimal lights from outside because folate and vitamin

E are light sensitive. I transported the samples in a thermosafe box containing dry ice to the different nutritional biomarker laboratories (NBL) in the Clifford Allbutt Building where the aliquots were stored at -80°C till analyses.

- **Analytical step**

Serum 25(OH)D concentrations were measured at the NIHR Cambridge BRC Nutritional Biomarker Laboratory (NBL) using ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Quality assessment of the assay was performed regularly as part of the Vitamin D External Quality Assessment Scheme (www.deqas.org) and performance was additionally assessed against National Institute of Standards and Technology Standard Reference Material 972a.

Serum samples stored at -80°C were removed and thawed and stable isotope-labelled internal standards were added to normalise the sample preparation process and instrument detection variability. Protein precipitation was performed with methanol followed by liquid-liquid extraction in hexane. Samples were dried and then reconstituted in 73% methanol. Samples were injected onto the UPLC (Waters Acquity, Waters UK, Herts, UK) and chromatographic separation was performed with a Hypersil GOLD PFP 2.1 x 100mm 1.9µm column (Thermo Scientific, UK) at 40°C and with a 74% methanol +0.1% formic acid isocratic mobile phase at 0.25mL/min. Detection was accomplished by an AB Sciex QTrap 4000 mass spectrometer (AB Sciex, Warrington, UK). The ratio of the signal of the metabolite to the internal standard obtained was compared against that of a calibration curve to determine the concentration of $25(OH)D_2$, $25(OH)D_3$ and C3 epimer of -$25(OH)D_3$ (epi-$25(OH)D_3$).

Total 25(OH)D was calculated from the sum of $25(OH)D_2$ and $25(OH)D_3$ and was used in subsequent data analysis. The inter-assay coefficient of variation for all metabolites ranged between 5.1% and 7.8%. The limit of detection (LOD) was 2, 3 and 4 nmol/L for $25(OH)D_3$, $25(OH)D_2$ and epi-$25(OH)D_3$ respectively. The limit of quantification (LOQ) was 6 nmol/L for all the metabolites. Over 90 % of participants had non-detectable levels of $25(OH)D_2$. I imputed a random number between 3 and 6 for $25(OH)D_2$ results which were below the LOQ but above the LOD (n=27). In total 586 participants had blood samples available for vitamin D analyses.

3.4.2 Outcomes

Outcomes were a continuous metabolic syndrome score and markers of glucose homeostasis (fasting glucose, 2-h glucose and HOMA-IR). The metabolic syndrome score was calculated by summing the sex-specific standardised values of waist circumference, blood pressure, fasting glucose, triglycerides and inverted HDL cholesterol as described in chapter 2.

3.5 Statistical analyses

Statistical analyses were performed using Stata 15 (StataCorp, College Station, TX). Descriptive statistics are presented as means ± SD (or median [25^{th}-75^{th} percentile] for non-normally distributed data) or numbers and percentages. Linear trends across quarters of serum 25(OH)D concentration were assessed by linear regression for continuous variables and chi-squared test for trend for categorical variables (Cochran-Armitage test or Cochran-Mantel-Haenszel test for categorical variables with 2 or ≥ 3 levels respectively). I reported the proportion of participants with deficient, insufficient and adequate vitamin D status using cut-offs suggested by the Institute of Medicine (deficient < 30 nmol/L, inadequate but not deficient: 30-49.9 nmol/L, adequate: 50-74.9 nmol/L) (121) and "optimal" vitamin D status as suggested by the Endocrine Society (≥ 75 nmol/L) (125).

Multiple linear regression adjusted for age and sex was used to identify predictors of continuously distributed serum 25(OH)D concentration. To estimate associations of vitamin D status with outcome variables, I fitted 5 models that were incrementally adjusted to account for potential confounding variables based on biological plausibility and previous research. Model 1 was unadjusted, model 2 was adjusted for age (continuous), sex, smoking (never, past or current), alcohol intake (never, past or current) and level of education (less than primary school, completed primary school, secondary school and university). Model 3 was additionally adjusted for season of blood draw (long dry, short rainy, short dry and long rainy) and residential site (4 sites). Model 4 was additionally adjusted for BMI (continuous) and model 5 for objectively measured PAEE (continuous). I investigated non-linear associations of serum 25(OH)D with outcomes by fitting restricted cubic splines with 3 knots corresponding to the 25^{th}, 50^{th} and 75^{th} percentile of continuous serum 25(OH)D, and tested for non-linearity using the Wald test. As there was no evidence of a non-linear association with any of the outcomes, I fitted linear regression models. With missing information observed (metabolic syndrome score, n=12; 2-h glucose, n=9; PAEE, n=49), complete-case analyses were performed with

further sensitivity analysis implementing multiple imputation. I tested interactions of 25(OH)D with sex, rural-urban residence and BMI categories (normal weight, overweight and obese) using model 5. When the p-value for interaction was < 0.05, I reported results within the relevant strata.

I performed sensitivity analyses (a) using multiple imputation to investigate the impact of missing data. With less than 10% of missing data, missing data were imputed under the assumption of missing at random with multiple imputation by chained equations to create 10 imputed datasets and combining estimates across the imputed datasets using Rubin's rule (127). (b) Replaced BMI by body fat in models 4 and 5; (c) included self-reported fruit and vegetable intake as a proxy for overall dietary quality in model 5 (d) used a metabolic syndrome score calculated without the waist circumference to explore the effect of adjusting for BMI when the abdominal obesity component was omitted from the outcome score.

3.6 Results

3.6.1 Descriptive characteristics

Mean ± SD serum 25(OH)D concentration was 51.7 ± 12.5 nmol/L with higher concentrations in men (53.5 ± 13.8 nmol/L) compared to women (50.6 ± 11.6 nmol/L), p-value = 0.008 and rural (53.4 ± 12.8 nmol/L) compared to urban (50.2 ± 12.1 nmol/L) participants (p-value = 0.002) (Figures 3.3). This is depicted in the rural-to-urban left shift in the distribution of serum 25(OH)D concentrations (Figure 3.4). The rural-urban difference in serum 25(OH)D concentration was consistent throughout the seasons except during the heavy rainy season when urban participants had higher concentrations of serum 25(OH)D (Figures 3.5 and 3.6). Participants with overweight and obesity had lower concentrations of 25(OH)D (49.9 nmol/L) than those with normal weight (53.4 nmol/L), p=0.001 (Figure 3.7). 4.4% of participants had serum 25(OH)D concentrations that were defined as deficient (< 30 nmol/L), 41.3% had inadequate but not deficient concentrations (30-49.9 nmol/L), 50.2% had sufficient concentrations (50-74.9 nmol/L) (24), and 4.1% had "optimal" concentrations (≥ 75 nmol/L). The mean of the metabolic syndrome score was higher in urban (0.36 ± 2.7) than in rural (-0.41 ± 2.27), p-value = 0.0003. This is depicted by the rural-to-urban right shift in the distribution of the metabolic syndrome score (Figure 3.4).

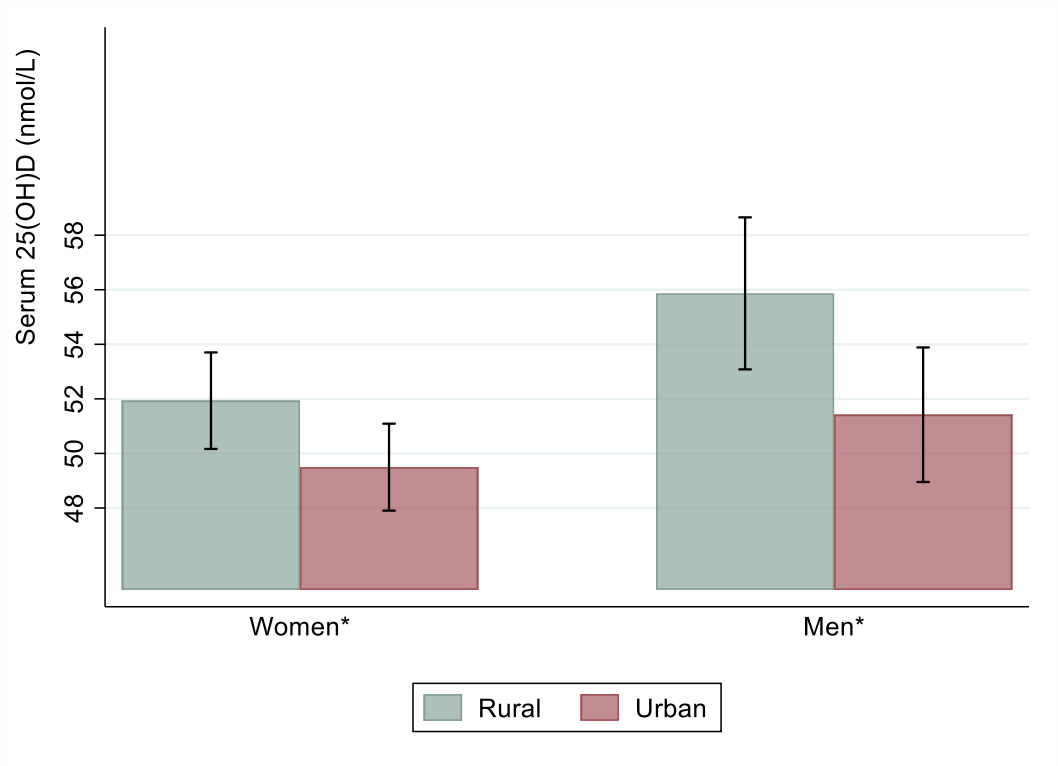

Figure 3.3 : Comparison of mean serum 25(OH)D concentration by sex in rural and urban dwellers (Cameroon study, n = 586).

Bars are means and error bars are 95% confidence intervals around the mean values. *p-value < 0.05 for a difference in mean levels of serum 25(OH)D between participants living in rural and urban areas.

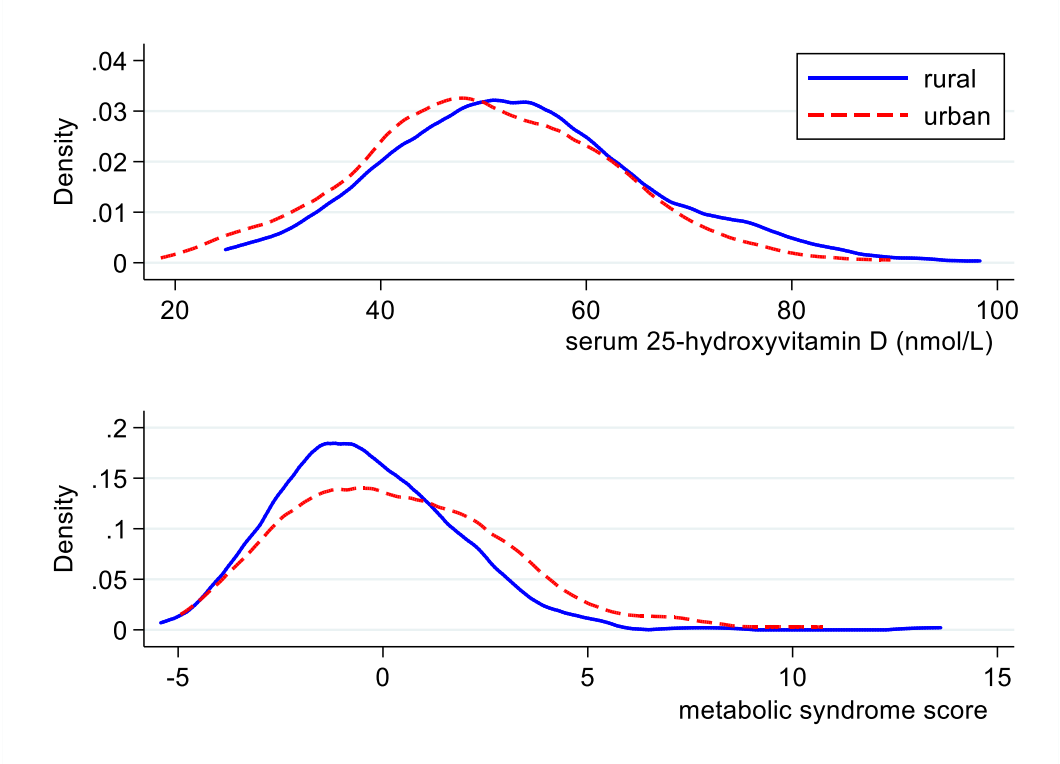

Figure 3.4 : Distribution of serum 25(OH)D concentration and metabolic syndrome score by rural and urban residence (Cameroon study, n = 574)

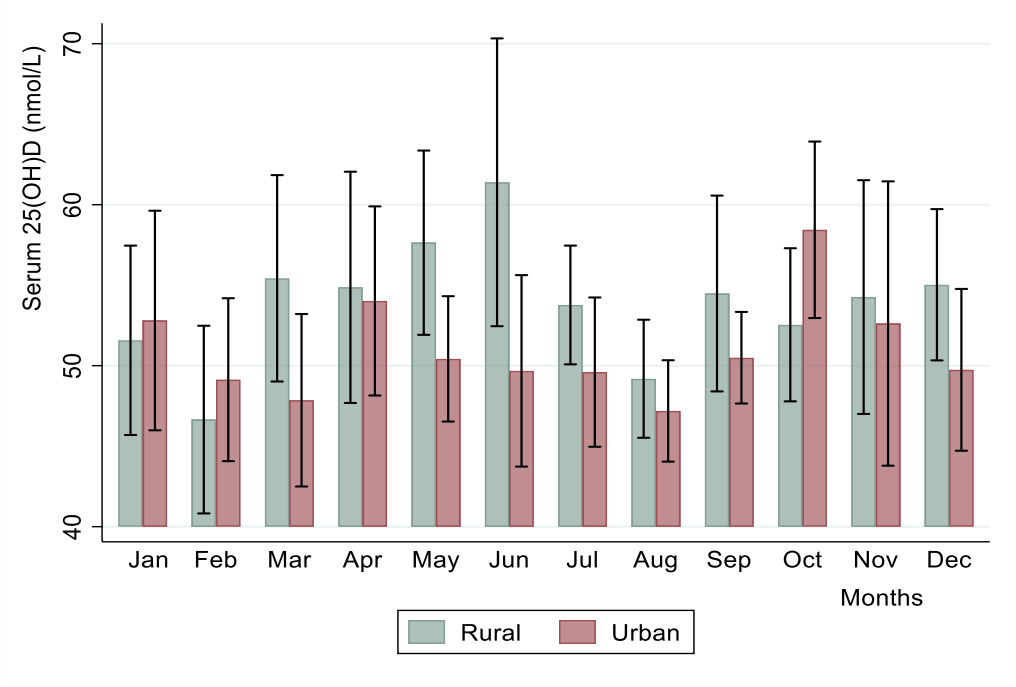

Figure 3.5 : Distribution of serum 25(OH)D concentration by month in rural and urban residents. Month 1 is January and month 12 is December

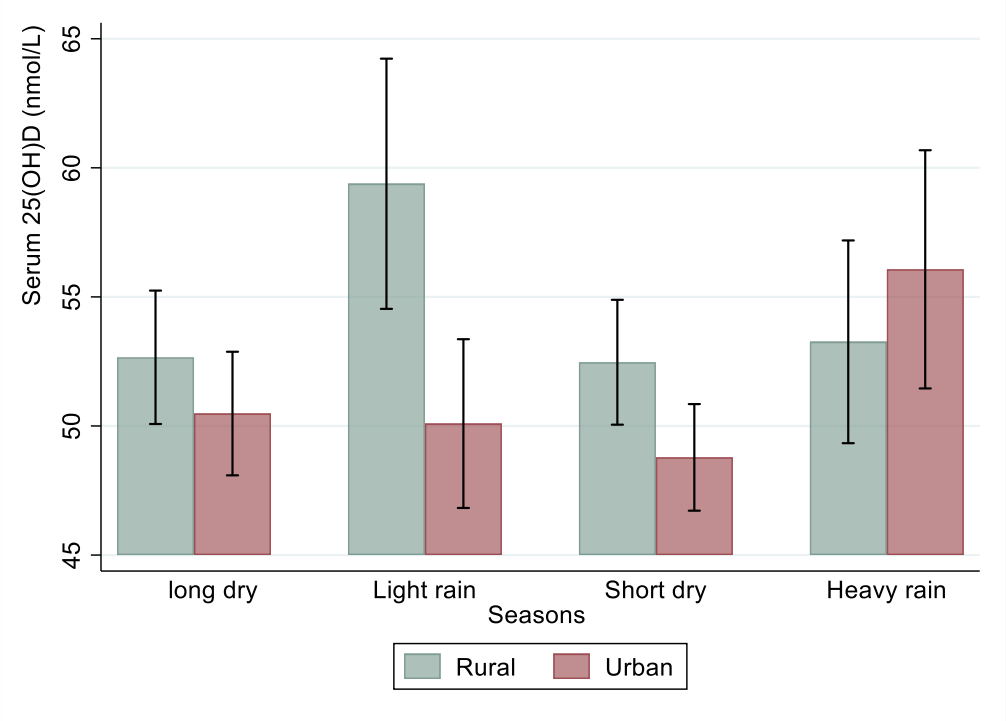

Figure 3.6 : Distribution of serum 25(OH)D concentration by seasons in rural and urban dwellers.

Seasons are: long dry; December-April, light rain; May-June; short dry; July-September and heavy rain; October-November

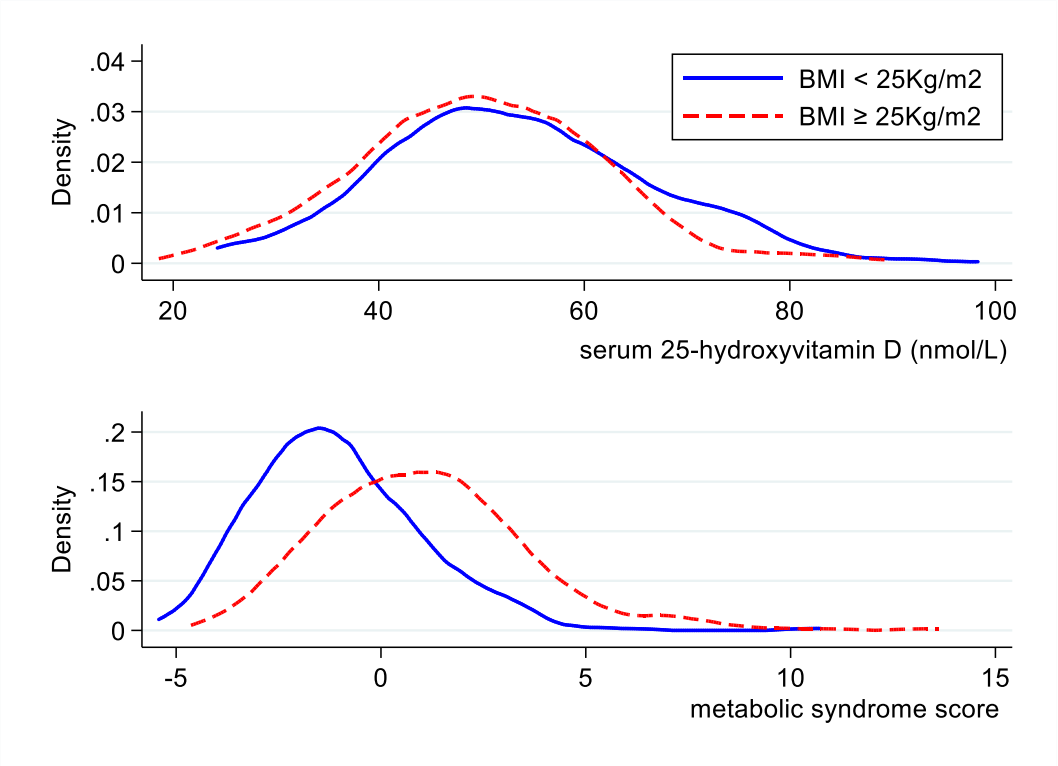

Figure 3.7 : Distribution of serum 25(OH)D concentration and metabolic syndrome score by BMI category (Cameroon study, n = 574)

3.6.2 Factors affecting vitamin D status

Education level, BMI, measures of adiposity (waist circumference, BMI and body fat percentage), alcohol intake, fasting blood glucose, PAEE and sedentary time were all significantly associated with serum 25(OH)D concentration. There was no evidence of a linear trend in blood pressure, lipids and the metabolic syndrome score across quarters of serum 25(OH)D concentration (Tables 3.4 and 3.5).

I also examined sociodemographic, behavioural and anthropometric correlates of serum 25(OH)D in models adjusted for age and sex and stratified by rural/urban site (Table 3.6). Variables that had a positive association with serum 25(OH)D concentration were male sex, PAEE (with stronger associations for objectively measured PAEE) and season of blood draw (light rain in rural and heavy rain in urban). Time spent being sedentary, measures of adiposity (BMI, body fat percentage and waist circumference), and level of education were inversely associated with serum 25(OH)D concentration.

Table 3.4 : Sociodemographic and health-related behavioural characteristics by quarters of serum 25(OH)D (Cameroon study: n = 586)

Characteristics	Quarters (Q) of serum 25(OH)D				p-value for linear trend
	Q1	Q2	Q3	Q4	
25(OH)D (nmol/L)	23.3 – 44.0	44.1 - 51.2	51.3 - 59.8	60.0 - 98.3	
Sex, n(%)					
Female	103(70.1)	91(61.9)	95(65.1)	83(56.9)	0.04
Age (years)	39.4 ± 8.5	37.4 ± 9.0	38.3 ± 8.3	38.05 ± 8.6	0.32
Education, n(%)					
< primary school	24(16.4)	22(14.9)	29(19.9)	28(19.2)	
Primary school	60(41.1)	58(39.5)	61(41.8)	80(54.8)	0.003
Secondary school	39(26.7)	41(27.9)	39(26.7)	28(19.2)	
University	23(15.8)	26(17.7)	17(11.6)	10(6.8)	
Smoking status, n(%)					
Never	113(76.9)	114(77.6)	117(80.1)	102 (75.0)	
Past	23(15.7)	18(12.2)	19(13.0)	17(12.5)	0.57
Current smoker	11(7.4)	15(10.2)	10(6.9)	17(12.5)	
Alcohol intake, n(%)					
Never	22(14.9)	18(12.2)	15(10.3)	11(7.5)	
Past	25(17.0)	12(8.2)	5(3.4)	14(9.6)	0.001
Current	100(68.1)	117(79.6)	126(86.3)	121(82.9)	
PAEE (KJ/Kg/day)	43.8 ± 22.4	51.1 ± 22.7	51.1 ± 22.7	55.4 ± 24.7	<0.001
Sedentary time (min/day)	999.8 ± 159.6	947.9 ± 146.32	941.6 ± 150.5	941.6 ± 150.5	0.001
LPA time (min/day)	337.5 ± 112.1	375.8 ± 105.9	375.8 ± 105.9	376.0 ± 98.7	0.007
MVPA time (min/day)	102.7 ± 83.7	114.2 ± 76.7	122.6 ± 85.4	130.4 ± 97.1	0.008
GPAQ PAEE (KJ/Kg/day)	13.3(3.5 - 104.9)	23.7(3.3-112.4)	27.2(4.2-106.6)	27.9(3.5-141.1)	0.036
GPAQ work (MET-min/week)	0(0-8640)	240(0-9600)	0(0-10080)	600(0-12240)	0.55
GPAQ leisure (MET -min/week)	0(0-0)	0(0-0)	0(0-0)	0(0-0)	
GPAQ travel (MET - min/week)	840(420-1680)	840(280-3360)	960(420-3360)	1120(420-3360)	0.205
Fruit (times/week)	3(1-5)	2(1-5)	2(1-4)	2(1-5)	0.729
Vegetables (times/week)	4(2-7)	4(2-6)	4(2-7)	4(2-8)	0.489

Results are presented as arithmetic mean ± SD [or median (25^{th}-75^{th} percentile) for non-normally distributed variables] or n (%). p-values for trend are from a chi-squared test for trend for categorical variables or from a linear regression model for continuous variables including quartile of serum 25(OH)D as a continuous exposure. n=586, except for PAEE where n=537.

PAEE, physical activity energy expenditure; LPA, light physical activity; MVPA, moderate to vigorous physical activity

Table 3.5 : Metabolic characteristics by quarters of serum 25(OH)D (Cameroon study: n=586)

Characteristics	Quarters (Q) of serum 25(OH)D				p for linear trend
	Q1	Q2	Q3	Q4	
Waist circumference (cm)	91.3 ± 13.5	86.7 ± 12.4	90.0 ± 11.8	86.5 ± 10.7	0.013
BMI (kg/m^2)	27.1 ± 6.0	25.7 ± 5.0	26.8 ± 5.3	24.9 ± 4.4	0.004
Body fat (%)	30.6 ± 11.1	27.5 ± 10.7	29.2 ± 11.4	25.6 ± 10.6	0.001
Systolic BP (mmHg)	121.8 ± 19.1	123. 3 ± 21.1	121.8 ±19.5	123.2 ± 23.0	0.74
Diastolic BP (mmHg)	76.6 ± 12.2	75.8 ± 14.3	77.5 ± 12.3	75.9 ± 14.6	0.94
Fasting BG (mmol/L)	5.0 ± 1.63	4.8 ± 1.49	4.74 ± 1.32	4.55 ± 0.74	0.004
2-hour BG (mmol/L)	6.42 ± 1.88	6.37 ± 1.80	6.25 ± 1.95	6.04 ± 1.73	0.065
Fasting insulin (pmol/L)	22.0(13.3 - 32.3)	19.3(11.7 - 33.6)	24.1(10.6-43.1)	19.8(12.1-30.4)	0.83
HOMA-IR index	0.75(0.45-1.29)	0.7(0.37-1.19)	0.78(0.33-1.62)	0.69(0.37-1.04)	0.59
Total cholesterol (mmol/L)	3.78 ± 0.89	3.95 ± 0.96	3.86 ± 0.94	3.80 ± 1.1	0.96
HDL cholesterol (mmol/L)	1.23 ± 0.32	1.25 ± 0.32	1.19 ± 0.30	1.21 ± 0.37	0.28
LDL cholesterol (mmol/L)	2.14 ± 0.78	2.34 ± 0.84	2.27 ± 0.78	2.21 ± 0.94	0.60
Triglycerides (mmol/L)	0.73(0.59-0.98)	0.74(0.58-0.95)	0.76(0.58-0.96)	0.74(0.6-0.91)	0.51
CRP (mg/L)	4.94(2.42-9.8)	4.6(2.55-8.59)	4.61(2.46-8.35)	5.28(2.65-8.37)	0.64
Metabolic syndrome score	0.39 ± 2.94	-0.27 ± 2.42	0.19 ± 2.39	-0.31 ± 2.31	0.087

n= 586, except for metabolic syndrome score, triglycerides and HDL where n= 574; 2-h glycaemia (n=577) HOMA-IR (n=582)

Results are presented as arithmetic mean ± SD [or median (25^{th}-75^{th} percentile) for non-normally distributed variables] or n (%). p-values for trend are from a chi-squared test for trend for categorical variables or from a linear regression model for continuous variables including quartile of serum 25(OH)D as a continuous exposure.

BMI, body mass index; BP, blood pressure; BG, blood glucose; CRP, C-reactive protein

Table 3.6 : Socio demographic, behavioural and anthropometric correlates of vitamin D status (Cameroon study: n = 586)

Correlates	Total (586)		Rural (n=273)		Urban (n=313)	
	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value
Age (years)	-0.06(-0.18 to 0.06)	0.32	-0.04(-0.22 to 0.15)	0.68	-0.1(-0.25 to 0.05)	0.21
Male (vs female)	2.72(0.60 to 4.84)	0.012	3.84(0.67 to 7.0)	0.018	1.69(-1.13 to 4.51)	0.24
Education level						
< Primary education (ref)						
Primary education	-0.79(-3.65 to 2.05)	0.58	0.84(-2.78 to 4.46)	0.649	-3.01(-8.05 to 2.02)	0.240
Secondary and high school	-4.29(-7.45 to -1.14)	0.008	-3.60(-8.57 to 1.37)	0.155	-4.99(-10.1 to 0.10)	0.06
University	-7.29(-11.06 to -3.52)	<0.001	-3.59(-13.40 to 6.62)	0.472	-8.37(-13.88 to -2.86)	0.003
Smoking status						
Never smoked (ref)						
Former	-1.85(-4.9 to 1.30)	0.25	0.58(-4.36 to 5.52)	0.818	-2.95(-7.0 to 1.11)	0.154
Current	-1.09(-5.05 to 2.87)	0.59	-2.46(-8.51 to 3.59)	0.424	0.45(-4.76 to 5.67)	0.86
Alcohol intake						
Never (ref)						
Former	-1.49(-5.91 to 2.93)	0.508	3.53(-3.40 to 10.46)	0.317	-4.23(-10.08 to 1.63)	0.156
Current	3.20(0.14 to 6.42)	0.051	5.12(0.63 to 9.59)	0.025	1.58(-3.04 to 6.19)	0.502
Season of blood draw						
Short dry (ref)						
Long dry	1.15(-1.17 to 3.47)	0.332	-0.17(-3.7 to 3.37)	0.926	2.26(-0.87 to 5.38)	0.156
Light rain	-2.97(-0.33 to 6.26)	0.077	6.75(1.45 to 12.04)	0.013	0.69(-3.44 to 4.82)	0.741
Heavy rain	4.32(0.99 to 7.66)	0.011	1.24(-3.28 to 5.78)	0.589	7.55(2.56 to 12.54)	0.003
Fruit (times/week)	0.06(-0.23 to 0.35)	0.71	-0.07(-0.46 to 0.31)	0.706	0.09(-0.361 to 0.55)	0.697

Vegetables (times/week)	0.30(-0.05 to 0.56)	0.02	0.37(-0.01 to 0.72)	0.043	0.08(-0.30 to 0.47)	0.669
PAEE (KJ/kg/day)	0.1(0.05 to 0.14)	<0.001	0.02(-0.05 to 0.08)	0.656	0.17(0.09 to 0.22)	<0.001
Objective sedentary time (min/day)	-0.01(-0.02 to -0.005)	0.001	-0.001(-0.01 to 0.01)	0.859	-0.02 (-0.03 to -0.01)	0.001
Objective LPA (min/day)	0.01(-0.001 to 0.02)	0.027	0.002(-0.02 to 0.02)	0.834	0.01(-0.001 to 0.03)	0.061
Objective MVPA (min/day)	0.02(0.01 to 0.03)	0.004	0.001(-0.02 to 0.02)	0.949	0.03(0.01 to 0.05)	0.001
GPAQ PAEE (KJ/Kg/day)	0.02(0.007 to 0.03)	0.002	0.01(-0.005 to 0.03)	0.157	0.02(0.001 to 0.04)	0.043
GPAQ work (MET-h/week)	0.01(0.003 to 0.02)	0.007	0.005(-0.005 to 0.015)	0.348	0.01(0.003 to 0.025)	0.044
GPAQ leisure (MET-h/week)	0.04(-0.003 to 0.07)	0.07	0.04(-0.006 to 0.09)	0.087	0.02(-0.04 to 0.09)	0.410
GPAQ travel (MET-h/week)	0.03(0.005 to 0.06)	0.019	0.03(-0.006 to 0.07)	0.119	0.02(-0.03 to 0.06)	0.427
BMI (kg/m^2)						
Continuous	-0.31(-0.51 to -0.11)	0.003	0.008(-0.34 to 0.36)	0.964	-0.40(-0.67 to -0.12)	0.005
<25 (ref)						
25-29.9	-2.23(-4.68 to -0.23)	0.076	-1.36(-5.05 to 2.34)	0.470	-2.23(-5.71 to 1.25)	0.208
≥ 30	-3.59(-6.30 to -0.89)	0.009	-0.58(-5.58 to 4.42)	0.820	-3.91(-7.59 to -0.23)	0.037
Body fat (%)	-0.21(-0.34 to -0.08)	0.002	0.004(-0.21 to 0.21)	0.970	-0.31(-0.50 to -0.11)	0.002
Waist circumference (cm)	-0.13(-0.21 to -0.04)	0.005	0.05(-0.10 to 0.20)	0.498	-0.18(-0.30 to -0.07)	0.002

β-coefficient represents the difference in serum 25(OH)D in nmol/L per unit difference in the predictor. Estimates are adjusted for age and sex (except for age adjusted for sex only and sex adjusted for age only)

Ref, reference category; PAEE, physical activity energy expenditure; LPA, Light physical activity; MVPA, moderate to vigorous PA; BMI, body mass index

3.6.3 Vitamin D status and cardiometabolic risk factors

The associations between serum 25(OH)D concentration and outcomes are shown in table 3.7. There was an inverse association between serum 25(OH)D and the metabolic syndrome score in the unadjusted model (β-coefficient -0.30, 95% CI -0.55 to -0.05 per 1 SD (12.5 nmol/L) of 25(OH)D). This was attenuated and became non-significant after adjusting for age, smoking status, alcohol intake and education level (model 2). For the glycaemic markers, an inverse association between serum 25(OH)D concentration and fasting blood glucose was observed, which remained significant after adjusting for age, sex, smoking status, alcohol intake and education level, residential site, season of data collection, BMI and PAEE (β-coefficient -0.17, 95% CI -0.29 to -0.06 per 1 SD of 25(OH)D) (model 5). Serum 25(OH)D concentration was also inversely associated with 2-h blood glucose and HOMA-IR but this was attenuated and became non-significant after adjusting for potential confounders (model 2 for HOMA-IR and model 4 for 2-h glucose). There was no evidence of a non-linear association between serum 25(OH)D concentrations and metabolic syndrome score (Figure 3.8)

There was evidence of interaction between serum 25(OH)D and rural/urban residential site on the metabolic syndrome score (p-value for interaction = 0.016 in model 5). In stratified analysis, a significant inverse association between serum 25(OH)D concentration and metabolic syndrome score was observed in the urban subgroup (β -0.52, 95% CI -0.87 to -0.16, per 1 SD of 25(OH)D) but not in the rural subgroup (+0.21, -0.06 to +0.49) in model 3 adjusted for age, sex, smoking status, alcohol intake, education level, residential site and season (Table 3.8). The inverse association in the urban subgroup was attenuated and became non-significant after adjusting for BMI and objectively measured physical activity. No evidence of interaction was observed by sex or obesity status (p-value > 0.05).

Results were unchanged in the sensitivity analyses in which I a) used multiple imputation to investigate the impact of missing data; b) replaced BMI with body fat in models 4 and 5; and c) adjusted for self-reported fruit and vegetable intake as a proxy for overall dietary quality in model 5. There was no association between the serum 25(OH)D and the metabolic syndrome computed without the abdominal obesity component.

Table 3.7 : Associations between serum 25(OH)D and cardiometabolic risk factors (Cameroon study)

Difference in outcome per 12.5 nmol/L (1 SD) of 25(OH)D	Model 1		Model 2		Model 3		Model 4		Model 5	
	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value
Metabolic syndrome score (n=528)	-0.30 (-0.55 to -0.05)	0.020	-0.23 (-0.47 to 0.01)	0.065	-0.18 (-0.42 to 0.05)	0.130	0.06 (-0.27 to 0.15)	0.587	0.03 (-0.24 to 0.18)	0.783
Fasting BG (mmol/L, n=537)	-0.21 (-0.34 to -0.08)	0.0003	-0.18 (-0.29 to -0.07)	0.002	-0.19 (-0.30 to -0.08)	0.001	-0.17 (-0.29 to -0.06)	0.003	-0.17 (-0.29 to -0.06)	0.003
2-h BG (mmol/L, n=530)	-0.20 (-0.34 to -0.05)	0.009	-0.17 (-0.32 to -0.02)	0.027	-0.16 (-0.30 to -0.01)	0.041	-0.14 (-0.29 to 0.01)	0.063	-0.14 (-0.29 to 0.01)	0.061
HOMA-IR index (n=534)	-0.12 (-0.19 to -0.04)	0.003	-0.07 (-0.14 to 0.02)	0.128	-0.06 (-0.14 to 0.02)	0.127	-0.03 (-0.11 to 0.05)	0.425	-0.02 (-0.10 to 0.06)	0.569

BG, blood glucose; HOMA-IR, homeostatic model assessment for insulin resistance

Model 1: Unadjusted

Model 2: Adjusted for age, sex, smoking status, alcohol intake and education level

Model 3: Model 2 + residential site (4 sites) and season (4 seasons)

Model 4: model 3 + BMI (continuous)

Model 5: model 4 + PAEE (continuous)

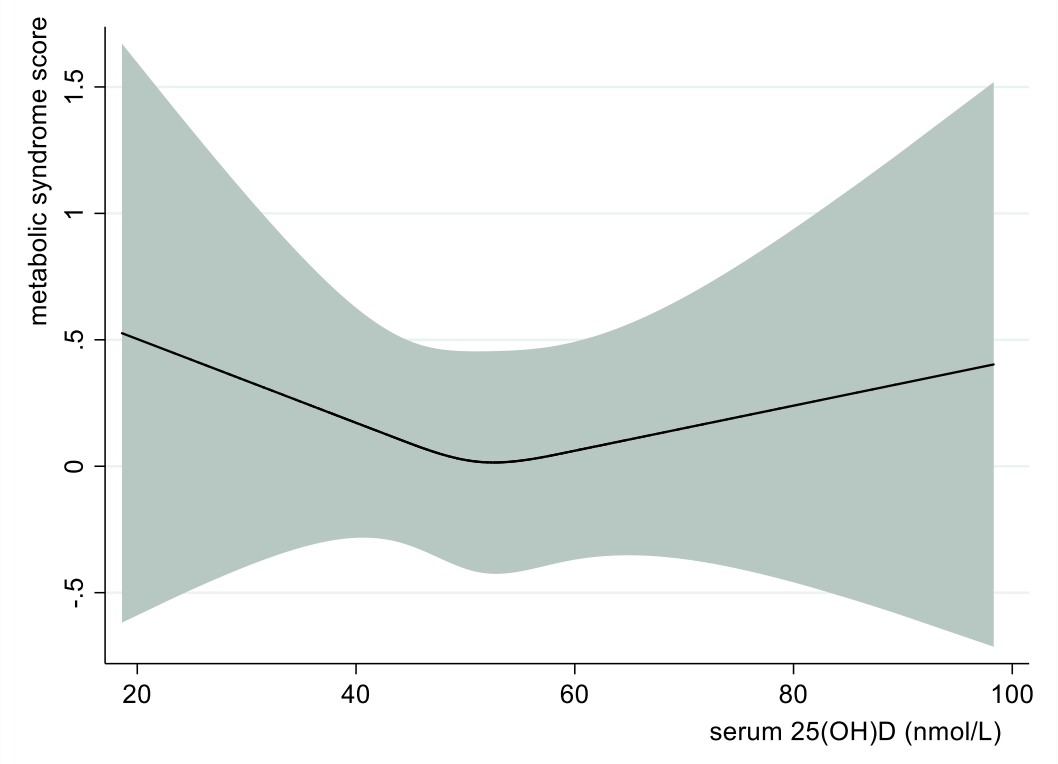

Figure 3.8: Association between serum 25(OH)D concentration and metabolic syndrome score (Cameroon Study, n = 528)

The shaded area represents the 95% confidence interval

Modelled using a restricted cubic spline function with 3 knots placed at 25th, 50th and 75th percentile. Covariates included age, sex, smoking status, alcohol intake and education level, residential site, season of data collection, BMI and PAEE.

Table 3.8 : Associations between serum 25(OH)D and metabolic syndrome score stratified by rural/urban residential site (Cameroon study, n = 528)

Difference in metabolic syndrome score per 1 SD of serum 25(OH)D	Rural (n = 245)		Urban (n = 283)	
	β (95% CI)	p-value	β (95% CI)	p-value
Model 1	0.22(-0.04 to 0.48)	0.097	-0.68(-1.06 to -0.29)	0.001
Model 2	0.22(-0.04 to 0.48)	0.101	-0.51(-0.87 to -0.15)	0.005
Model 3	0.21(-0.06 to 0.49)	0.130	-0.52(-0.87 to -0.16)	0.005
Model 4	0.23(-0.02 to 0.47)	0.074	-0.27(-0.60 to 0.05)	0.99
Model 5	0.23(-0.01 to 0.47)	0.06	-0.23(-0.55 to 0.09)	0.163

Model 1: Unadjusted

Model 2: Adjusted for age, sex, smoking, alcohol intake, education level

Model 3: Model 2 + residential site (2 sites), season (4 seasons)

Model 4: model 3 + BMI (continuous)

Table 3.9: Associations between serum 25(OH)D and cardiometabolic risk factors using multiple imputation for missing data (Cameroon study: n=586)

Difference in outcome per 1 SD difference in 25(OH)D	Model 1		Model 2		Model 3		Model 4		Model 5	
	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value
Metabolic syndrome z-score	-0.24 (-0.47 to 0.01)	0.039	-0.19 (-0.41 to 0.03)	0.098	-0.16 (-0.38 to 0.06)	0.151	-0.04 (-0.24 to 0.15)	0.664	-0.01 (-0.21 to 0.18)	0.884
Fasting BG (mmol/L,	-0.18 (-0.30 to -0.06)	0.003	-0.16 (-0.26 to -0.05)	0.003	-0.17 (-0.27 to -0.06)	0.002	-0.15 (-0.25 to -0.04)	0.008	-0.15 (-0.25 to -0.04)	0.007
2-h BG (mmol/L)	-0.19 (-0.35 to -0.04)	0.013	-0.14 (-0.29 to 0.003)	0.055	-0.15 (-0.30 to -0.003)	0.054	-0.13 (-0.28 to 0.02)	0.096	-0.13 (-0.28 to 0.02)	0.082
HOMA-IR index	-0.11 (-0.17 to -0.04)	0.004	-0.06 (-0.13 to 0.02)	0.139	-0.05 (-0.12 to 0.03)	0.203	-0.02 (-0.10 to 0.06)	0.555	-0.01 (-0.09 to 0.07)	0.778

Model 1: Unadjusted

Model 2: Adjusted for age, sex, smoking, alcohol intake, education level

Model 3: Model 2 + residential site (4 sites) and season (4 seasons)

Model 4: model 3 + BMI (continuous)

Model 5: model 4 + PAEE (continuous)

3.7 Discussion

The present analysis based on a cross-sectional population-based study of adults in rural and urban Cameroon demonstrates inverse associations between vitamin D status, assessed by serum 25(OH)D concentration, and a composite score of the metabolic syndrome and glycaemic markers. The association with fasting glucose was independent of age, sex, education, smoking, alcohol intake, season of data collection, BMI and objectively measured physical activity. Although adequate vitamin D status is crucial for bone and muscular health, the association with cardiometabolic health is still debated. These findings provide evidence that vitamin D may modulate glucose homeostasis in people in SSA who tend to have low concentrations of serum 25(OH)D.

In this study, 45.7% of the participants had vitamin D insufficiency (25(OH)D < 50 nmol/L), which is higher than the prevalence reported in Europe (40.4%) (88) and the US (23.3%) (128). Differences in assay methods for 25(OH)D limit our ability to compare the prevalence of vitamin D insufficiency with previous studies in Africa. A meta-analysis of vitamin D status in Africa reported a prevalence of vitamin D insufficiency (< 50 nmol/L) of 34% with a high variation between countries ranging from 4% in Ghana to 99% in Sudan (86). However, this reported prevalence is likely to be underestimated as over 90% of the studies used immunoassay-based techniques. Immunoassays have been shown to yield 25(OH)D concentrations that are ~ 50% higher than LC-MS/MS due to the high cross-reactivity with 24,25(OH)D_2 (120). While sunshine is abundant in Africa, several factors may account for the high prevalence of low vitamin D status in our population such as dark skin pigmentation, limited skin exposure to sunshine due to clothing and cultural practices, low dietary calcium intakes and a high burden of infectious disease (126). Moreover, participants living in rural areas had higher serum 25(OH)D concentration than those living in urban areas. Farming is the main activity in these rural areas and travelling to farms is mainly done by walking resulting in higher sunlight exposure. Similarly, higher education was associated with lower serum 25(OH)D concentration, perhaps because people with a higher level of education are more likely to have white-collar jobs which is associated with lower sunshine exposure.

In this study, I observed an inverse association between vitamin D status and metabolic syndrome score which became non-significant after adjusting for sociodemographic and health-behavioural characteristics, which is consistent with previous observational studies (80), (89), (90), (129). Other studies found an inverse association between vitamin D status and

metabolic syndrome independently of potential confounders (78), (130), (131). However, these associations could be the result of residual confounding due to the imprecise measurement of covariates such as physical activity and adiposity. Most previous studies either did not adjust for physical activity or adjusted only for self-reported physical activity, thus creating the potential for residual confounding even after adjustment. An inverse association between serum 25(OH)D concentration and fasting glucose was observed in unadjusted analysis, which remained unchanged after adjusting for potential confounders including adiposity and physical activity. This finding supports previous research suggesting that the association between vitamin D and metabolic syndrome risk is largely driven by the glycaemic parameters (80). A meta-analysis of prospective studies showed that lower serum 25(OH)D concentration was associated with higher diabetes risk (132).

Physical activity has been shown to be a strong correlate of vitamin D status, with similar associations for self-reported outdoor and indoor physical activity (93). This suggests the association between vitamin D and physical activity may be independent of sunlight exposure (115). Results from this study show that the association between serum 25(OH)D concentration and objectively measured PAEE was stronger than that with self-reported physical activity. Higher BMI, waist circumference and body fat percentage were all associated with lower 25(OH)D concentration in this analysis which is consistent with previous studies (110). Vitamin D is a fat-soluble vitamin and may be sequestrated in adipose tissues leading to lower vitamin D bioavailability. This may also explain the lower serum 25(OH)D concentration in women than in men. I accounted for adiposity by using BMI and our results were unchanged in a sensitivity analysis in which I replaced BMI with body fat % measured by bioimpedance analysis. After adjusting for adiposity measures, the inverse association of serum 25(OH)D and metabolic syndrome score was further attenuated to the null, in particular in the urban population, suggesting adiposity is an important source of confounding in the inverse association observed in the unadjusted analysis.

Mechanisms by which vitamin D may reduce the risk of cardiometabolic diseases have not been fully elucidated. Vitamin D appears to play a role directly or indirectly in insulin secretion, insulin sensitivity and systemic inflammation. Evidence from animal studies suggests a direct effect of $1,25(OH)_2D$, the active form of vitamin D on insulin secretion via the activation of vitamin D receptors expressed in the pancreatic β-cells and on insulin sensitivity by stimulating the expression of insulin receptors in peripheral target tissues (105). Indirectly, low vitamin D status could stimulate parathyroid hormone release, which has been shown to be associated

with insulin resistance. Furthermore, low vitamin D status appears to activate macrophages suggesting a role in inflammatory processes. The identification of vitamin D receptors in over 30 different tissues has led to the hypothesis of its effect on several health outcomes (107). However, in a mendelian randomisation study, genetically predicted higher 25(OH)D concentration was not associated with type 2 diabetes risk (132). In this study, the findings of an inverse association between vitamin D status and fasting glucose and the null association with HOMA-IR suggest an effect via insulin secretion rather than insulin resistance.

Most RCTs have consistently shown a null association between vitamin D supplementation and cardiometabolic endpoints including a recent trial examining intermediate endpoints (high-sensitivity CRP and N-terminal pro-B-type natriuretic peptide) (84), (85), (98), (100), (96). However, some of these trials were of small sample size and short duration, used a low dose of vitamin D, were powered to detect large effect sizes, and were not designed to study cardiometabolic diseases as primary endpoints. In addition, participants in most of these trials already had an adequate vitamin D status at baseline. In the vitamin D and type 2 diabetes study, specifically designed to test diabetes as an endpoint, vitamin D supplementation lowered diabetes risk in people with low baseline vitamin D status only (< 30 nmol/L) (84).

Considering the evidence from reviews suggesting that vitamin D status is worse in some parts of Africa compared with other parts of the world, understanding the link between vitamin D status and metabolic risk factors in this part of the world is important (94). If supported by further evidence from prospective studies and RCTs, strategies to improve vitamin D status in populations with a high burden of vitamin D deficiency may provide a cheap and feasible public health approach to improve cardiometabolic health in these populations.

3.8 Strengths and limitations

The strengths of this paper rely in the population-based nature of the study including participants from both rural and urban settings of Cameroon and considers the issue of confounding by physical activity and adiposity using objective measures. Serum 25(OH)D concentration was measured using the gold standard method leading to greater precision in the exposure. Nonetheless, some limitations warrant attention. In addition to the limitations of the Cameroon study related to the non-probability sampling used and generalisability of the findings, as discussed in Chapter 2, the major limitation of these aetiological analyses is the cross-sectional design of our study, which limits the ability to establish temporality and the possibility of reverse causation cannot be excluded. It is possible that poor metabolic health

could have led to dietary changes, reduced sun exposure, physical activity or increased inflammation, all of which could affect serum 25(OH)D concentration (reverse causality). The sample size of this study is small and therefore the subgroup analysis stratified by rural/urban area of residence should be interpreted with caution. Data were not available on sun exposure or dietary intake of vitamin D from natural, fortified food or dietary supplements. However, it is widely known that the contribution of vitamin D from natural food sources is low. Because there is no mandatory vitamin D fortification of foods and the use of dietary supplements among healthy African adults is low, sunlight exposure is likely to be the main source of vitamin D in this population (133). The proportion of participants in our study with serum 25(OH)D concentrations below 30 nmol/L or above 75 nmol/L was low, which limited my ability to investigate the relationship at the extremes of the range. Future prospective studies and trials are needed to confirm the role of vitamin D on metabolic health in populations at high risk of vitamin D deficiency or insufficiency.

3.9 Conclusion

This population-based study showed an inverse association between serum 25(OH)D concentration and a composite score of the metabolic syndrome, which was confounded by socio-demographic characteristics. The inverse association of serum 25(OH)D with fasting glucose was independent of potential confounders including objectively measured physical activity and adiposity. This suggests that public health interventions to improve vitamin D status may improve glucose metabolism in this population and warrant further investigation in prospective studies.

Chapter 4 : Associations of plasma carotenoids, vitamin C, tocopherol and fasting glucose

Publication

Mba CM, Koulman A, Forouhi NG, Imamura F, Assah F, Mbanya JC, Wareham JN. The associations of plasma carotenoids and α-tocopherol concentrations with fasting glucose in adults in Cameroon (Manuscript under review by co-authors)

Summary

Background: Dietary approaches to prevent type 2 diabetes include recommendations to increase intake of fruits and vegetables. However, epidemiological evidence of the associations between biomarkers estimating intake of fruits and vegetables and type 2 diabetes show mixed results. I aimed to examine the associations of plasma carotenoids and α-tocopherol with fasting glucose in adults in Cameroon.

Methods: This was a population-based cross-sectional study of 592 adults from rural and urban areas of Cameroon. Self-reported intake of fruits and vegetables was assessed using the WHO STEPS questionnaire. The exposures were plasma total and individual carotenoids and α-tocopherol. Linear regression models were fitted to assess the associations between the exposures and fasting glucose.

Results: The mean ± SD age of participants was 38.5 ± 8.6 years (63.7% women). Plasma total carotenoids was positively correlated with self-reported intake of fruits (r = 0.13) and vegetables (r = 0.29), both p-value < 0.01. Plasma total carotenoids and α-carotene were inversely associated with fasting glucose. In unadjusted analysis, the difference in fasting glucose comparing the highest against the lowest tertile of the biomarkers concentrations was -0.28(95% CI -0.56 to -0.001) mmol/L for total carotenoids and -0.31(-0.59 to -0.03) mmol/L for plasma α-carotene. The inverse associations became stronger after adjusting for age, sex, education level, residential site, smoking status, alcohol intake, season, objectively measured physical activity, BMI and total cholesterol (-0.36(-0.73 to -0.002) mmol/L for total carotenoids and -0.41(-0.79 to -0.03) mmol/L for α-carotene). There was no evidence of an association between α-tocopherol and fasting glucose.

Conclusion: In this population-based study with the inclusion of participants from rural and urban settings, there was an inverse association of fruit and vegetables intake assessed using objectively measured biomarkers with fasting glucose. This contributes to the growing evidence that a higher intake of fruits and vegetables is likely to be beneficial for diabetes prevention in this population.

4.1 Introduction

The 2017 global burden of disease study estimated that 11 million deaths globally were attributable to dietary risk factors, with CVDs and diabetes among the leading causes of diet-related deaths. Specifically, low intake of fruits and vegetables accounted for 3.9 million global deaths (134). The incidence of type 2 diabetes has risen substantially over the past 3 decades and identifying modifiable risk factors for interventions is a major public health priority (4).

A higher intake of fruit and vegetables is widely promoted for the prevention of NCDs including type 2 diabetes (72). However, epidemiological studies on the associations between fruit and vegetable intake are inconclusive partly owing to the use of traditional dietary assessment methods, which are prone to measurement error and recall bias (135), (136), (137). In a meta-analysis of 23 cohort studies, high compared with low intake of fruit and vegetables combined was associated with a 7% lower risk of type 2 diabetes, but a higher diabetes risk was observed for intakes of some subtypes of fruits and vegetables (138). Low consumption of fruits and vegetables is common worldwide (~ 100g/day of fruits and 200g/day of vegetables) (134). The Prospective Urban Rural Epidemiology study showed that fruits and vegetables are less affordable in low and middle-income countries than in high-income countries (139).

Objectively measured nutritional biomarkers offer a complementary approach to assessing dietary intake since they do not rely on the participant's memory. Circulating carotenoids and vitamin C widely found in fruits and vegetables have been proposed as objective indicators of intake of fruits and vegetables with evidence from observational and experimental studies (140), (141), (142), (143), (144). In a meta-analysis of randomised controlled feeding trials, study arms provided with more fruits and vegetables showed an increase in plasma vitamin C and individual carotenoids concentrations (140). Circulating α-tocopherol has also been suggested as a biomarker of intake of fruit and vegetables, although less consistently than circulating vitamin C and carotenoids (141). Results from cross-sectional and prospective studies on the association between circulating carotenoids, vitamin C and α-tocopherol estimating intake of fruits and vegetables and type 2 diabetes are inconsistent indicating null (145), (146), inverse (147), (148), (149), (150) or positive associations (150). I did not find any study conducted in Africa where 79.1% of adults consume less than the recommended five portions a day of fruits and vegetables (151). The apparent discrepancy in study findings may suggest differences in the fruits and vegetables sources of the biomarkers, food matrices and food preparation, which vary across populations.

Improving understanding of the association between intake of fruits and vegetables and type 2 diabetes, particularly in populations where there is a high proportion of inadequate intake of fruit and vegetables using objective assessment methods to estimate intakes is important. Results from the Cameroon WHO STEPS in 2003 showed that fruits and vegetables were consumed only ~ 3 days a week (152).

4.2 Study aims

This study aimed to identify factors that affect the nutritional biomarkers that reflect intake of fruits and vegetables (plasma carotenoids, vitamin C and α-tocopherol) and to examine the associations between the nutritional biomarkers and fasting glucose in rural and urban settings of Cameroon.

4.3 Background on carotenoids, tocopherol and vitamin C

4.3.1 Food sources, metabolism and functions of carotenoids, tocopherol and vitamin C

- **Food sources of carotenoids and tocopherol**

Carotenoids are phytochemicals found in many fruits and vegetables and are responsible for their yellow to reddish pigment. Carotenoids are hydrophobic molecules and are broadly classified into 2 groups based on their chemical structures: carotenes and xanthophylls. Carotenes are made up of hydrocarbons only while xanthophylls contain both hydrocarbons and oxygen atoms.

To date, over 750 different carotenoids have been identified, but only ~ 40 have been detected in human samples (blood, milk and tissues) (153). Of these, six carotenoids have been found at a higher concentration than the rest and received most of the research attention. These are three carotenes: α- and β-carotene and lycopene, and three xanthophylls: β-cryptoxanthin, lutein and zeaxanthin. Different fruits and vegetables contain different amounts of carotenoids. Table 4.1 shows some of the fruits and vegetables sources of the six most studied carotenoids. The content of fruits and vegetables in carotenoids may vary depending on the size, degree of maturity, growing conditions, food processing, storage, and cooking methods.

- Alpha- and β-carotene

β-carotene is the most abundant of all the carotenoids. α-carotene is usually present in the same foods as β-carotene but in lower amounts. α- and β-carotene are present in substantial amounts in orange coloured fruits and vegetables such as mango, carrot, pumpkin and apricot.

- Lycopene

Lycopene is the phytochemical that gives the red coloured pigment to fruits and vegetables and is found in high amounts in tomato, and tomato products. Other rich sources include watermelon and pink grapefruit.

- β-cryptoxanthin

β-cryptoxanthin is present in orange coloured fruits and vegetables and rich sources include citrus fruits such as mandarin, tangerines, oranges, and clementine

- Lutein and zeaxanthin

Lutein and zeaxanthin are the most abundant carotenoids found in dark green leafy vegetables and rich sources include spinach, kale, broccoli and lettuce. Some fruits like kiwi and grapes and egg yolk can also contribute to the dietary intake of lutein and zeaxanthin (154). Lutein is more abundant than zeaxanthin in most foods with a ratio of ~ 5:1.

Table 4.1 : Some food sources of carotenoids (153, 154)

	Carotenoids	Food sources
Carotenes	α- and β-carotene	- Carrots - Mango - Pumpkin - Apricot
	Lycopene	- Tomatoes - Tomatoes products - Watermelon
Xanthophylls	β-cryptoxanthin	Orange coloured fruits and vegetables such as: - Mandarin - Tangerine - Clementine
	Lutein and zeaxanthin	Green leafy vegetables such as: - Broccoli - Kale - Spinach - Lettuce

Alpha-carotene, β-carotene and β-cryptoxanthin are important sources of vitamin A, given that they have provitamin A activity. These carotenoids are provitamin A that can be cleaved into retinal which is further metabolised into retinol (vitamin A) and retinoic acid. The conversion efficacy of β-carotene is higher than that of α-carotene and β-cryptoxanthin.

- Tocopherol

Vitamin E occurs in nature in different structurally related forms of which α- and γ-tocopherols have been most widely studied. α-tocopherol is the most abundant form of vitamin E in the diet. Humans and animals do not synthesize vitamin E which must be acquired from plant diets. Tocopherols are mostly found in plant seeds and products that derive from them such as vegetable oils (soybean, cottonseed, corn and sesame oils) (155). Fruits and vegetables such as avocado mango, pumpkin and spinach contain some amount of tocopherol.

- **Metabolism of carotenoids and tocopherol**

Both carotenoids and tocopherols are lipophilic molecules, so following the ingestion of a meal, carotenoids and tocopherols are emulsified with fat and then incorporated into the lipid micelles at the level of the small intestines. The food matrix affects the bioavailability of carotenoids and tocopherols. For example, the amount of fat in food appears to increase the bioavailability of carotenoids and tocopherols by facilitating absorption, thereby increasing plasma concentrations of these biomarkers (156).

Absorption of carotenoids and tocopherols occurs in the intestines similar to dietary fat and mainly via passive diffusion. Carotenoids and tocopherols are packed into chylomicrons alongside other fat-soluble nutrients inside the intestinal cells. Carotenoids and tocopherols are transported to the liver via the lymphatic system where some are stored and others released into the general circulation. In circulation, carotenoids and tocopherols are transported by HDL, LDL and VLDL cholesterol which is responsible for the distribution to other tissues (157). A positive correlation has been shown between plasma total cholesterol and plasma total carotenoids *(r* = 0.57) (158) and α-tocopherol (*r* = 0.78) (157).

- **Food sources and metabolism of vitamin C**

Vitamin C also known as ascorbic acid is a water-soluble vitamin that can only be obtained from the diet given that humans lack the enzyme which catalyses the final step of synthesis of vitamin C. Vitamin C is found in a wide variety of fruits and vegetables and good sources include citrus fruits, strawberries, brussels, broccoli and other leafy vegetables (159).

Upon ingestion of food, vitamin C is released from the food matrix and absorbed by the epithelial cells of the small intestines mainly by active transport via active sodium-dependent vitamin C transporters. The efficiency of absorption of vitamin C varies depending on the amount of vitamin C in the diet. At low doses, almost 100% of vitamin C is absorbed, but at high doses, only ~ 20% is absorbed. Vitamin C in circulation is mainly unbound and in the oxidation forms ascorbyl radical, monodehydro-L-ascorbic acid. During oxidative stress, these oxidation forms are converted to dehydroascorbic acid. Dehydroascorbic acid is transported on glucose transporters into the cells where it is recycled into vitamin C. Because dehydroascorbic acid is transported by glucose transporters, uptake of the vitamin C into the cells is reduced during hyperglycaemia due to saturation of glucose transporters (159). Therefore, low plasma concentrations of vitamin C may be a consequence of hyperglycaemia.

- **Functions of vitamin C, carotenoids and tocopherol**

Most of the health-related benefits of vitamin C, carotenoids and tocopherols are attributable to their anti-oxidative properties. These biomarkers are potent antioxidants that have the ability to scavenge toxic free reactive oxygen and nitrogen species. Reactive oxygen species can cause oxidative damage to cell proteins, lipids and nucleic acid in the DNA and RNA material. Vitamin C, carotenoids and tocopherol also have anti-inflammatory properties (160). Both oxidative stress and systemic inflammation are associated with the development of type 2 diabetes (161).

Some carotenoids have provitamin A activity and can be converted to vitamin A when vitamin A levels are low. Lutein and zeaxanthin concentrated in the macula of the eye help to absorb high UV wavelength thereby preventing damage to the eye (162). Vitamin C is an important cofactor for many enzymes, including enzymes involved in collagen synthesis. Severe vitamin C deficiency causes scurvy. Vitamin C also facilitates iron absorption in the intestines by maintaining iron ion in the ferrous state (160).

4.3.2 Plasma vitamin C, carotenoids and tocopherol as biomarkers of intake of fruits and vegetables

Biomarkers offer a complimentary objective approach to the traditional dietary assessment methods which rely on memory as discussed in chapter 2. Specifically, social desirability bias has been shown to occur in the assessment of fruit and vegetables intake resulting in an

overestimation of self-reported intakes of fruits and vegetables (44). Plasma vitamin C, carotenoids and tocopherol widely found in fruits and vegetables have been proposed as objective indicators of intake of fruits and vegetables.

Most observational studies have reported a modest positive correlation between self-reported intake of fruits and vegetables and vitamin C and individual carotenoids, probably because of measurement errors of self-reports and issues related to the linkage of nutrient intakes to food composition databases (163), (143), (164). A meta-analysis showed a positive correlation between dietary and plasma vitamin C with a correlation coefficient of 0.35 when the FFQ was used and 0.46 with 24-h recall (163). More robust evidence comes from intervention studies with direct observations or provision of foods to participants. In a meta-analysis of RCTs, groups provided with more fruits and vegetables showed an increase in blood concentrations of plasma vitamin C and individual carotenoids (140). Although circulating α-tocopherol has also been postulated as an objective indicator of intake of fruit and vegetables studies reporting on the association of fruit and vegetable intake with tocopherol are less consistent (165), (142).

Plasma vitamin C, carotenoids and tocopherol are concentration biomarkers and as discussed in Chapter 2, they do not reflect absolute intake given that they are affected by stress, alcohol intake, smoking and physical activity (163). In particular, the lower plasma carotenoids concentrations in smokers have been widely described (146), (166). Cigarette contains free radicals, which induce oxidative stress leading to increase utilisation of anti-oxidants such as carotenoids.

4.3.3 Dietary requirements for vitamin C, carotenoids and tocopherol

There are no reference nutrient intakes for carotenoids probably because deficiencies in carotenoids intake have not been associated with specific diseases per se. Vitamin C deficiency is associated with scurvy. In the UK, the RNI for vitamin C for people aged 15 years and above is 40 mg/day; 35 mg/day in adolescents aged 11-14 years, and 30 mg/day in children aged 1-10 years (167). For vitamin E, the RNI for people aged 14 years and above is 15 mg/day in the form of α-tocopherol; 11 mg/day in adolescents aged 9-13 years, 7 mg/day in children aged 4-8 years and 6 mg/d in children aged 1-3 years (168).

4.3.4 Recommendation for intake of fruits and vegetables

The WHO recommends the consumption of at least five 5 servings of fruits and vegetables (400 g) per day as part of a healthy diet (72). A high intake of fruits and vegetables has been

suggested to play a role in the prevention of non-communicable diseases including diabetes and CVDs (138), (169), (170). Observational studies of the association between intake of fruits and vegetables and diabetes and CVDs show inconsistent findings, with some studies showing a weak or modest inverse association (171), (136), (169), (172) and others showing no evidence of an association (173), (174). Other studies have shown a positive association between fruit juice and diabetes risk (171). The inverse association of fruit intake with diabetes has been slightly more consistent than for vegetables (170).

Several factors may explain the potential cardiometabolic benefits associated with intake of fruits and vegetables. Apart from being rich sources of vitamin C, carotenoids and tocopherol, fruits and vegetables are also high in dietary fibres, minerals like magnesium and non-nutritive compounds like polyphenols. Higher intake of dietary fibres has been associated with weight loss partly owing to increase satiety (175). Moreover, there is evidence suggesting that dietary fibres and polyphenols modulate the gut microbiome composition (176). An altered gut microbiome has been associated with diabetes and CVDs.

4.4 Methods

The study design, setting and other common methods and procedures used in this cross-sectional study are reported in Chapter 2.

4.4.1 Measurement of biomarkers

- **Measurement of plasma carotenoids and tocopherol**

A total of 592 participants had plasma samples for the measurement of the biomarkers. Plasma carotenoids and tocopherol were analysed on the same samples at the NIHR Cambridge BRC Nutritional Biomarker Laboratory using high-performance liquid chromatography coupled with a photodiode array detector (HPLC-PDA) using analytical methods described previously (20).

Briefly, plasma samples were deproteinised with methanol followed by liquid-liquid extraction in hexane. The samples were dried and reconstituted in ethanol and acetonitrile in 2 steps. The samples were injected onto HPLC (Waters Acquity, Waters UK, Herts, UK) and chromatographic separation was performed with a YMC-pack pro C18 analytical column; 3µm, 4.6mm ID x 150mm and with a 25:75 Ethanol + 0.1% triethylamine mobile phase at 1.2mL/min. Detection was accomplished by a photodiode array detector. Plasma concentrations of six carotenoids (α- and β-carotene, lycopene, β-cryptoxanthin, lutein and

zeaxanthin) and 2 tocopherols (α and γ-tocopherol) were determined. Lutein and zeaxanthin were not differentiated. Carotenoids were quantified at 450 nm and tocopherol at 300 nm. The stable isotope-labelled internal standard added during the extraction step helps to normalise the sample preparation process and instrument detection variability. The ratio of the signal of the metabolite to the internal standard obtained was compared against that of a calibration curve to determine the concentration of the individual biomarkers. The sample preparation and laboratory analysis were conducted in a darkened room under non-actinic lighting to prevent degradation of tocopherol due to its photosensitive nature.

I imputed random values between zero and the lower limit of quantification (LOQ) for biomarkers with < 5% of missing data (0.17% α-tocopherol, 2.87% lutein + zeaxanthin, 4.9% for lycopene). β-cryptoxanthin was not included in the analysis because of the high proportion of values below the lower LOQ (> 30%).

- **Measurement of plasma vitamin C**

Fluorometric assay of vitamin C in plasma samples was done based on a procedure described by Vuilleumier and Keck (177). Plasma samples were stabilised by the addition of an equal volume of 10% metaphosphoric acid as described in the pre-analytical step in Chapter 2. Ascorbate oxidase was added to the plasma sample to convert ascorbic acid in the sample to dehydroascorbic acid. The resulting dehydroascorbic acid was coupled with o-phenylene diamine to form a fluorescent derivative quinoxaline which was measured on BMG Labtech FLUOstar OPTIMA plate reader. The amount of quinoxaline formed is linearly related to the amount of vitamin C in the sample.

Concentrations of plasma vitamin C were undetectable in all the samples, probably because the samples were not initially collected in tubes containing metaphosphoric acid. In the absence of stabilising agents such as metaphosphoric acid, perchloric acid or dithiothreitol, vitamin C is rapidly oxidised in sample extracts leading to its irreversible degradation. This makes analyses of vitamin C challenging in large-scale epidemiological studies where samples are not always collected specifically for vitamin C measurements.

I initially intended to include plasma vitamin C in these analyses; due to the undetectable levels of plasma vitamin C in all the samples, this aim could not be satisfied. Therefore, the results presented below are only for plasma carotenoids and α-tocopherol.

4.4.2 Outcome

The outcome for this analysis was fasting glucose.

4.5 Statistical analyses

All statistical analyses were performed using STATA 15 (StataCorp, College Station, TX). I calculated plasma total carotenoids as the sum of plasma α- and β-carotene, lycopene, lutein and zeaxanthin. The Spearman correlation coefficient was calculated between plasma concentrations of total and individual carotenoids, α-tocopherol and self-reported intake of fruits and vegetables. Multiple linear regression models adjusted for age and sex were fitted to identify the potential correlates of continuously distributed plasma carotenoids and α-tocopherol after log transforming the values of plasma total carotenoids, β-carotene and lycopene to account for their skewed distributions.

Plasma total and individual carotenoids, and α-tocopherol were the exposures of interest. I fitted restricted cubic splines with 3 knots corresponding to the 25^{th}, 50^{th} and 75^{th} percentile of continuously distributed biomarkers using model 4 to model any non-linear association between the biomarkers and fasting glucose. Non-linearity was tested using the Wald test. I further categorised plasma carotenoids and α-tocopherol into tertiles and fitted linear regression models to assess their associations with fasting glucose. Using a block-wise approach, four models, incrementally adjusted for potential confounding variables based on biological plausibility were fitted. After fitting crude regression models (model 1), I further adjusted for age (continuous) and sex (model 2), and then for smoking (never, past or current), alcohol intake (never, past or current), level of education (less than primary school, completed primary school, secondary school or university), residential site (4 sites), PAEE (continuous) and BMI (model 3) and total cholesterol (model 4). P-values for trend were obtained from linear regression models including the biomarkers as an ordinal variable across tertile categories. With missing information observed for some covariates (PAEE, n= 50; total cholesterol, n= 26) complete case analyses were performed.

I tested for effect modification by sex, rural-urban area of residence, BMI categories and smoking status on the associations of plasma carotenoids and α-tocopherol with fasting glucose and performed subgroup analysis if the p-value for interaction term was < 0.05. In sensitivity analyses, I imputed missing values of covariates using multiple imputation by chained equations to assess the impact of the missing data on the associations of plasma carotenoids

and α-tocopherol with fasting glucose. I used multiple imputation by chained equations to create 10 multiply imputed data sets and used Rubin's rule to combine estimates across the imputed datasets (127).

4.6 Results

4.6.1 Descriptive characteristics

The median (25^{th}-75^{th} percentile) of plasma total carotenoids was 4.5 (2.9-6.4) μmol/L and the mean ± SD of α-tocopherol was 19.7 ± 5.6 μmol/L. Participants living in urban areas had higher plasma concentrations of total carotenoids (4.9(3.1-6.8) μmol/L and α-tocopherol (20.3 ± 5.9 μmol/L), than those living in rural areas (total carotenoids: 4.2(2.5-6.1) μmol/L, α-tocopherol: 19.0 ± 5.2 μmol/L), p-value <0.01 for both comparisons. Women had higher plasma total carotenoids (4.9(3.2-6.9) μmol/L) and α-tocopherol (20.4 ± 5.9 μmol/L) than men (total carotenoids: 3.9(2.3-6.0) μmol/L, α-tocopherol: 18.6 ± 5.0 μmol/L), p-value <0.001 for both comparisons. The distribution of the individual plasma carotenoids is shown in Figure 4.1.

The median (25^{th}-75^{th} percentile) number of times participants self-reported consuming fruits in a typical week was 2(1-5) times/week and the comparable figure for vegetable intake was 4(2-7) times/week. Participants living in rural areas and women reported a higher frequency of fruit consumption (3(1-6) times/week) than urban residents and men (2(1-4) times/week), p-value for both comparisons < 0.01. Similarly, the frequency of self-reported vegetable intake was higher in rural residents (5(2-9) times/week) and women (4(2-8) times/week) than in urban residents (3.5(2-6) times/week) and men (3(2-6) times/week), p-value < 0.001 for both comparisons.

Plasma total carotenoids, individual carotenoids and α-tocopherol were positively correlated with each other (Table 4.2). Plasma β-carotene was the highest contributor to total carotenoids (58.9%) followed by α-carotene (25.5%) (Figure 4.2). Plasma total carotenoids, α- and β-carotene were positively correlated with both self-reported intake of fruits and vegetables with correlation coefficients ranging from 0.13 to 0.35. Plasma lutein-zeaxanthin showed a positive correlation with self-reported vegetable intake only, while plasma lycopene was negatively correlated with self-reported intake of fruits and vegetables.

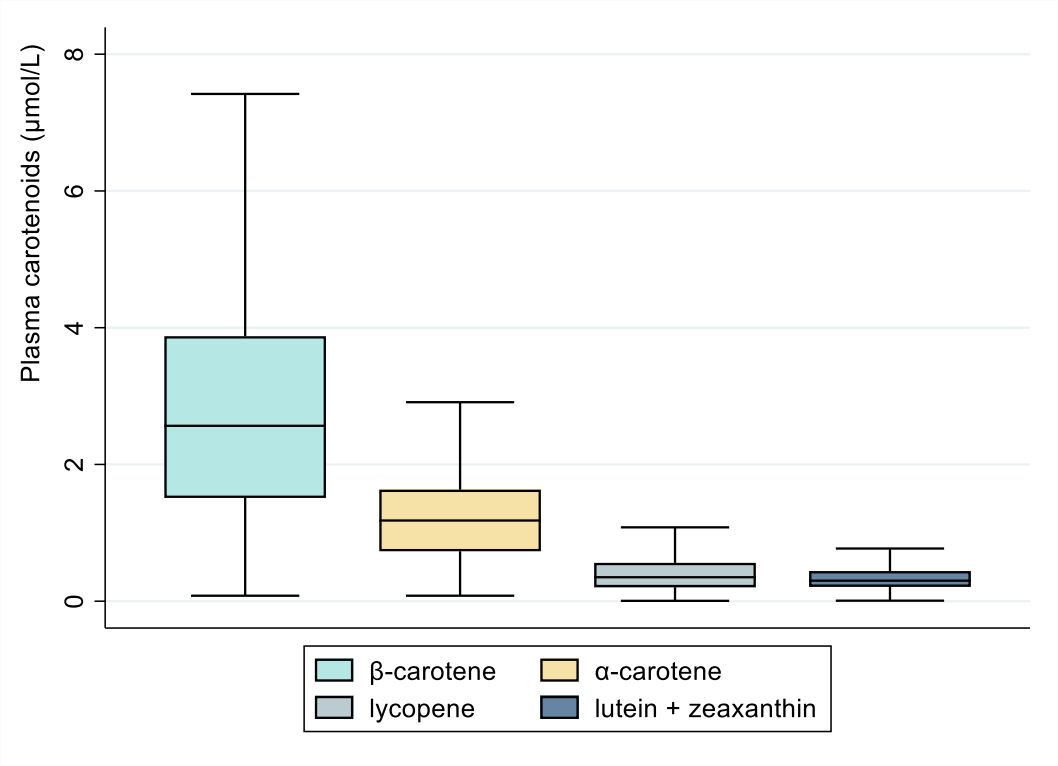

Figure 4.1 : Distribution of plasma individual carotenoids concentrations (µmol/L) Cameroon study, n = 592)

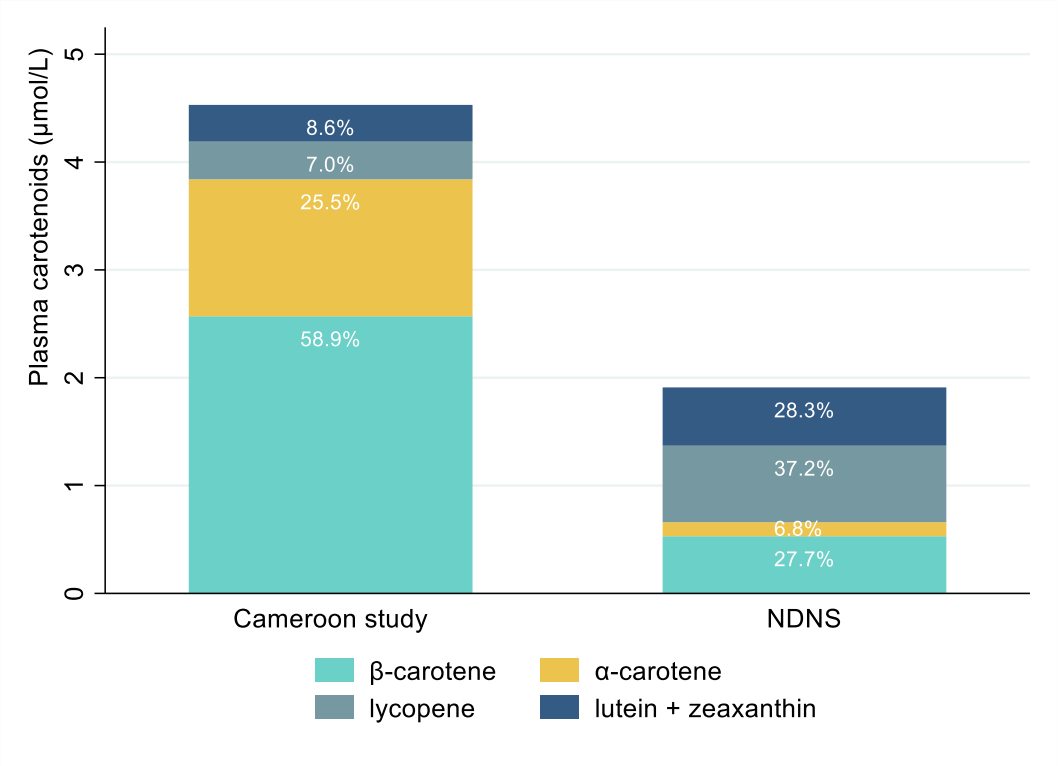

Figure 4.2 : Contribution of plasma individual carotenoids to total carotenoids in the Cameroon study (Compared with the National Diet and Nutrition Examination Survey year 1-4 combined (178))

Table 4.2 : Pairwise correlation between individual biomarkers and self-reported fruit and vegetables intake (Cameroon study, n = 592)

	α-carotene	β-carotene	Lycopene	Lutein + zeaxanthin	α-tocopherol	Fruit intake	Vegetable intake
Total carotenoids	0.97‡	0.98‡	0.46‡	0.62‡	0.47‡	0.13†	0.29‡
α-carotene		0.95‡	0.38‡	0.56‡	0.41‡	0.14†	0.32‡
β-carotene			0.32‡	0.53‡	0.39‡	0.17‡	0.35‡
lycopene				0.42‡	0.57‡	-0.12*	-0.29‡
Lutein-zeaxanthin					0.47‡	0.06	0.13†
α-tocopherol						-0.04	-0.03
Fruit intake							0.32‡

Results are spearman correlation coefficients

* p-value < 0.05; †p-value < 0.01; ‡p-value < 0.001

4.6.2 Factors affecting plasma carotenoids and tocopherol concentrations

After adjusting for age and sex, factors that were positively associated with plasma total carotenoids and α-tocopherol were age (adjusted for sex only), living in urban areas, level of education, the season of light rain and self-reported vegetable intake, self-reported physical activity and plasma cholesterol concentrations (Table 4.3). The male sex and season of heavy rain were inversely associated with both plasma total carotenoids and α-tocopherol. Current smoking status was inversely associated with plasma total carotenoids only while self-reported intake of fruit and vegetables was inversely associated with plasma α-tocopherol. Factors positively associated with self-reported intake of fruits and vegetables were the season of light rain and physical activity and factors associated inversely were the male sex (adjusted for age only), living in urban areas and BMI (Table 4.4).

Table 4.3 : Factors affecting plasma carotenoids and α-tocopherol concentrations (Cameroon study, n=592)

	β-coefficient (95 % confidence interval)					
	Total carotenoids	α-carotene	β-carotene	Lycopene	Lutein + zeaxanthin,	α-tocopherol
Age (10 y)	0.60(0.30 to 0.90)	0.10(0.05 to 0.20)	0.20(0.09 to 0.30)	0.07(-0.009 to 0.20)	0.05(0.03 to 0.07)	1.8(1.3 to 2.3)
men (vs women)	-0.89(-1.37 to -0.40)	-0.16(-0.28 to -0.04)	-0.40(-0.55 to -0.24)	0.11(-0.03 to 0.25)	-0.02(-0.06 to 0.01)	-1.29(-2.16 to -0.44)
Urban (vs rural)	0.75(0.28 to 1.23)	0.22(0.10 to 0.33)	0.49(0.12 to 0.98)	0.50(0.37 to 0.63)	0.11(0.07 to 0.14)	1.57(0.70 to 2.44)
Education level						
<primary school (ref)						
Primary school	0.96(0.31 to 1.61)	0.24(0.09 to 0.40)	0.27(0.06 to 0.48)	0.32(0.13 to 0.51)	0.07(0.03 to 0.11)	1.23(-0.01 to 2.48)
Secondary school	0.99(0.27 to 1.72)	0.24(0.06 to 0.41)	0.27(0.05 to 0.49)	0.62(0.41 to 0.83)	0.11(0.06 to 0.16)	2.71(1.25 to 4.17)
University	1.57(0.76 to 2.37)	0.39(0.19 to 0.60)	0.46(0.20 to 0.71)	0.79(0.56 to 1.03)	0.13(0.07 to 0.18)	2.27(0.80 to 3.74)
Smoking status						
Never (ref)						
Former	-0.23(-0.95 to 0.49)	-0.04(-0.22 to 0.14)	-0.02(-0.25 to 0.20)	-0.17(-0.36 to 0.02)	-0.03(-0.08 to 0.03)	-0.27(-1.56 to 1.01)
Current	-0.97(-1.78 to -0.16)	-0.22(-0.44 to -0.003)	-0.41(-0.75 to -0.08)	-0.41(-0.74 to -0.08)	-0.05(-0.11 to 0.006)	-1.06(-2.66 to 0.54)
Alcohol intake						
Never (ref)						
Former	0.29(-0.71 to 1.29)	0.09(-0.16 to 0.34)	0.06-0.21 to 0.34)	-0.04(-0.34 to 0.26)	0.04(-0.06 to 0.14)	-0.56(-2.43 to 1.31)
Current	0.24(-0.43 to 0.92)	0.14(-0.03 to 0.31)	0.08(-0.13 to 0.29)	-0.19(-0.38 to 0.01)	-0.02(-0.08 to 0.05)	-1.31(-2.59to -0.03)
Marital status						
Single (ref)						
Married	0.29(0.96 to 2.61)	0.10(-0.06 to 0.26)	0.16(-0.03 to 0.35)	0.01(-0.17 to 0.20)	0.03(-0.008 to 0.07)	0.66(-0.41 to 1.72)
Separated	0.22(-1.29 to 1.72)	0.06(-0.29 to 0.41)	0.02(-0.48 to 0.51)	-0.47(-0.99 to 0.06)	-0.02(-0.12 to 0.08)	-1.67(-4.83 to 1.48)
Widow	-0.007(-1.13 to 1.13)	0.03(-0.25 to 0.31)	0.09(-0.25 to 0.43)	-0.40(-0.77 to -0.04)	0.003(-0.08 to 0.08)	0.04(-2.19 to 2.27)
Season						
Long dry (ref)						
Light rain	1.78(0.96 to 2.61)	0.39(0.19 to 0.58)	0.41(0.22 to 0.61)	-0.06(-0.27 to 0.15)	0.13(0.07 to 0.18)	-0.66(-1.91 to 0.60)
Short dry	0.53(0.004 to 1.05)	0.09(-0.05 to 0.22)	-0.01(-0.16 to 0.14)	-0.04(-0.06 to 0.15)	0.09(0.05 to 0.13)	-0.42(-1.44 to 0.61)
Heavy rain	-1.01(-1.60 to -0.41)	-0.24(-0.40 to -0.08)	-0.34(-0.56 to -0.11)	-0.36(-0.58 to -0.14)	-0.05(-0.09 to -0.02)	-1.91(-3.30 to -0.53)
Fruit (times /week)	0.04(-0.03 to 0.11)	0.01(-0.006 to 0.03)	0.01(-0.008 to 0.03)	-0.04(-0.06 to -0.02)	-0.002(-0.01 to 0.003)	-0.16(-0.29 to -0.04)
Vegetable (times /week)	0.12(0.05 to 0.18)	0.03(0.02 to 0.05)	0.04(0.02 to 0.06)	-0.06(-0.07 to -0.04)	-0.001(-0.005 to 0.003)	-0.22(-0.33 to -0.11)

PAEE (MJ/kg/d)	1.08(-9.7 to 11.8)	1.04(-1.63 to 3.72)	2.82(-0.04 to 5.70)	-6.87(-9.75 to -4.0)	-2.1(-4.31 to 0.30)	-39.0(-58.4 to -19.6)
Objective sedentary time (h/day)	0.05(-0.05 to 0.15)	0.007(-0.002 to 0.04)	-0.02(-0.04 to 0.01)	0.04(0.01 to 0.07)	0.02(-0.003 to 0.04)	0.23(0.05 to 0.41)
Objective LPA (h/day)	-0.12(-0.27 to 0.03)	-0.003(-0.07 to 0.01)	-0.008(-0.05 to 0.03)	-0.03(-0.07 to 0.01)	-0.008(-0.02 to 0.003)	-0.07(-0.33 to 0.19)
Objective MVPA (h/day)	0.03(-0.13 to 0.18)	0.02(-0.002 to 0.007)	0.05(0.001 to 0.09)	-0.07(-0.11 to -0.03)	-0.03(-0.07 to 0.006)	-0.60(-89 to -0.30)
GPAQ PAEE (MJ/kg/d)	5.75(2.76 to 8.74)	1.68(0.96 to 2.39)	2.22(1.49 to 0.2.95)	-1.92(-2.63 to -1.21)	-0.66(-1.24 to -0.08)	-11.5(-16.9 to -6.13)
GPAQ work	0.08(0.04 to 0.12)	0.002(0.001 to 0.003)	0.03(0.02 to 0.04)	-0.03(-0.04 to -0.02)	-0.01(-0.02to -0.003)	-0.14(-0.22 to -0.06)
GPAQ leisure	-0.07(-0.21 to 0.06)	-0.001(-0.03 to 0.07)	-0.007(-0.05 to 0.04)	0.03(-0.004 to 0.06)	0.02(-0.009 to 0.05)	-0.14(-0.47 to 0.19)
GPAQ travel	0.25(0.08 to 0.42)	0.003(0.001 to 0.005)	0.10(0.05 to 0.15)	-0.10(-0.14 to -0.06)	-0.02(-0.05 to 0.02)	-0.70(-0.98 to -0.42)
BMI (kg/m^2)	-0.009(-0.06 to 0.04)	-0.005(-0.01 to 0.01)	-0.004(-0.02 to 0.01)	0.03(0.02 to 0.05)	0.003(-0.0002 to 0.006)	0.18(0.08 to 0.29)
Body fat %	0.006(-0.02 to 0.04)	0.004(-0.004 to 0.01)	0.002(-0.006 to 0.01)	0.02(0.01 to 0.03)	0.002(0.0004 to 0.005)	0.12(0.06 to 0.18)
WC (cm)	-0.004(-0.02 to 0.02)	-0.003(-0.05 to 0.01)	-0.002(-0.07 to 0.04)	0.01(0.005 to 0.02)	0.002(0.001 to 0.0003)	0.07(0.02 to 0.11)
HDL cholesterol (mmol/L)	2.97(2.24 to 3.71)	0.75(0.57 to 0.93)	0.84(0.62 to 1.07)	0.26(0.19 to 0.33)	0.18(0.13 to 0.24)	5.63(4.29 to 7.0)
LDL cholesterol	0.81(0.45 to 1.17)	0.17(0.08 to 0.26)	0.21(0.12 to 0.30)	0.15(0.1 to 0.19)	0.05(0.03 to 0.07)	2.8(2.16 to 3.45)
Total cholesterol (mmol/L)	0.92(0.62 to 1.23)	0.21(0.14 to 0.29)	0.25(0.17 to 0.33)	0.13(0.10 to 0.17)	0.05(0.04 to 0.07)	2.80(2.29 to 3.31)

β-coefficient represents the difference in serum biomarkers in μmol/L per unit difference in the predictor. Estimates are adjusted for age and sex (except for age adjusted for sex only and sex adjusted for age only)

Ref, reference category; WC, waist circumference; PAEE, physical activity energy expenditure; LPA, Light physical activity; MVPA, moderate to vigorous PA; BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein

Table 4.4 : Correlates of self-reported fruit and vegetable intake (Cameroon study, n=592)

	Self-reported fruit intake (times/week) β (95% confidence interval)	Self-reported vegetable intake (times/week) β (95% confidence interval)
Age (10 y)	0.04(-0.3 to 0.4)	0.8(0.5 to 1.2)
men	-0.85(-1.42 to -0.28)	-1.11(-1.75 to -0.47)
Urban (vs rural)	-1.01(-1.58 to -0.44)	-1.54(-2.18 to -0.89)
Education level		
<primary school (ref)		
primary school	0.11(-0.75 to 0.98)	-0.69(-1.69 to 0.31)
Secondary school	-0.09(-0.97 to 0.79)	-1.19(-2.26 to -0.12)
University	0.22(-0.84 to 1.29)	-0.95(-2.07 to 0.17)
Smoking		
Never (ref)		
Former	-0.11(-0.91 to 0.70)	-0.71(-1.53 to 0.11)
Current	0.38(-0.74 to 1.50)	-0.52(-1.59 to 0.55)
Alcohol		
Never (ref)		
Former	0.93(-0.29 to 2.15)	0.18(-1.13 to 1.49)
Current	0.95(0.07 to 1.83)	0.55(-0.49 to 1.59)
Marital stat		
Single (ref)		
Married	0.40(-0.37 to 1.18)	0.68(-0.10 to 1.47)
Separated	1.11(-0.40 to 2.62)	2.25(0.11 to 4.38)
Widow	-0.23(-1.54 to 1.07)	2.49(0.92 to 4.07)
Family size		
<3 (ref)		
3-5	0.45(-0.21 to 1.11)	-0.28(-1.05 to 0.49)
>5	0.69(-0.22 to 1.61)	-0.79(-1.76 to 0.18)
Season		
Long dry (ref)		
Light rain	1.65(0.57 to 2.72)	1.74(0.64 to 2.84)
Short dry	0.46(-0.15 to 1.08)	0.31(-0.41 to 1.03)
Heavy rain	0.30(-0.58 to 1.17)	0.51(-0.70 to 1.72)
PAEE (MJ/kg/d)	22.0(7.80 to 36.2)	38.1(22.6 to 53.5)
Objective sedentary (h/day)	-0.13(-0.26 to -0.009)	-0.22(-0.36 to -0.09)
Objective LPA (h/day)	0.10(-0.07 to 0.28)	0.12(-0.06 to 0.31)
Objective MVPA (h/day)	0.25(0.02 to 0.48)	0.50(0.26 to 0.74)
GPAQ PAEE (MJ/kg/d)	4.63(1.09 to 8.16)	15.5(11.2 to 19.9)
GPAQ work	0.06(0.01 to 0.11)	0.19(0.13 to 0.26)
GPAQ leisure	-0.18(-0.30 to -0.05)	-0.08(-0.30 to 0.13)
GPAQ travel	0.21(0.009 to 0.41)	0.91(0.68 to 1.14)
BMI (kg/m^2)	-0.06(-0.11 to -0.006)	-0.14(-0.20 to -0.08)
Body fat %	-0.03(-0.07 to 0.005)	-0.08(-0.12 to -0.04)
WC (cm)	-0.02(-0.04 to 0.007)	-0.04(-0.06 to -0.01)

β-coefficient represents the difference in the frequency of intake of fruits and vegetables per unit difference in the predictor. Estimates are adjusted for age and sex (except for age adjusted for sex only and sex adjusted for age only). Ref, reference category; WC, waist circumference; PAEE, physical activity energy expenditure; LPA, Light physical activity; MVPA, moderate to vigorous PA; BMI, body mass index

4.6.3 Plasma carotenoids, tocopherol and fasting glucose

The associations of plasma carotenoids and α-tocopherol concentrations with fasting glucose are shown in Table 4.5. Plasma total carotenoids and α-carotene were inversely associated with fasting glucose. Comparing the highest with the lowest tertile of the biomarkers, fasting glucose was lower by -0.28(95% CI -0.56 to -0.001) mmol/L for plasma total carotenoids and -0.31(-0.59 to -0.03) mmol/L for plasma α-carotene in unadjusted analysis. The inverse associations became stronger after adjusting for age, sex, education level, residential site, smoking status, alcohol intake, season, objectively measured physical activity, BMI and total cholesterol (-0.36(-0.73 to -0.002) mmol/L for total carotenoids and -0.41(-0.79 to -0.03) mmol/L for α-carotene). There was no evidence of an association of plasma concentrations of lycopene, lutein-zeaxanthin and α-tocopherol with fasting glucose. I did not find evidence of a non-linear association between any of the biomarkers and fasting glucose. The shapes of the associations are presented in Figure 4.3.

There was no evidence of an effect modification by sex, rural-urban area of residence, BMI categories and smoking status on the associations of plasma carotenoids and α-tocopherol with fasting glucose. Results were unchanged in sensitivity analyses using multiple imputation to account for the effect of missing covariates.

Table 4.5: Associations of plasma carotenoids and α-tocopherol categorised by tertiles (T) with fasting glucose (mmol/L) (Cameroon study, n=542)

Biomarkers	β-coefficient (95% confidence interval)			p-value for linear trend
	Thirds (T) of biomarkers			
	T 1	T 2	T 3	
Total carotenoids (range, µmol/L)	0.27, 3.39	3.4, 5.74	5.75, 18.94	
Model 1	1.0 (ref)	-0.22(-0.52 to 0.08)	-0.28(-0.56 to -0.001)	0.048
Model 2	1.0 (ref)	-0.27(-0.56 to 0.03)	-0.36(-0.65 to -0.08)	0.013
Model 3	1.0 (ref)	-0.26(-0.55 to 0.02)	-0.40(-0.71 to -0.08)	0.013
Model 4	1.0 (ref)	-0.28(-0.59 to 0.03)	-0.36(-0.73 to -0.002)	0.04
α-carotene (range, µmol/L)	0.08, 0.9	0.91, 1.47	1.48, 4.71	
Model 1	1.0 (ref)	-0.21(-0.51 to 0.09)	-0.31(-0.59 to -0.03)	0.029
Model 2	1.0 (ref)	-0.24(-0.54 to 0.06)	-0.38(-0.67 to -0.10)	0.008
Model 3	1.0 (ref)	-0.27(-0.56 to 0.02)	-0.46(-0.79 to -0.14)	0.005
Model 4	1.0 (ref)	-0.26(-0.59 to 0.08)	-0.41(-0.79 to -0.03)	0.03
β-carotene (range, µmol/L)	0.08, 1.8	1.81, 3.39	3.4, 11.03	
Model 1	1.0 (ref)	-0.05(-0.35 to 0.26)	-0.23(-0.51 to 0.06)	0.12
Model 2	1.0 (ref)	-0.11(-0.40 to 0.19)	-0.32(-0.61 to -0.03)	0.029
Model 3	1.0 (ref)	-0.12(-0.40 to 0.16)	-0.33(-0.66 to -0.01)	0.045
Model 4	1.0 (ref)	-0.13(-0.44 to 0.18)	-0.29(-0.67 to 0.07)	0.12
Lycopene (range, µmol/L)	0.005, 0.24	0.25, 0.47	0.48, 2.45	
Model 1	1.0 (ref)	-0.20(-0.47 to 0.08)	-0.17(-0.47 to 0.12)	0.246
Model 2	1.0 (ref)	-0.19(-0.48 to 0.09)	-0.18(-0.49 to 0.12)	0.251
Model 3	1.0 (ref)	-0.23(-0.53 to 0.07)	-0.31(-0.75 to 0.12)	0.157

Model 4	1.0 (ref)	-0.19(-0.52 to 0.15)	-0.25(-0.76 to 0.25)	0.32
Luteine + zeaxanthin (range, µmol/L)	0.007, 0.24	0.25, 0.38	0.39, 1.58	
Model 1	1.0 (ref)	-0.19(-0.47 to 0.08)	-0.13(-0.40 to 0.15)	0.515
Model 2	1.0 (ref)	-0.23(-0.52 to 0.07)	-0.22(-0.52 to 0.08)	0.265
Model 3	1.0 (ref)	-0.14(-0.42 to 0.13)	-0.19(-0.47 to 0.08)	0.165
Model 4	1.0 (ref)	-0.11(-0.40 to 0.18)	-0.16(-0.46 to 0.14)	0.28
α-tocopherol (range, µmol/L)	0.68, 17.24	17.25, 21.67	21.68, 48.01	
Model 1	1.0 (ref)	-0.17(-0.45 to 0.11)	-0.12(-0.40 to 0.15)	0.301
Model 2	1.0 (ref)	-0.23(-0.52 to 0.06)	-0.26(-0.60 to 0.08)	0.099
Model 3	1.0 (ref)	-0.27(-0.52 to -0.03)	-0.33(-0.67 to 0.01)	0.053
Model 4	1.0 (ref)	-0.22(-0.47 to 0.02)	-0.29(-0.67 to 0.09)	0.14

p-values for trend are from a linear regression model for continuous variables including thirds of the biomarkers as a continuous exposure.

Model 1: Unadjusted

Model 2: Adjusted for age and sex,

Model 3: Model 2 + education level, residential site (4 sites), smoking, alcohol intake, season of data collection (4 seasons), objectively measured physical activity (continuous) and BMI (continuous)

Model 4: model 3 + total cholesterol

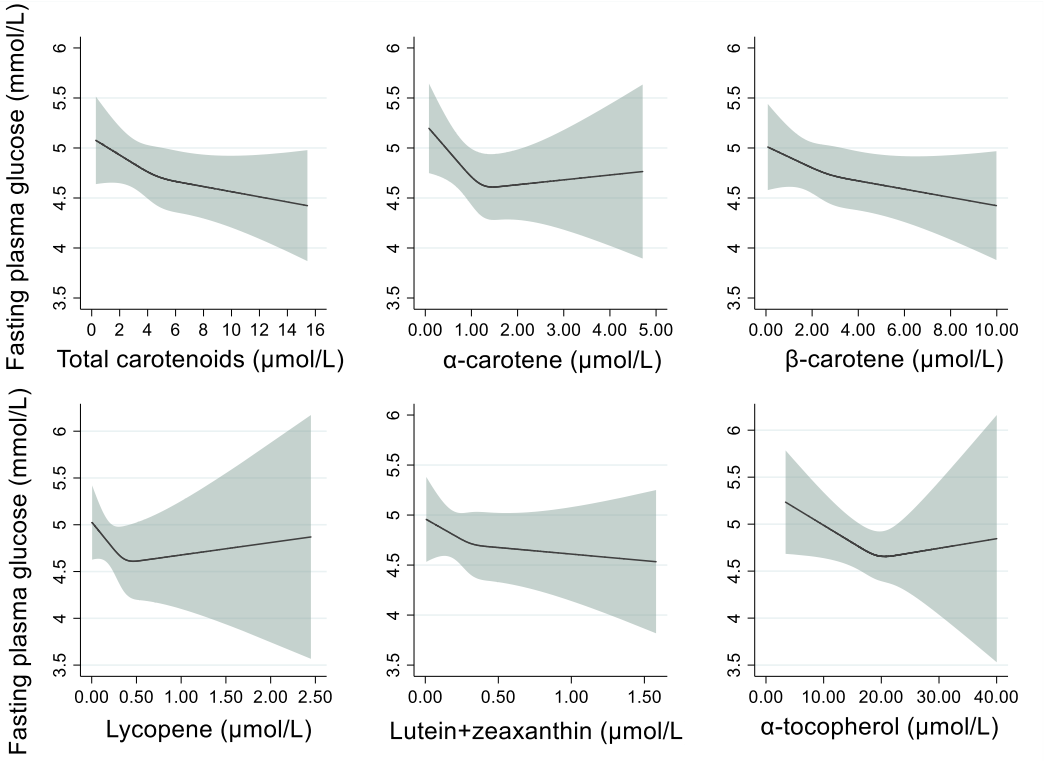

Figure 4.3 : Associations of plasma carotenoids and α-tocopherol with fasting glucose (Cameroon Study, n=542)

The shaded area represents the 95% confidence interval

Modelled using a restricted cubic spline function with 3 knots placed at 25th, 50th and 75th percentile. Covariates included age, sex, smoking status, alcohol intake, level of education, residential site, PAEE, BMI and total cholesterol. There was no evidence of a non-linear association between any of the biomarkers and fasting glucose (p-value for non-linearity > 0.05)

4.7 Discussion

In this population-based cross-sectional study in rural and urban Cameroon, higher concentrations of plasma total carotenoids, and α-carotene were associated with lower fasting glucose independently of socio-demographic characteristics and health-related behaviours. This study provides for the first time, to our knowledge, the relationship between intake of fruits and vegetables estimated objectively using biomarkers and fasting glucose in a population-based study in an African setting.

The distribution of plasma carotenoids in this study differed from those in previous studies. While plasma α- and β-carotene constituted ~ 85% of total carotenoids in our study, they contributed only ~35% in the National Diet and Nutrition Survey (NDNS) in the UK. In absolute concentrations, the mean of plasma α- and β-carotene in this study were over four folds higher than those reported in the NDNS and National Health and Nutrition Examination Survey (NHANES) in the US (179), (178), but were lower than α- and β-carotene concentrations reported in a study in pregnant women in Nigeria (180). The mean of plasma lycopene, lutein and zeaxanthin concentrations were similar to those reported in the NHANES but lower than in the NDNS, where lycopene, lutein and zeaxanthin were the leading contributors to plasma total carotenoids concentrations (179), (178). Consistent with previous studies reporting smoking as a major determinant of carotenoids concentrations, this study showed that current smokers had lower plasma total carotenoids concentration than participants who had never smoked (181), (166). Cigarette contains free radicals, which induce oxidative stress leading to increase utilisation of anti-oxidants such as carotenoids.

Our findings of an inverse association of plasma total carotenoids and α-carotene with fasting glucose are consistent with previous observational studies (182), (183), (184), (185). In a meta-analysis of 13 prospective observational studies, higher circulating total carotenoids was associated with lower risk of type 2 diabetes (184). In contrast, other cross-sectional and prospective cohort studies reported a positive association between β-carotene and fasting glucose (150) or no evidence of an association between circulating carotenoids and glycaemic markers or type 2 diabetes risk (181), (186), (166).

In this study, plasma total carotenoids, α- and β-carotene were positively correlated with self-reported fruit and vegetable intake and the magnitude was comparable to those reported in previous studies (140), (143). For instance, I found a positive correlation (r=0.35) between self-reported vegetable intake and plasma β-carotene, which is comparable to the correlation

between intake of vegetables assessed using the FFQ and β-carotene (r=0.21) reported in the European Prospective Investigation into Cancer and Nutrition study (143).

Therefore, these finding of an inverse association between plasma total carotenoids and fasting glucose suggests an inverse association between intake of fruits and vegetables and fasting glucose. A meta-analysis of 23 prospective observational studies showed that higher intake of fruits and vegetables was associated with lower risk of type 2 diabetes (138). I observed an inverse association between plasma α-carotene and fasting glucose, which may suggest an association of carrots and root vegetables with fasting glucose, given that plasma α-carotene has been shown to highly correlate with intake of carrots and root vegetables (143).

The inverse association of plasma lycopene with self-reported intake of fruits and vegetables is not well understood. In a meta-analysis of randomised controlled feeding trials, study arms provided with more fruits and vegetables showed an increase in plasma vitamin C and individual carotenoids concentrations except for lycopene (140). In Western settings, tomato and tomato-based food products are the main sources of lycopene (187). However, nutritional biomarker concentrations are affected by food storage and cooking methods. A study amongst African Americans showed that cooked tomatoes and watermelon contributed to a substantial proportion of dietary lycopene intake and raw tomatoes contributed little (187). In addition to the potential misreporting of tomato intake in this study, the inverse association between plasma lycopene and self-reported intake of fruits and vegetables may suggest the contribution of other food sources to plasma lycopene concentrations in this population. I observed an inverse association of intakes of fruit and vegetables with α-tocopherol after adjusting for age and sex. Although considerable amounts of α-tocopherol are found in certain fruit and vegetables, their main dietary sources are vegetable oils (155).

The potential mechanisms of action by which high intakes of fruits and vegetables may prevent diabetes could be that fruits and vegetables help in weight loss since they are rich in fibres and help to increase satiety and are low energy density foods (175). This is consistent with our results showing an inverse association between self-reported fruit and vegetables intake and BMI. Besides being rich sources of carotenoids and tocopherol, fruits and vegetables are also rich in other vitamins like vitamin C, folate, minerals like magnesium and phytochemicals like polyphenols all of which have been shown to lower systemic inflammation and oxidative stress; processes involved in the development and progression of type 2 diabetes (188) (189). Dietary

fibres and polyphenols found in fruits and vegetables may modulate the gut microbiome (176). An altered gut microbiome has been associated with higher risk of type 2 diabetes (190).

I did not find evidence of associations of plasma lycopene, lutein-zeaxanthin and α-tocopherol with fasting glucose, which is consistent with previous studies (191), (183), (145), (181), (182). Similarly, in prospective cohort studies in the US and Finland, there was no evidence of an association between dietary lycopene or intake of tomato-based food and type 2 diabetes risk (135), (192). I observed a significant inverse association between α-carotene and fasting glucose and a borderline inverse association for β-carotene, which are provitamin A. Previous studies have also reported an inverse association between provitamin A and diabetes but no association for non-provitamin A (148), (183). This could be because provitamin A carotenoids are converted in the body to retinol and may improve insulin sensitivity by binding to retinol-binding protein 4 (RBP4) the only specific binding protein for retinol. High RBP4 has been associated with higher resistance (193). Although the test for non-linearity for the associations of plasma lycopene, lutein-zeaxanthin and α-tocopherol with fasting glucose were not significant, Figure 4.3 suggest any inverse associations are likely to be observed in participants who have low concentrations of biomarkers.

Evidence of an inverse association between plasma carotenoids and type 2 diabetes comes mainly from observational studies whereas intervention studies of micronutrient supplementation mainly show no beneficial effect. In the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study, supplementation with β-carotene at 20 mg/d, or α-tocopherol at 50 mg/d in male smokers did not reduce the risk of type 2 diabetes after 6.1 years (194). Compared with placebo, supplementation with β-carotene (50 mg every other day), α-tocopherol (600 IU every other day), or vitamin C (500 mg/d) in the Women's Antioxidant Cardiovascular Study had no effect on type 2 diabetes risk after 9.2 years (195). Similarly, supplementation with β-carotene at 50 mg every other day did not reduce the risk of diabetes in the Physician's Health Study after 12 years (196). This could be because carotenoids are biomarkers of intake of fruit and vegetables and the inverse associations between circulating carotenoids and type 2 diabetes reported in observational studies reflect the associations of intake of fruit and vegetables with type 2 diabetes rather than the micronutrients in isolation. Fruits and vegetables are rich in different nutrients and non-nutritive compounds like flavonoids and it is possible that the benefits observed result from a synergistic effect. This could also provide an explanation for the null association observed in some prospective observational studies of dietary carotenoids intake and risk of type 2 diabetes (135), (146). Moreover, studies using Mendelian

randomisation approach suggest no causal association between circulating β-carotene or vitamin C and type 2 diabetes (197), (198). Future studies are needed to identify the contributions of different foods to carotenoids concentrations in this population.

4.8 Strengths and limitations

The main strength of this study relies on the use of objectively measured biomarkers to assess intake of fruits and vegetables to reduce the measurement error and recall bias associated with the self-reports. The major limitation of this study is the cross-sectional study design, which limits our ability to infer a causal effect due to the possibility of reverse causation. I used plasma carotenoids and α-tocopherol as objective indicators of intake of fruits and vegetables, but their bioavailability may be influenced by intrinsic and extrinsic factors such as genetic variations, food composition, processing and storage. The concentration of the pro-vitamin A carotenoids may also be influenced by the rate of conversion to vitamin A which is dependent on vitamin A status. Moreover, carotenoids and tocopherol are antioxidants and their concentrations may be affected by underlying health conditions (156).

4.9 Conclusion

In this population-based study, higher total carotenoids and α-carotene as objective indicators of fruit and vegetables intake were associated with lower fasting glucose independently of socio-demographic characteristics and health-related behaviours. These findings add to the evidence in favour of the public health recommendation to increase intake of fruits and vegetables to prevent diabetes in this population.

Chapter 5 : Associations of serum folate and holotranscobalamin with cardio-metabolic risk factors

Publication

Mba CM, Koulman A, Forouhi NG, Imamura F, Assah F, Mbanya JC, Wareham NJ. Associations of Serum Folate and Holotranscobalamin with Cardiometabolic Risk Factors in Rural and Urban Cameroon. Nutrients. 2021;14(1):178

Summary

Background: Epidemiological evidence has suggested that a low intake of fruit and vegetables and high intake of meat is associated with higher cardiometabolic disease risk but may have relied on subjective methods for dietary assessment and focused on Western populations. I aimed to investigate the association of blood folate concentration as an objective marker of fruit and vegetables intake and holotranscobalamin (holoTC) as a marker of animal-sourced foods with cardiometabolic risk factors in adults in Cameroon.

Method: In a population-based cross-sectional study of 578 adults. Serum folate and holoTC concentrations were measured by liquid chromatography-tandem mass spectrometry and "sandwich" ELISA respectively. Self-reported fruit and vegetable intake was assessed using the WHO STEPS. The primary outcome was a continuous metabolic syndrome score computed by summing the sex-specific z-scores risk components of central adiposity, blood pressure, fasting glucose, triglycerides and HDL cholesterol (with the latter included in the score in an opposite direction to the other factors). I fitted linear regression models to assess potential correlates of serum folate and holoTC and independent associations of these B-vitamins with cardiometabolic risk factors.

Results: Mean ± SD age was 38.2±8.6 years and 64% of the participants were women. Median (25^{th}-75^{th} percentile) serum folate was 12.9 (8.6 - 20.5) nmol/L and the mean holoTC was 75 ± 34.3 pmol/L. Rural residents had higher serum folate concentrations (15.9 (9.8 - 25.9) nmol/L) than urban residents (11.3 (7.9 - 15.8) nmol/L), but lower serum holoTC concentrations (rural: 69.8 ± 32.9 pmol/L; urban: 79.8 ± 34.9), p-value < 0.001 for both comparison. There was an inverse association between serum folate and metabolic syndrome score by -0.20 in the score (95% CI, -0.38 to -0.02) per 1 SD (10.8 nmol/L) of folate) in a model adjusted for socio-demographic factors, smoking status, alcohol intake, residential site, BMI and physical activity. Serum holoTC was positively associated with the metabolic syndrome score in unadjusted analysis (0.33 (95% CI, 0.10 to 0.56)) but became non-significant (0.17(-0.05 to 0.39)) after adjusting for socio-demographic and behavioural characteristics.

Conclusion: Serum folate and holoTC were associated with the metabolic syndrome score in opposite directions. The positive association between serum holoTC and the metabolic syndrome score was partly dependent on sociodemographic characteristics. These findings suggest that based on these biomarkers reflecting dietary intakes, public health approaches

promoting a higher intake of fruits and vegetables and lower animal-sourced foods may lower cardiometabolic risk factors in this population.

5.1 Introduction

According to the 2017 global burden of disease study, low intake of fruits and vegetables was amongst the leading dietary risk factors for NCD deaths in SSA (134). In addition, a meta-analysis of nine prospective studies showed that higher meat consumption (particularly red and processed meat) was linked to a higher risk of metabolic syndrome (199). A few cross-sectional studies in SSA have reported an inverse association between self-reported intake of fruits and vegetables and hypertension (200), (201), (172) and no association between meat intake and hypertension (200), (172). In Cameroon, a cross-sectional study of 571 members of defence forces showed that a dietary pattern high in fruit and vegetables intake was associated with a lower prevalence of hypertension but there was no association with meat intake (172). Results from a meta-analysis of 47 studies from 22 SSA countries suggest fruit and vegetables intake is low in SSA with 79.1% of adults reporting intakes of fruits and vegetables below the WHO recommended minimum daily consumption of 400 g while meat consumption is high (51% of adults consuming > 70 g daily) (151). However, these studies have relied on self-reported dietary assessment methods, which are subject to recall bias and measurement error.

Circulating plasma vitamin C and carotenoids have been widely investigated as objective biomarkers of fruit and vegetables intake (140), (144), (142) and applied to test diet-disease association (147). However, fruits and vegetables are also rich sources of folate. Plasma folate has been shown to be correlated with self-reported intake of fruits and vegetables in observational studies (202). In short-term (4 days to 3 weeks) intervention studies of fruit and vegetables intake compared to control conditions, plasma folate increased (203), (204), (205). Natural vitamin B12 is found almost exclusively in animal products. Intake of animal products has been shown to be positively correlated with total vitamin B12 and holotranscobalamin (holoTC) in observational and intervention studies (206), (207). HoloTC is vitamin B12 bound to transcobalamin II and is the metabolically active fraction of vitamin B12 (the fraction that can readily enter the cells). Some studies suggest blood holoTC is a better indicator of vitamin B12 status than blood total vitamin B12 (208). Taken together, this evidence suggests that blood folate may be a practical and valid biomarker of intake of fruits and vegetables and that

plasma vitamin B12 can be used as a marker of animal-sourced foods, especially in countries where food fortification with folate or vitamin B12 is not mandatory.

Epidemiological studies of associations between folate status and insulin resistance (209), (210), hypertension (211), (212) and dyslipidaemia (38), (213) are sparse and show inconsistent findings. Studies examining the association of vitamin B12 status with metabolic syndrome or its individual components have also shown mixed results (210), (214). Results from a prospective study of 8067 patients with type 2 diabetes in the US, showed a non-linear association between serum vitamin B12 and CVD mortality where both low and high concentrations of serum vitamin B12 were associated with higher CVD mortality (215). In another study in patients with obesity, HOMA-IR estimates were highest in patients with the lowest concentrations of serum vitamin B12 and highest concentrations of serum folate (210). In SSA, only a few studies have examined the association between dietary intake of folate and vitamin B12 and individual cardiometabolic risk factors (36), (37), (38). A study in 498 South African women found a positive association between an animal-derived nutrient pattern and adiposity (36). In another study, there was no association between folate and starch-rich nutrient pattern and obesity (37). In Benin, intake of vitamin B12 was positively associated with HDL cholesterol, but there was no association between dietary folate intake and HDL (38). Other studies in Africa have examined the link between homocysteine a metabolic indicator of folate and vitamin B12 deficiency and metabolic risk factors and showed a positive association between homocysteine and hypertension and dyslipidaemia (216), (217). In a nationally representative survey of women aged 15-49 years in Cameroon, 17.3% and 28.8% of women had low folate and total vitamin B12 concentrations respectively (218), but the association of folate/vitamin B12 status with metabolic risk factors was not examined.

Although serum folate and vitamin B12 have been proposed as objective indicators of intake of fruits and vegetables and animal-sourced foods respectively, I did not find any previous study examining the association between these biomarkers and a clustered score of the metabolic syndrome in SSA. Our study aimed to examine how serum folate and holoTC would be related to the clustering of cardiometabolic risk factors in adults in rural and urban Cameroon. Given that food fortification with folate or B12 was not mandatory in Cameroon at the time of data collection, this study offers the possibility to examine the associations between foods for which these B-vitamins are objective indicators and cardiometabolic risk factors.

5.2 Study aims

This study aimed to identify factors that affect serum folate and holoTC concentrations and examine the associations of serum folate and holoTC as objective indicators of intake of fruits and vegetables and intake of meat respectively with a clustered score of the metabolic syndrome in rural and urban settings of Cameroon.

5.3 Background on folate and vitamin B12

5.3.1 Sources, metabolism and functions of folate and vitamin B12

- **Folate**

The term folate refers to a family of B-group vitamins that are naturally occurring in a wide variety of foods such as green leafy vegetables, citrus fruits, legumes, beans, whole grains and liver. Folic acid on the other hand is the synthetic form of folate and is widely used as supplements and in food fortification (e.g. breakfast cereals, flour) because folic acid has higher chemical stability and is more easily absorbed than folate. Folate is very unstable and is rapidly broken down within a few days or weeks to biological compounds (pteridine and p-aminobenzoylglutamate) which have no biological activity. As a result, processes such as harvesting, processing, storage and preparation may lead to ~ 60% loss in the biochemical activity of folate found in foods. Only ~ 50% of dietary folate is absorbed in the intestines (219).

Dietary folate is absorbed in the small intestines after hydroxylation of the polyglutamyl THF, which is the predominant form of folate found in foods to monoglutamyl THF by folate hydroxylase. THF is absorbed at the level of the small intestines. THF is converted to 10-formyl THF and then to 5,10-methylene THF by a vitamin B6-dependent enzyme methylenetetrahydrofolate dehydrogenase. 5,10-methylene THF is then irreversibly reduced to 5-MTHF by a vitamin B2-dependent enzyme methylenetetrahydrofolate reductase. 5-MTHF is the main form of folate found in the blood. Folate forms circulate in blood freely, loosely bound to albumin and a small proportion is bound to folate binding protein. THF is the active form of folate in the body (220).

Folate is required as a cofactor for enzymes involved in the one-carbon metabolism. The main function of folate is to donate or accept one-carbon unit in the one-carbon metabolism which is a network of pathways involving the transfer and utilisation of one-carbon unit required for

several processes including methylation, amino acid metabolism, and DNA and RNA synthesis (221).

- **Vitamin B12**

Vitamin B12 is naturally found in animal-sourced foods only and good sources include meat, fish, eggs, cheese and milk. Because vitamin B12 can only be synthesised by microorganisms, it is not found naturally in plant-based foods.

The primary site of absorption for vitamin B12 is the terminal ileum. For absorption to occur, vitamin B12 must bind to the intrinsic factor secreted by the gastric parietal. In circulation, 70-90% of vitamin B12 is transported bound to haptocorrin (a complex known as holohaptocorrin) and the remaining 10-30% bound to transcobalamin II (a complex known as holoTC). Transcobalamin II is responsible for the delivery of vitamin B12 to all the required tissues. HoloTC is therefore referred to as the active vitamin B12, given that it is the only form that can be taken up by the cells (208), (222). Vitamin B12 is absorbed in the ileum and is transported in the form of holoTC into the cells where it is used as a cofactor for metabolic reactions such as DNA and RNA synthesis.

The main function of vitamin B12 is to act as a co-factor for enzymes involved in protein and DNA synthesis. Deficiency of vitamin B12 is commonly associated with megaloblastic anaemia and peripheral neuropathy. Vitamin B12 is required by methylmalonyl-CoA-mutase and methionine synthase for the conversion of methylmalonyl-CoA to succinyl-CoA and for the remethylation of homocysteine to methionine respectively. Elevated levels of methylmalonic acid and homocysteine have been associated with higher risk of CVDs (223), (224).

5.3.2 Vitamin B12-folate interrelationship

The methionine synthase reaction is the only common metabolic pathway between folate and vitamin B12. Folate and vitamin B12 play a key role in the remethylation of homocysteine to methionine. In the methionine synthase reaction, 5-MTHF provides a methyl group required by methionine synthase for the vitamin B12-dependent conversion of homocysteine to methionine. In case of vitamin B12 deficiency, the vitamin B12-dependent methionine synthase reaction is halted and folate is trapped in the form of 5-MTHF which results in functional folate deficiency. This is known as the “methyl trap hypothesis” (225).

5.3.3 Plasma folate and vitamin B12 as biomarkers and dietary requirements

- **Plasma folate as a biomarker of intake of fruits and vegetables and holotranscobalamin as a biomarker of animal-based products**

Fruits and vegetables are amongst the main natural sources of folate intake and have been suggested as objective indicators of intake of fruits and vegetables. In a large population-based study in Norway where food fortification with folic acid is limited, fruits and vegetables were the highest contributors to folate intake and intake of fruits and vegetables showed a positive correlation with plasma folate concentrations (r=0.22) (202). However, the weak correlation may be because of the measurement errors commonly associated with self-reports. In feeding controlled trials where fruits and vegetables were provided to participants, the groups with higher intake of fruits and vegetables showed higher serum folate concentrations (144), (226), (227), (228). Although plasma homocysteine has also been proposed as a biomarker of intake of folate-rich foods, plasma homocysteine is not specific to folate as homocysteine concentrations are also affected by inadequate intake of vitamin B2, B6 and B12 (227), (228), (229).

Animal-based foods are the only natural sources of dietary intake of vitamin B12. Low vitamin B12 concentration is common in populations with low or no consumption of animal-based foods such as vegetarians and vegans (230). Observational studies have consistently shown higher serum total vitamin B12 and holoTC in groups with higher intake of animal-based products than those with low intakes (207), (231). Evidence from intervention studies also shows higher serum vitamin B12 and holoTC in groups allocated to meat-rich diets compared with vegan diets (206), (232).

- **Plasma folate and holotranscobalamin as biomarkers of folate and vitamin B12 intake/status respectively**

Serum folate and holoTC concentrations are sensitive biomarkers of folate and vitamin B12 intake/status respectively. Serum folate reflects short-term folate intake and status as it responds rapidly to changes in dietary folate intake (229). The Food Standards Agency recommends the use of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as the method of choice to assess serum folate. The LC-MS/MS has the advantage of capturing the major forms of folate in circulation including free folic acid (from supplements or fortified food), which is not the case with other methods such as microbiological assay. Red

blood cell (RBC) folate is another biomarker of folate, which reflects medium-term folate intake/status, given that it reflects folate body stores over the preceding 120 days (lifespan of the RBCs) (229). Serum holoTC has a short half-life and appears to be one of the earliest indicators of vitamin B12 deficiency (208). Some studies suggest that plasma holoTC is a better indicator of plasma vitamin B12 intake and status than total vitamin B12 given that it is the only form that is readily available for uptake into the cells (233).

In 2008, the WHO suggested cut-offs for folate and vitamin B12 deficiency. These cut-offs are derived from the 3rd National Health and Nutrition Examination Survey and are based on the concentrations of blood folate and vitamin B12 below which concentrations of homocysteine and methylmalonic acid respectively are likely to become elevated (234). The cut-offs for blood folate and vitamin B12 deficiency are:

- Serum folate deficiency < 10 nmol/L
- Red blood cell folate < 340 nmol/L
- Plasma vitamin B12 < 150 pmol/L using total vitamin B12 (or < 50 nmol/L using holoTC) (235).

- **Dietary requirements for folate and vitamin B12**

The primary biomarker used to derive dietary reference values for folate is RBC folate concentration given that it reflects medium-term folate stores and for vitamin B12 is plasma total vitamin B12 (229). Table 5.1 provides reference nutrient intakes for folate and vitamin B12 according to different guidelines.

Table 5.1 : Reference nutrient intakes (RNI) for folate and vitamin B12

Age	Folate RNI (µg/d)		Vitamin B12 RNI (µg/d)	
	WHO/FAO DFE (236)	UK Folate (237)	WHO/FAO (236)	UK (167)
1-3	160	70	0.9	0.5
4-6	200	100	1.2	0.8
7-10	(7-9 y) 300; ≥ 10 y 400	150	(7-8 y) 1.2; (9-10 y) 1.8	1.0
11-13	400	200	1.8	1.2
≥ 14	400	200	2.4	(14 y) 1.2; (≥ 15 y) 1.5
Pregnancy	600	300	2.6	1.5

The reference nutrient intake is the amount of intake of folate or vitamin B12 required to meet the needs of 97.5% of the population.

DFE, dietary folate equivalent; WHO, World Health Organisation; FAO, Food and Agricultural Organisation

In the table above, the units to express intake of folate differ between the WHO/FAO and UK. The Institute of Medicine introduced the term “dietary folate equivalents (DFE)” in 1998 to account for the differences in absorption between folate and folic acid and defines DFE as: 1 µg of DFE = 0.6 µg of folic acid consumed with food (or 0.5 µg of folic acid if taken on an empty stomach) (238).

For dietary intakes of a mixed food that contains both natural folate and folic acid, the DFE can be computed as follows:

DFE (µg) = food folate (µg) + (folic acid (µg) x 1.7)

Food fortification can add substantial amounts of folic acid and vitamin B12 to the diet (229). In Cameroon, a national mandatory fortification of wheat flour in folic acid (5.0 mg/kg), vitamin B12 (0.04 mg/kg), zinc (95 mg/kg) and iron (60 mg/kg) was launched in August 2011. A study in ~ 300 women of reproductive age showed that in 2012 compared with 2009, mean concentrations of plasma folate and vitamin B12 were higher by 200% and 50% respectively (239).

5.4 Methods

The study design, setting and other common methods and procedures used in this cross-sectional study are reported in Chapter 2.

5.4.1 Measurement of serum folate and holotranscobalamin concentration

Frozen serum samples stored at -80°C were defrosted for analyses of folate and holoTC at the Nutritional Biomarker Laboratory at the MRC Epidemiology Unit, University of Cambridge, UK. Serum folate concentration was measured using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) (20). This allowed for the highly specific detection of six folate forms: 5-methyltetrahydrofolate (5-methylTHF), tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-formylTHF), free folic acid, 5,10 methenyltetrahydrofolate (5,10 methenylTHF) and an oxidation product of 5-methyltetrahydrofolate (pyrazino-s-triazine derivative (meFox)). Solid-phase extraction with phenyl columns was used to isolate the folate forms in serum samples. The analytes were analysed using reversed UPLC on a column (Waters Acquity UPLC® HSS T3 C8 1.7 μm 2.1 x 100 mm, Wilmslow, United Kingdom) at 30 °C before mass spectrometry analysis. The addition of stable isotopes labelled internal standards during the extraction, which undergo identical processing helps to normalise for sample preparation and instrument variability. The concentrations of the analytes were determined by comparing the analyte/internal standard signal to that of the calibration curve.

As > 90% of the results were below the limit of detection (< 0.05 nmol/L) for 5,10 methenylTHF and 5-formylTHF and that meFox lacks biological activity, I calculated total serum folate as the sum of three folate forms (5-methylTHF, free folic acid and THF) and used it in subsequent analysis. To enable comparability with previous studies that used measurement methods that do not separately identify the different folate forms, I also calculated "total serum folate including meFox", which included the oxidation product meFox. Throughout the text, I use "serum folate" to refer to total folate calculated without the oxidation product meFox. In total, 578 participants had blood samples available for folate analysis.

Serum holoTC concentration was measured by sandwich enzyme-linked immunosorbent assay (ELISA) manufactured by Axis-Shield, Dundee, Scotland. A specific antibody coated on the plate reacted with holoTC. A detection antibody added directly to the plate reacted to form a "sandwich" to produce a colour change. The absorbance was read in a microwave

spectrophotometer and the concentration was extrapolated from the calibration curve. Blood samples for 547 participants were available for holoTC biochemical analysis.

5.4.2 Outcomes

The primary outcome was a continuous metabolic syndrome score computed by summing sex-specific standardised values of waist circumference, fasting glycaemia, blood pressure, triglycerides and HDL cholesterol as described in Chapter 2. Secondary outcomes were individual components of the score (fasting glucose, systolic and diastolic blood pressure, waist circumference, triglycerides and HDL cholesterol).

5.5 Statistical analyses

All statistical analyses were performed in Stata 15 (StataCorp, College Station, TX). Data are presented as the mean±SD for continuous variables (or median (25^{th}-75^{th} percentile) for non-normally distributed variables) and percentages for categorical variables. Using previously suggested cut-offs, I reported the proportion of participants with folate deficiency (serum folate < 10 nmol/L) and vitamin B12 deficiency (holoTC < 50 pmol/L) (234), (235). I tested differences in means using the t-test (or differences in medians using the Mann Whitney test) and differences in proportions using the chi-squared test. Spearman coefficient was used to assess pairwise correlations between serum folate and holoTC and between serum folate and self-reported fruit and vegetable intake. I fitted linear regression models adjusted for age and sex to identify predictors of serum folate and holoTC after log transformation of serum folate to account for its skewed distribution.

I investigated for non-linear relationships between serum folate, holoTC and outcomes by fitting restricted cubic splines with 3 knots at the 25^{th}, 50^{th} and 75^{th} percentiles and tested for non-linearity using the Wald test. As the tests for non-linearity were non-significant, I fitted multiple linear regressions using a block-wise selection approach to estimate β coefficients and 95% confidence intervals per 1 SD of serum B-vitamins concentrations. Three models with incremental adjustment for potential confounders were used. Model 1 was unadjusted; model 2 was adjusted for age, sex, level of education (less than primary school, completed primary school, secondary school and university), smoking status (never smoked, past and current smoker), alcohol intake (never, past and current) and residential site (4 sites); Model 3 was additionally adjusted for BMI (continuous) and PAEE (continuous). With missing information observed (metabolic syndrome, n=12; HDL cholesterol, n=12; PAEE, n=49), complete-case

analyses were performed with further sensitivity analysis implementing multiple imputation. I used multiple imputation by chained equations under the assumption of missing at random and created 10 multiply imputed datasets and then used Rubin's combination rules to combine estimates (127). I tested interactions between the B-vitamins and sex, BMI categories and residential site, using model 3 and performed subgroup analyses if the p-value for interaction was < 0.05.

In sensitivity analyses, I: a) fitted linear regression models using serum folate including the oxidation product meFox; b) mutually adjusted for the other B-vitamins in model 3 and tested for interaction between the B-vitamins; c) used a metabolic syndrome score computed without waste circumference to adjust for adiposity when waist circumference was not included in the outcome score. Throughout, a two-sided α-level of 0.05 was used to test for statistical significance.

5.6 Results

5.6.1 Descriptive characteristics

In total, 578 and 547 participants had serum samples available for the analysis of folate and holoTC respectively. The mean age ± SD of the participants was 38.2 ± 8.6 years. The distribution of serum folate and holoTC by rural-urban area of residence is shown in Figure 5.1. The median concentration for serum folate was 12.9 (8.6 - 20.5) nmol/L and the mean of holoTC was 75 ± 34.3 pmol/L. Rural residents had higher concentrations of serum folate (15.9 (9.8 - 25.9 nmol/L) than those living in urban areas (11.3 (7.9 - 15.8 nmol/L), p-value < 0.0001. The distributions of both B-vitamins were similar in men and women in the entire sample, but serum folate was higher amongst rural men (18.1(11.3 - 27.1) nmol/L than rural women (14.4(9.3 - 24.3) nmol/L. Participants living in urban areas had higher serum holoTC concentrations (79.8 ± 34.9 pmol/L) than rural residents (69.8 ± 32.9 pmol/L), p-value = 0.0006. There was a negative correlation between serum folate and holoTC (*r*: -0.12 (p-value = 0.007)). 35.3% of participants were deficient for folate (serum folate < 10 nmol/L) and 26.5% deficient for vitamin B12 (serum holoTC < 50 pmol/L). The characteristics of participants with both folate and holoTC deficiency were similar to those of the rest of the sample, except for the area of residence. A higher proportion of participants with combined folate and holoTC deficiency lived in the urban areas than those without (Table 5.2).

The median (25th-75th percentile) number of times people self-reported consuming fruit in a typical week was 2(1 - 5) times/week and the comparable figure for vegetables was 4(2 - 7) times/week. Rural residents reported higher frequency of fruit consumption (3(1 - 6) times/week) than urban residents (2(1 - 4) times/week), p-value = 0.005 (Figure 5.2). Similarly, the frequency of self-reported vegetables consumption was higher in rural residents (5(2 - 9) times/week) than in those living in urban areas (4(2 - 6) times/week), p-value < 0.0001 (Figure 5.2). Women reported higher frequency of consumption of both fruit (3(1 - 6) times/week) and vegetables (4(2 - 8) times/week) than men, in whom comparable frequencies were 1(2 - 4) times/week and 3(2 - 6) times/week for intake of fruits and vegetables respectively (p-value < 0.001 for both comparisons) (Figure 5.3).

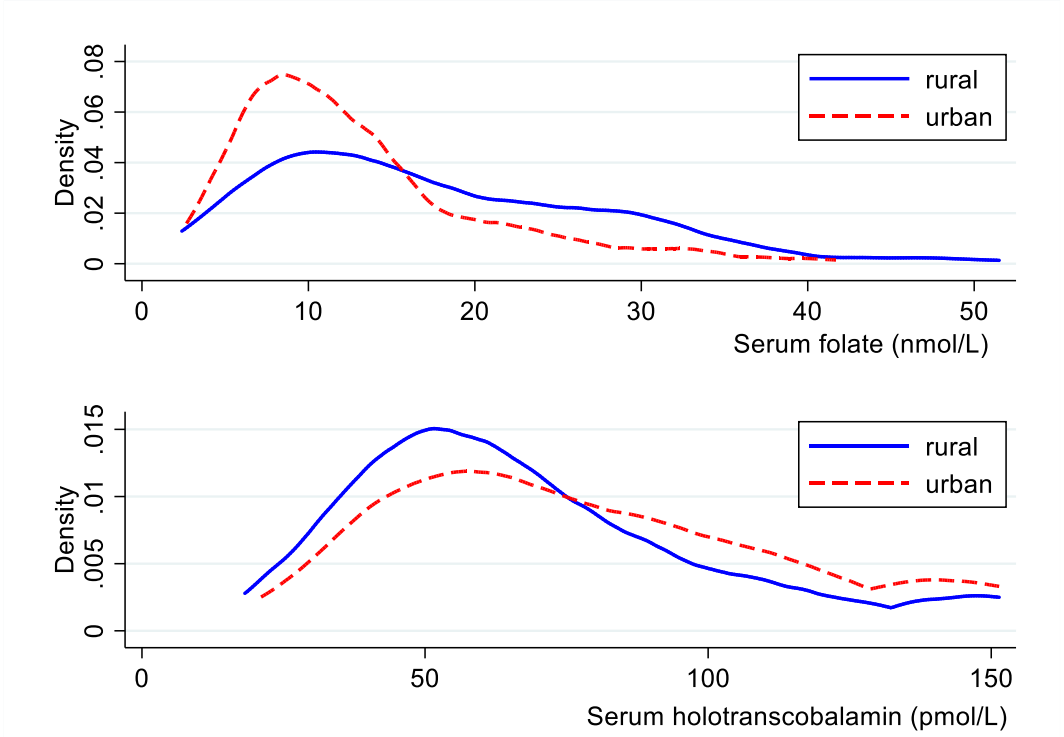

Figure 5.1 : Distribution of serum folate and holoTC in rural and urban residents (Cameroon study, n=547)

Table 5.2 : Characteristics of participants with vitamin B12 and folate deficiency (Cameroon study, n=547)

Characteristics	Combined deficiency, n=37 (6.8%)	Others, n=510 (93.2%)	p-value
Age (years)	36.3± 9.1	38.4 ± 8.5	0.15
Sex			
Male, n(%)	16(43.2)	177(34.7)	0.29
Education level, n(%)			
< primary education	5(13.5)	95(18.7)	
Primary	16(43.2)	225(44.2)	0.814
Secondary and high school	10(27.1)	125(24.5)	
University	6(16.2)	64(12.6)	
Site			
Urban, n(%)	27(73.0)	260(51.0)	0.01
Alcohol consumption (%):			
Never	3(8.1)	57(11.2)	
Past	4(10.8)	50(9.8)	0.850
Current	30(81.1)	403(79.0)	
Smoking status, n(%):			
Never	26(70.3)	396(77.7)	
Past	7(18.9)	69(13.5)	0.572
Current	4(10.8)	45(8.8)	
PAEE (KJ/Kg/day)	50 ± 18.1	50.9 ± 23.4	0.417
GPAQ PAEE (KJ/Kg/day)	6.5(3.5-75.3)	25.5(4.2-128.9)	0.166
Fruit (times/week)	3(1-6)	2(1-5)	0.539
Vegetable (times/week)	4(2-8)	4(2-7)	0.914
BMI (Kg/m^2)	26.1 ± 4.8	26.1 ± 5.3	0.97
Waist (cm)	89.6 ± 11.3	88.5 ± 12.2	0.617
Systolic blood pressure (mmHg)	123.3 ± 24.9	122.3 ± 20.2	0.786
Diastolic blood pressure (mmHg)	77.8 ± 16.3	76.3 ± 12.9	0.529
Fasting blood glucose (mmol/L)	4.9 ± 1.3	4.8 ± 1.4	0.67
2-h blood glucose (mmol/L)	6.15 ± 1.46	6.28 ± 1.87	0.682
HOMA-IR	0.45(0.87-1.41)	0.71(0.38-1.23)	0.27
Metabolic syndrome z-score	0.18 ± 2.7	-0.007 ± 2.5	0.672

Results are presented as arithmetic mean [or median (25^{th}-75^{th} percentile) for non-normally distributed variables] or n (%). p-values are from a t-test for normally distributed continuous variables (or Mann Whitney test for non-normally distributed variables) and a chi squared test (or Fisher exact test) for categorical variables.

PAEE, physical activity energy expenditure; GPAQ, Global Physical Activity Questionnaire, BMI, body mass index; HOMA-IR, homeostatic model assessment for insulin resistance

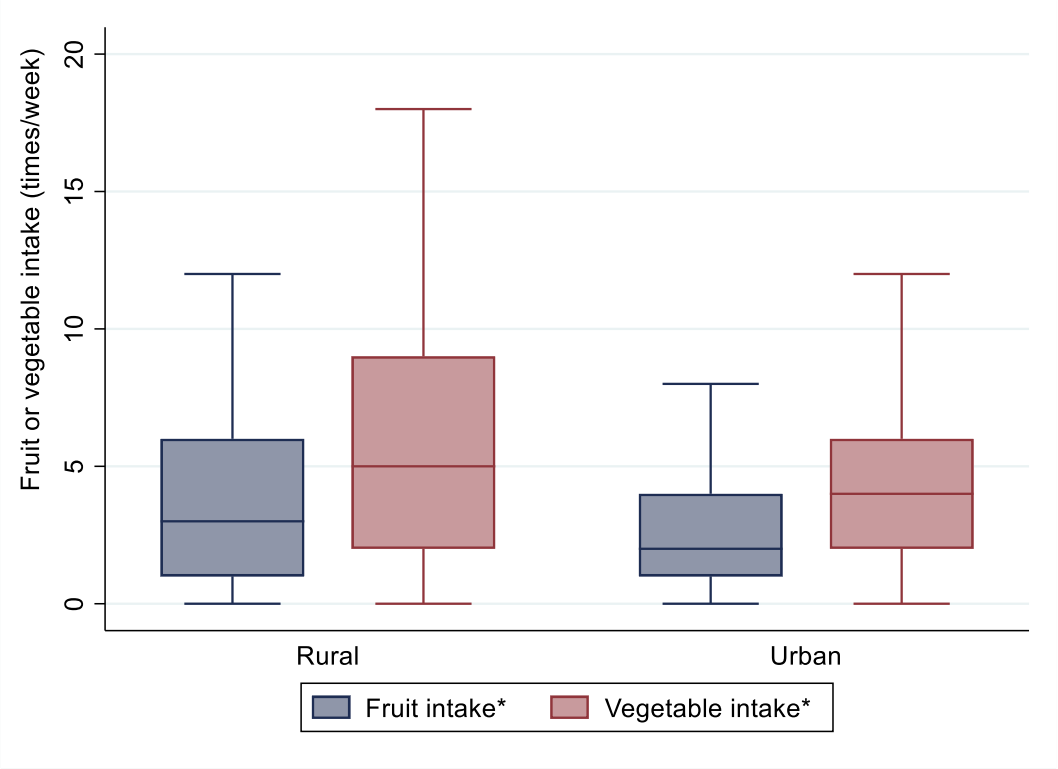

Figure 5.2 : Distribution of self-reported fruit and vegetable intake in rural and urban residents (Cameroon study, n = 578)

Median (25^{th}-75^{th} percentile)

*p-value < 0.05 for the difference in self-reported intake between participants living in rural and urban areas using a Mann Whitney test

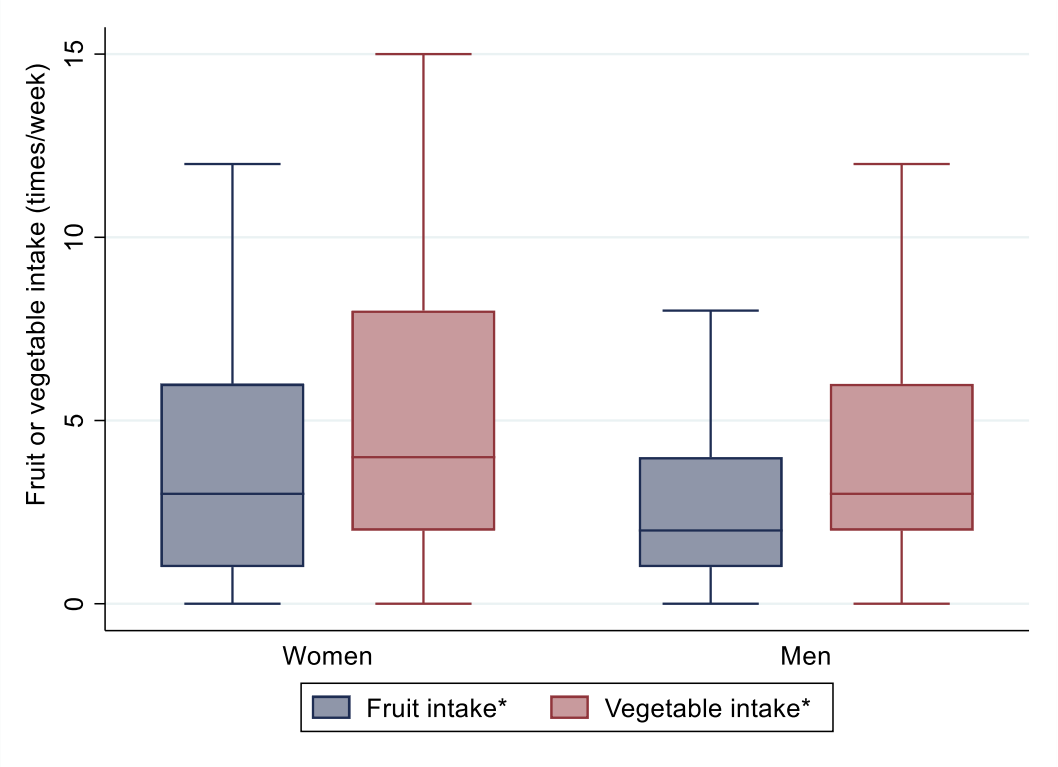

Figure 5.3 : Distribution of self-reported fruit and vegetable intake in women and men (Cameroon study, n = 578)

Median (25^{th}-75^{th} percentile)

*p-value < 0.05 for the difference in self-reported intake between women and men using a Mann Whitney test

5.6.2 Factors affecting serum folate and holotranscobalamin concentrations

After controlling for age and sex, I observed positive correlations between serum folate and alcohol intake and PAEE while education level and BMI were inversely correlated. For serum holoTC, positive associations were found for age, education level and BMI while PAEE was inversely associated (Table 1). Serum folate was not correlated with self-reported intake of fruit (*r*: 0.003 (p-value = 0.95)) and vegetable (*r*: 0.05 (p-value = 0.24)).

Table 5.3 : Factors associated with serum folate (n = 578) and holotranscobalamin (n = 547); Cameroon study

Characteristics	Folate β (95 confidence interval)	Holotranscobalamin β (95 confidence interval)
Age (10 years)	0.10 (0.04 to 0.16)	5.5 (2.1 to 8.9)
Men (vs women)	0.06 (-0.05 to 0.18)	0.36 (-5.71 to 6.43)
Education level		
< primary education (ref)		
Primary	0.04 (-0.11 to 0.19)	4.15 (-3.58 to 11.88)
Secondary school	-0.12 (-0.29 to 0.05)	9.9 (1.29 to 18.69)
University	-0.37 (-0.57 to -0.18)	13.83 (3.35 to 24.31)
Urban (vs rural)	-0.29(-0.39 to -0.19)	10.8(5.1 to 16.4)
Smoking status		
Never smoked (ref)		
Former smoker	0.08 (-0.08 to 0.24)	0.78 (-8.07 to 9.63)
Current smoker	0.02 (-0.21 to 0.25)	4.73 (-7.34 to 16.79)
Alcohol drinking		
Never (ref)		
Former	0.01 (-0.21 to 0.22)	-3.82 (-14.62 to 6.99)
Current	0.19 (0.04 to 0.34)	7.50 (-0.99 to 15.99)
Fruit intake (times/week)	0.003 (-0.01 to 0.02)	0.28 (-0.61 to 1.16)
Vegetable intake (times/week)	0.001 (-0.01 to 0.01)	-0.46 (-1.21 to 0.29)
PAEE (KJ/Kg/day)	0.005 (0.003 to 0.008)	-0.18 (-0.32 to -0.04)
Objective sedentary (hour/day)	-0.04 (-0.06 to -0.02)	0.81 (-0.48 to 2.1)
Objective LPA (hour/day)	0.03 (0.001 to 0.07)	0.003 (-0.03 to 0.04)
Objective MVPA (hour/day)	0.06 (0.03 to 0.10)	-0.04 (-0.08 to -0.009)
GPAQ PAEE (KJ/Kg/day)	0.001 (0.0002 to 0.001)	-0.01 (-0.05 to 0.02)
GPAQ work (MET-h/week)	0.0005 (0.0001 to 0.0008)	-0.004 (-0.02 to 0.02)
GPAQ leisure (MET-h/week)	0.0005 (-0.002 to 0.003)	-0.03 (-0.11 to 0.05)
GPAQ travel (MET-h/week)	0.001 (-0.0004 to 0.003)	-0.06 (-0.13 to 0.008)
BMI (Kg/m^2)	-0.01 (-0.02 to -0.004)	1.21 (0.65 to 1.77)
BMI categories		
<25 (ref)		
25-29.9	-0.15(-0.26 to -0.03)	5.17 (-1.81 to 12.15)
≥ 30	-0.17(-0.29 to -0.04)	13.22 (5.63 to 20.82)
Body fat (10 %)	-0.09(-0.20 to -0.03)	10.0 (6.8 to 14.1)
Waist circumference (10 cm)	-0.06(-0.10 to -0.02)	4.1 (1.6 to 6.7)

Estimates were adjusted for age and sex (except for age adjusted for sex only and sex adjusted for age only). β-coefficient represents the difference in holotranscobalamin in pmol/L or the log-transformed value of folate in nmol/L per unit difference in the predictor. For example, a β-coefficient of 0.01 for folate means that, for a year difference in age, folate concentration changes by exp (0.01) = 1.01 nmol/L, which corresponds to an increase of 1.0%.

PAEE, physical activity energy expenditure; LPA, Light physical activity; MVPA, moderate to vigorous physical activity; GPAQ, global physical activity questionnaire; BMI, body mass index.

5.6.3 Serum folate, holotranscobalamin and metabolic syndrome score

Serum folate was inversely associated with the metabolic syndrome score in unadjusted analysis (β: -0.30 (95% CI, -0.51 to -0.09) per 1 SD (10.8 nmol/L) of serum folate). This remained significant in model 3 adjusted for age, sex, education level, smoking, alcohol intake, area of residence, BMI and PAEE (β: -0.20 (95% CI, -0.38 to -0.02) per 1 SD of serum folate) (Table 5.3). For individual risk factors, serum folate was inversely associated with diastolic blood pressure (-1.13 (-2.04 to -0.21) per 1 SD of folate) and positively associated with HDL cholesterol (0.04 (0.005 to 0.07) in model 3.

For serum holoTC, positive associations were observed with the metabolic syndrome score (β: 0.33 (95% CI, 0.10 to 0.56) per 1 SD (34.3 pmol) of holoTC), diastolic BP (1.50 (0.39 to 2.62)) and 2-h glucose (0.22 (0.05 to 0.40)) in unadjusted analysis (Table 5.4). These associations were attenuated and became non-significant after adjusting for socio-demographic and behavioural characteristics (model 2). There was no evidence of interaction between sex, rural/urban residence, BMI categories and B-vitamins on any outcome.

In sensitivity analyses, the inverse association between serum folate and the metabolic syndrome score was attenuated and became non-significant when I additionally adjusted for serum holoTC in model 3 (β: -0.15 (95% CI, -0.34 to 0.03) per 1 SD of serum folate). There was no evidence of interaction between serum folate and holoTC on the metabolic syndrome score in model 3 (p-value for interaction = 0.39). The inverse association between serum folate and the metabolic syndrome score was similar when I used total folate including the oxidation product meFox. The overall results were unchanged when I used multiple imputation to investigate the impact of missing data or a computation of the metabolic syndrome score omitting the waist circumference.

Table 5.4 : Associations between serum folate and metabolic risk factors, Cameroon study

Outcome	Difference in outcome per 1 SD (10.8 nmol/L) of serum folate					
	Model 1		Model 2		Model 3	
	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value
Metabolic syndrome score (n=520)	-0.30 (-0.51 to 0.09)	0.005	-0.30 (-0.50 to -0.10)	0.004	-0.20 (-0.38 to -0.02)	0.029
Systolic blood pressure (mmHg), (n=529)	-1.51 (-3.18 to 0.16)	0.076	-1.58 (-3.29 to 0.12)	0.069	-1.25 (-2.89 to 0.39)	0.135
Diastolic blood pressure (mmHg), (n=529)	-1.57 (-2.58 to -0.56)	0.002	-1.42 (-2.39 to -0.45)	0.004	-1.13 (-2.04 to -0.21)	0.016
Fasting blood glucose (mmol/L), (n=529)	-0.003 (-0.10 to 0.10)	0.957	0.01 (-0.08 to 0.11)	0.807	0.03 (-0.08 to 0.12)	0.629
2-h blood glucose (mmol/L) (n=522)	-0.01 (-0.17 to 0.15)	0.865	0.01 (-0.15 to 0.18)	0.881	0.02 (-0.15 to 0.19)	0.809
HOMA_IR (n=526)	-0.004 (-0.09 to 0.09)	0.932	0.02 (-0.06 to 0.11)	0.603	0.05 (-0.03 to 0.14)	0.224
HDL cholesterol (mmol/L) (n=520)	0.03 (0.002 to 0.06)	0.037	0.04 (0.006 to 0.07)	0.018	0.04 (0.01 to 0.07)	0.023
Triglycerides (mmol/L) (n=520)	0.0004 (-0.04 to 0.04)	0.983	-0.01 (-0.05 to 0.03)	0.525	-0.003 (-0.04 to 0.04)	0.883

HOMA-IR, homeostatic model assessment for insulin resistance; β, β-coefficient; CI, confidence interval

Model 1: Unadjusted

Model 2: Adjusted for age, sex, education level, smoking, alcohol intake, residential site

Model 3: model 2 + body mass index + objectively measured physical activity

Table 5.5 : Associations between serum holotranscobalamin (holoTC) and metabolic risk factors, Cameroon study

Outcome	Difference in outcome per 1 SD (34.3 pmol/L) of serum holoTC					
	Model 1		Model 2		Model 3	
	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value
Metabolic syndrome score, (n=491)	0.33 (0.10 to 0.56)	0.004	0.17 (-0.05 to 0.39)	0.127	0.04 (-0.16 to 0.23)	0.70
Systolic blood pressure (mmHg), (n=500)	1.72 (0.001 to 3.44)	0.050	0.49 (-1.16 to 2.14)	0.561	0.08 (-1.60 to 1.76)	0.923
Diastolic blood pressure (mmHg), (n=500)	1.50 (0.39 to 2.62)	0.008	0.65 (-0.42 to 1.71)	0.234	0.32 (-0.75 to 1.38)	0.561
Fasting blood glucose (mmol/L), (n=500)	0.10 (-0.03 to 0.22)	0.121	0.07 (-0.07 to 0.21)	0.323	0.05 (-0.09 to 0.18)	0.502
2-h blood glucose (mmol/L), (n=494)	0.22 (0.05 to 0.40)	0.014	0.12 (-0.05 to 0.30)	0.162	0.12 (-0.06 to 0.29)	0.187
HOMA-IR (n=497)	0.09 (-0.003 to 0.18)	0.059	0.12 (0.03 to 0.23)	0.012	0.08 (-0.01 to 0.18)	0.075
Triglycerides (mmol/L) (n=491)	0.03 (-0.006 to 0.07)	0.10	0.02 (-0.02 to 0.06)	0.313	0.01 (-0.03 to 0.05)	0.475
HDL cholesterol (mmol/L) (n=491)	0.02 (-0.01 to 0.05)	0.165	0.01 (-0.02 to 0.03)	0.678	0.01 (-0.02 to 0.04)	0.647

HOMA-IR, homeostatic model assessment for insulin resistance; β, β-coefficient; CI, confidence interval

Model 1: Unadjusted

Model 2: Adjusted for age, sex, education level, smoking, alcohol intake and residential site

Model 3: model 2 + body mass index and objectively measured physical activity

5.7 Discussion

In this cross-sectional population-based study, serum folate and holoTC were associated with the clustered score of cardiometabolic risk factors in opposite directions. The positive association between serum holoTC and the metabolic syndrome score was confounded by socio-demographic and behavioural characteristics. The inverse association between serum folate and metabolic syndrome score was independent of age, sex, education level, smoking, alcohol intake, residential site, BMI and physical activity. Moreover, higher serum folate was associated with higher HDL and lower diastolic blood pressure independently of potential confounders. As serum folate and holoTC status are dependent on dietary patterns, the observed associations reflect the role of dietary patterns for which folate and vitamin B12 are objective indicators. To my knowledge, this is the first study from SSA to examine the associations of serum folate and holoTC, with the metabolic syndrome score,

Previous observational studies mostly in Western and Eastern populations have examined the associations of blood concentrations of folate and vitamin B12 with metabolic syndrome. In a cross-sectional population-based study of 2201 adults in the US, participants with metabolic syndrome had higher serum folate and lower serum vitamin B12 concentrations than those without metabolic syndrome (240). Similarly, a study of 524 adults in 9 Mesoamerican countries showed that RBC folate was positively associated with metabolic syndrome and plasma vitamin B12 was positively associated with fasting blood glucose and hypertension (241). In another study in obese individuals (BMI > 35kg/m^2) in France, plasma folate and vitamin B12 showed no association with the number of metabolic syndrome components (210). Other studies examining the association of dietary intake of folate and vitamin B12 with metabolic syndrome found an inverse association with folate intake (242), (243) and no association with vitamin B12 intake (243).

While the evidence from epidemiological studies on the association between these B-vitamins and metabolic syndrome is conflicting, the difference in the ascertainment of the exposure should be noted. Some studies measured serum (or plasma) folate/vitamin B12, others RBC folate and others dietary folate/vitamin B12 intake which may limit comparability across studies. For instance, RBC folate reflects body stores of folate over the lifespan of the RBCs and thus is an indicator of medium-term status (120 days), while serum folate responds rapidly to changes in diet. Moreover, the relationship between folate/vitamin B12 intake and status markers is influenced by several factors such as polymorphisms in metabolising enzymes,

smoking, physical activity, vitamin absorption, bioavailability from natural food sources, food fortification and food processing (244). The positive association between serum folate and the metabolic syndrome reported in the US may not reflect the association of dietary sources of folate with metabolic endpoints given that folic acid fortification of wheat flour is mandatory in the US since 1998 (245).

In this study, I observed an inverse association between serum folate and metabolic syndrome score and a positive association with serum holoTC, which reflects the associations of metabolic syndrome with diet. Green leafy vegetables, legumes and fruits are rich sources of folate and serum folate has therefore been proposed as an objective indicator of fruit and vegetables intake with evidence from observational and intervention studies (202), (203), (204), (205). These findings of an inverse association between serum folate and the metabolic syndrome score and diastolic blood pressure confirm previously reported cross-sectional studies in SSA showing an inverse association between a dietary pattern rich in fruit and vegetables intake and the metabolic syndrome (38) and hypertension (172), (200), (201), (246).

There are several potential mechanisms through which intake of fruits and vegetables may reduce metabolic syndrome risk. Firstly, apart from being rich in folate, fruit and vegetables are also rich sources of vitamin C, E, carotenoids, magnesium and phytochemicals like polyphenols. A higher fruit and vegetable intake has been associated with lower inflammatory markers and reactive oxygen species (188) thereby reducing oxidative stress and systemic inflammation, which are involved in the development and severity of the metabolic syndrome (189). Secondly, fruits and vegetables contain polyphenols and fibres, which modulate the gut microbiome composition and function (176) and metabolic syndrome has been linked with altered gut microbiota (190), moreover dietary fibres have benefits on individual components of the metabolic syndrome (247). Thirdly, increasing fruit and vegetable intake is associated with weight loss and adiposity has a significant pathophysiological role in the development of metabolic syndrome (175).

While serum folate may be an objective indicator of fruit and vegetables intake, the possibility that the observed association is due to the metabolic effects of folate via its lowering effect on plasma homocysteine cannot be excluded. Folate is required for the conversion of homocysteine to methionine, and in the case of folate deficiency, the reaction is inhibited leading to the accumulation of homocysteine. Elevated plasma homocysteine has been linked with a higher prevalence of metabolic syndrome (248), (249). High plasma homocysteine

levels can be lowered by folic acid supplementation but evidence of the efficacy of folic acid supplementation in lowering cardiometabolic risk is inconclusive (250), (251), (252). A large RCT in China amongst adults with hypertension showed that supplementation with folic acid and enalapril reduced the risk of first stroke compared with enalapril alone (253). In a small RCT in Tanzania, supplementation with folic acid and nitrate did not lower blood pressure compared with the placebo group (254). A meta-analysis of RCTs showed that supplementation with folic acid had no effect on CVD incidence or mortality (251) or diabetes risk (252). In mendelian randomisation studies, genetically predicted plasma concentrations of homocysteine, folate or vitamin B12 were neither associated with CVDs (255), (256) nor diabetes (257). In this study, the inverse association between serum folate and the metabolic syndrome score became non-significant after adjusting for serum holoTC. One reason for this could be because vitamin B12 together with folate act as cofactors in the remethylation of homocysteine to methionine, therefore the mutual adjustment of these B-vitamins in the analyses may attenuate or mask potential associations (258).

I observed a positive association between holoTC and the metabolic syndrome in unadjusted analysis, which could suggest a potential positive association of the metabolic syndrome with animal-sourced foods. Natural vitamin B12 is found almost exclusively in animal products, which are high in haem iron and fats especially saturated fats. High intake of animal-based fat has been linked with higher risk of obesity, hyperglycaemia and CVD. Moreover, iron has pro-oxidative properties and may contribute to increase oxidative stress by the generation of reactive oxygen species (259). The positive association between serum holoTC and the metabolic syndrome became non-significant after adjusting for potential confounders, suggesting that socio-demographic variations and health-related behaviours contributed to the association observed.

Serum folate concentration was higher in rural residents than in those living in urban areas, while serum holoTC was higher in urban areas than in rural areas. This is consistent with findings from a meta-analysis of 47 studies from 22 countries in SSA showing a higher meat intake in urban residents compared with those living in rural areas, and though not statistically significant, a tendency toward higher intake of fruit and vegetables in rural areas (151). Although there was no correlation between serum folate and frequency of self-reported intake of fruit and vegetables, the socio-demographic patterning of self-reported frequency of fruit and vegetables intake was similar to that of serum folate. I observed a higher self-reported frequency of fruit and vegetables intake in rural residents compared with those living in urban

areas. The frequency of fruit and vegetables intake was based on self-reports, where participants reported the number of times in a typical week when they ate fruits and vegetables which could be influenced by recall bias and social desirability bias. Moreover, portion sizes were not estimated nor were detailed information available about the fruits and vegetables consumed (for instance, fresh vegetables, dried vegetables, cooked or raw vegetables). All these factors could affect the relationship between intake of fruits and vegetables and serum folate. In a nationally representative survey of women of reproductive age in Cameroon, fruits, vegetables, beans and legumes, grains, fruit, roots and tubers contributed 98% of the total folate intake (218).

Future studies are needed to determine what dietary changes can provide a sustainable change in folate status and if these changes have a positive effect on the reduction of metabolic risk in this population.

5.8 Strengths and limitations

The strengths of our study include the objective assessment of the B-vitamins in a population-based study. Folate status was measured using UPLC-MS/MS, which is regarded as the gold standard for serum folate and captures the individual folate forms separately, including free folic acid. We measured serum holoTC, which has been postulated as a better marker of vitamin B12 status than total vitamin B12 (208). Moreover, our study included participants from rural and urban areas and I adjusted for potential confounders including objectively measured physical activity. Physical activity may lead to a higher turnover of B-vitamins because of increased protein catabolism which is associated with an increase in muscular amino acid pools and homocysteine production (260). In this study, physical activity was positively associated with serum folate but inversely associated with serum holoTC, suggesting that the association of B-vitamins with physical activity may partly reflect differences in health-related behaviours rather than a mechanistic association. For example, a higher intake of fruits and vegetables and a lower intake of animal-sourced foods in more physically active individuals.

This study had some limitations related to the cross-sectional design of the study and sampling technique as discussed in Chapters 2 and 3. The samples used in this study were collected and stored from 2005-2006. However, both folate and vitamin B12 have been shown to be relatively stable after long-term storage (261). Moreover, because, these samples were collected before the mandatory wheat flour fortification in Cameroon in 2011, this study provides a unique opportunity to examine the associations of serum folate and vitamin B12 as

objective indicators of fruit and vegetables intake and animal-sourced foods intake respectively with cardiometabolic risk factors in the absence of food fortification. Detailed data on fruit and vegetables intake was not available and there was no data on animal product intake. However, previous observational and intervention studies have reported an association between serum folate and intake of fruits and vegetables and between serum holoTC and intake of animal products. This suggests that serum folate and vitamin B12 may be used as objective indicators of dietary intake, especially in settings where there is no food fortification with these B-vitamins.

5.9 Conclusion

In this population-based study, higher serum folate as an objective indicator of fruit and vegetables intake was associated with lower composite score of metabolic syndrome, diastolic blood pressure and higher HDL cholesterol independently of socio-demographic and health-related behaviours. Higher serum holoTC concentration as an indicator of intake of animal-sourced foods was associated with higher metabolic syndrome score, but this association was confounded by sociodemographic characteristics. These findings suggest that based on these biomarkers, public health approaches promoting a higher intake of fruits and vegetables may reduce the burden of cardiometabolic disease in this population. Future large prospective studies to investigate the associations of serum folate and vitamin B12 with metabolic risk in populations in SSA are needed as well as studies to explore the dietary determinants of serum folate and vitamin B12 concentrations.

Chapter 6 : Associations between plasma zinc and glycaemic markers

Publication

Mba CM, Jones K, Forouhi NG, Imamura F, Assah F, Mbanya JC, Wareham NJ. The association between plasma zinc concentrations and markers of glucose metabolism in adults in Cameroon. (Under review at Public Health Nutrition)

Summary

Background: An abnormal zinc status has been suggested to play a role in the pathogenesis of type 2 diabetes. However, epidemiological studies of the relationship between plasma zinc concentrations and type 2 diabetes are sparse and inconclusive. I aimed to investigate the association between plasma zinc concentration and glycaemic markers in rural and urban Cameroon.

Methods: This was a population-based cross-sectional study of 596 healthy adults (63.3% women) aged 25-55 years. Fasting plasma zinc concentrations were quantified using inductively coupled plasma mass spectrometer. Outcomes were glycaemic markers including fasting glucose, 2-h glucose and homeostatic model assessment for insulin resistance (HOMA-IR). I fitted linear regression models to assess the association between plasma zinc concentration and glycaemic markers.

Results: The mean ± SD plasma zinc concentration was 13.7 ± 2.7 µmol/L overall, with higher levels in men (14.4 ± 2.9 µmol/L) than in women (13.2 ± 2.6 µmol/L), p-value < 0.0001. There was a significant inverse association between tertiles of plasma zinc concentration and 2-h glucose concentration (p-value for linear trend = 0.002). The difference in 2-h glucose between those in the highest tertile of plasma zinc compared to the lowest was -0.63(95% CI -1.02 to -0.23) mmol/L in unadjusted analysis. This association was attenuated but remained significant after adjusting for socio-demographic characteristics and health-related behaviours -0.43(-0.82 to -0.04). Similar inverse associations were observed between plasma zinc concentration and fasting glucose and HOMA-IR when adjusted for confounding factors.

Conclusion: The current findings of an inverse association between plasma zinc concentration and several markers of glucose homeostasis, together with growing evidence from intervention studies suggest a role for zinc in glucose metabolism. If supported by further evidence, strategies to improve zinc status in populations may provide a cheap public health prevention approach for diabetes.

6.1 Introduction

The burden of diabetes has risen globally over the past three decades but at a faster rate in low and middle-income countries where 80% of people in the world with diabetes live (17). In Africa, an estimated 24 million adults had diabetes in 2021, and this has been projected to reach 55 million by 2045. Over 70% of deaths in people with diabetes in Africa occur in those who are in an economically productive age group, which has substantial implications at the individual, household and societal levels (1). Therefore, identifying the determinants of this growing diabetes burden is a major public health concern. In SSA, the rise in diabetes prevalence has been attributed to a shift in dietary patterns along with physical inactivity, driven in part by urbanisation (262). This dietary transition toward the consumption of processed foods is associated with diets that do not often meet recommended dietary intakes of some micronutrients (263).

Zinc is an essential trace element naturally found mainly in meat, poultry, dairy products, and seafood (264), (265). Fortified foods, dietary supplements and plant foods like legumes and grains are also good dietary sources of zinc (266). Although there is evidence to suggest that zinc deficiency is a public health issue globally (267), (268), the limited set of studies from Africa, which are mainly in children and women of reproductive age, suggest that the magnitude of zinc deficiency in Africa may be greater than in other parts of the world (267), (268), (269). Based on estimates of dietary intake of zinc using national food balance sheets, 26% of people in Africa have inadequate zinc intake (compared with 16% globally) (268).

There is evidence that zinc plays a role in glucose metabolism (270). Findings from large prospective observational studies suggest an inverse association between dietary zinc intake and type 2 diabetes risk (271), (272), (273). However, these studies relied on self-reports to assess dietary zinc intake which is subject to measurement error and recall bias. Measurement of blood zinc concentrations to assess zinc status provides an objective measure that complements data on dietary zinc intake (274). There are few studies on the association between blood zinc concentration and type 2 diabetes and the results of these are inconsistent. In a previous cross-sectional study in the US and a prospective study in Finland, plasma zinc concentration was positively associated with diabetes prevalence or risk (275), (276). However, these studies were conducted in a context where the prevalence of zinc deficiency is low (268). In other studies in China where low plasma zinc concentration is more common, plasma zinc concentration was either inversely associated with diabetes prevalence (277) or not markedly

associated with diabetes (278). Thus, it is possible that observations of the association between plasma zinc concentrations and diabetes could be affected by the frequency of low plasma zinc concentrations in the population studied.

I did not find any previous population-based study in an African population linking dietary zinc intake or zinc biomarkers to glycaemic markers. Dietary patterns in many low and middle-income countries are rich in intake of phytates, which bind to zinc and inhibit its absorption (266). In such settings, blood zinc may be a better indicator of zinc exposure than dietary zinc intake. This study aimed to examine the independent associations between plasma zinc concentration and glycaemic markers in adults in Cameroon, a country with a high prevalence of low zinc status (268), (269).

6.2 Study aims

This study aimed to identify factors that affect plasma zinc concentrations and examine the associations between plasma zinc concentration and glycaemic markers in rural and urban settings of Cameroon.

6.3 Background on zinc

6.3.1 Dietary sources, metabolism and functions of zinc

Zinc is an essential trace element and good dietary sources of zinc include protein-rich foods such as fish, meat, poultry and oysters. Plant-based foods such as beans, legumes and whole grains are also high in zinc but are less efficiently absorbed than dietary zinc from animal-sourced foods due to the presence of dietary phytates in plant-based foods. Dietary phytates bind to zinc to form an insoluble complex that cannot be digested because humans lack the intestinal enzyme phytase to digest this complex, thereby inhibiting zinc absorption (274). Moreover, the content of plant-based foods in zinc may vary based on the concentration of zinc in the soil. This emphasises the need for locally adapted food composition tables when assessing dietary zinc using traditional dietary assessment instruments.

Following a meal, zinc is absorbed at the level of the small intestines primarily via transporter-mediated processes and enters the portal system. Zinc is transported to the liver and then released into the systemic circulation for distribution to the required tissues. In circulation, ~ 70% of zinc is bound to albumin. Therefore in the case of hypoalbuminaemia, plasma zinc concentration also drops. Other factors that affect plasma zinc concentration include stress,

infections and acute trauma, given that these conditions increase tissue uptake leading to reduced plasma zinc concentration. Physiologic conditions such as pregnancy also increase zinc turnover (274).

Zinc is an essential micronutrient that is required in numerous structural and biochemical processes including cell growth, immunity, DNA, RNA and protein synthesis, gene expression and enzyme function. Low zinc status is associated with multiple non-specific clinical manifestations including stunting and dysfunctions affecting the immune, reproductive and nervous systems (279). Zinc also functions as an important cell-signalling agent, influencing signalling pathways such as insulin receptor signalling. Accumulating evidence from epidemiological studies suggests the role of zinc in the development of diabetes (270).

6.3.2 Plasma zinc as a biomarker and dietary requirements

The Biomarkers of Nutrition for Development (BOND) zinc expert panel currently recommends 3 biomarkers to assess zinc intake or status at the individual or population level (280):

- Dietary zinc intake
- Plasma or serum zinc concentration
- Prevalence of stunting in children

The limitations of assessing dietary zinc intake using traditional dietary assessment methods include issues related to recall bias, social desirability bias and inadequate food composition databases leading to over or underestimation of dietary zinc intake as discussed in Chapter 2.

Plasma or serum zinc is the most widely used indicator of zinc status and reflects zinc status over the previous weeks (280). Plasma zinc concentrations have also been shown to respond in a dose-dependent manner to zinc intake (from supplements, natural or fortified foods) (281), (282) and can be used to evaluate the effectiveness of dietary zinc intervention programmes. In a RCT in healthy men in Senegal, plasma zinc concentration increased within 2 weeks of zinc supplementation compared with placebo (283). In Cameroon, following the mandatory fortification of wheat flour in some micronutrients including zinc, a study in ~ 300 women of reproductive age showed that compared with the pre-fortification period, the mean plasma zinc concentration was higher by 18% and the prevalence of zinc deficiency was almost halved at 1-year post fortification (284). However, plasma zinc concentrations do not reflect absolute

zinc intakes because of the influence of genetics, metabolism and lifestyle factors such as smoking and physical activity (280).

The suggested cut-offs to define zinc deficiency based on plasma zinc concentration according to age, sex and time of the day suggested by the International Zinc Nutrition Consultative Group are presented in Table 6.1:

Table 6.1: Cut-offs to define zinc deficiency based on plasma zinc concentration (280)

	Plasma zinc concentration (µmol/L)		
	Morning		PM
	Fasting	Non fasting	
< 10 years	NA	9.9	8.7
≥ 10 years			
Females	10.7	10.1	9.0
Males	11.3	10.7	9.3

The cut-off levels were derived from the National Health and Nutrition Examination Survey data using the mean - 2 SD to represent the cut-off levels below which zinc deficiency is likely to occur and taking into account the known effects of age, sex and fasting state on plasma zinc concentration.

The RNIs for zinc varies by age and sex and are shown in Table 6.2

Table 6.2: Reference nutrient intakes for zinc in the UK (167)

Age	Zinc RNI (mg/d)
1-3 years	5.0
4-6 years	6.5
7-10 years	7.0
11-14 years	9.0
≥ 15 years	
Male	9.5
Female	7.0

Other potential biomarkers of zinc status, which have been less studied, include concentrations of hair zinc, and nail zinc, which reflect long-term zinc status. However, despite the assessment of hair and nail zinc concentrations being non-invasive and the advantage of the stability of the specimen, lack of reference data and sensitive measurement techniques currently limit the use of hair or nail zinc as a marker of zinc status. Moreover, studies assessing the response of hair and nail zinc concentrations to zinc intake show inconsistent results (282), (285).

6.4 Methods

The study design, setting and other common methods and procedures used in this cross-sectional study are reported in Chapter 2.

6.4.1 Measurement of plasma zinc concentration

Plasma zinc concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS) using a Perkin Elmer NEXION 300D at Southampton University Hospitals. An internal standard (rhodium) was added to the plasma diluted in 1 in 50 in distilled water and to the quality control to normalise for sample preparation and instrument variability. Samples were run against matrix-matched calibration solutions prepared with bovine serum (Sigma-Aldrich). The zinc isotope signals (^{66}Zn) were compared against the internal standard to determine the concentration of plasma zinc. Quality control materials were run with each analytical batch and consisted of certified reference material (Sero, Norway) and an in-house material. The inter-batch coefficient of variation was less than 10.1% demonstrating acceptable analytical accuracy and precision. External quality assurance was performed as part of the Trace Element Quality Assurance Scheme (TEQAS) (UK NEQAS, Guildford, Surrey, United Kingdom).

6.4.2 Outcomes

Outcomes were markers of glucose homeostasis including fasting glucose, 2-h glucose and HOMA-IR.

6.5 Statistical analyses

All statistical analyses were performed using Stata 15 (StataCorp, Texas, United States). Descriptive statistics are presented as means ± SD (or median and (25^{th}-75^{th} percentile) for non-normally distributed data) or numbers and percentages. I tested differences in means using

the t-test. Using the sex-specific cut-offs for defining zinc deficiency recommended by the International Zinc Nutrition Consultative Group, I reported the proportion of participants with plasma zinc deficiency (< 10.7 µmol/L in women and < 11.3 µmol/L in men) (280). Linear trends across tertiles of plasma zinc concentration were obtained from a linear regression model for continuous variables including tertiles of plasma zinc concentration as a continuous exposure and chi-squared test for trend (Cochran-Armitage test or Cochran-Mantel-Haenszel test for categorical variables with 2 or ≥ 3 levels respectively). I fitted linear regression models adjusted for age and sex to identify potential correlates of plasma zinc concentration.

To examine the independent associations between plasma zinc and glycaemic markers, I categorised plasma zinc concentration by tertiles and fitted three statistical models incrementally adjusted for potential confounding variables. After fitting crude regression models, I further adjusted for age (continuous) and sex, and then for smoking (never, past or current), alcohol intake (never, past or current), level of education (less than primary school, completed primary school, secondary school and university), residential site (4 sites), PAEE (continuous) and BMI (continuous). P-values for trend were obtained from linear regression models including plasma zinc as an ordinal variable across tertile categories. HOMA-IR was log-transformed to account for its skewed distribution. Complete case analysis was performed.

In sensitivity analysis, a) with missing data (PAEE, n=53; 2-h glycaemia, n=9; HOMA-IR, n=5) assumed to be missing at random, I imputed missing data by using multiple imputation by chained equations to create 10 imputed datasets and using Rubin's rules to combine estimates (127); b) I further adjusted for self-reported fruit and vegetables intake as a proxy for overall dietary quality in model 3; and c) replaced BMI by body fat in model 3. I investigated non-linear associations of plasma zinc concentration with glycaemic markers by fitting restricted cubic splines with 3 knots corresponding to the 25^{th}, 50^{th}, and 75^{th} percentile of continuously distributed plasma zinc concentration using model 3. Non-linearity was tested using the Wald test. I tested for effect modification by sex, rural-urban residence and BMI categories on the association between plasma zinc concentration and glycaemic markers and subgroup analysis was performed if the p-value for interaction was < 0.05.

6.6 Results

6.6.1 Descriptive characteristics

The mean concentration of plasma zinc was 13.7 ± 2.7 µmol/L with higher levels in men (14.4 ± 2.9 µmol/L) than in women (13.2 ± 2.6 µmol/L), p-value <0.0001 (Figure 6.1). There was no evidence of a difference in the mean plasma zinc concentration between rural (13.5 ± 2.9 µmol/L) and urban (13.8 ± 2.6 µmol/L) participants, p-value = 0.35. Using pre-established cut-offs for plasma zinc deficiency, 13.8% of women had plasma zinc concentrations below 10.7 µmol/L and 11.9% of men below 11.3 µmol/L.

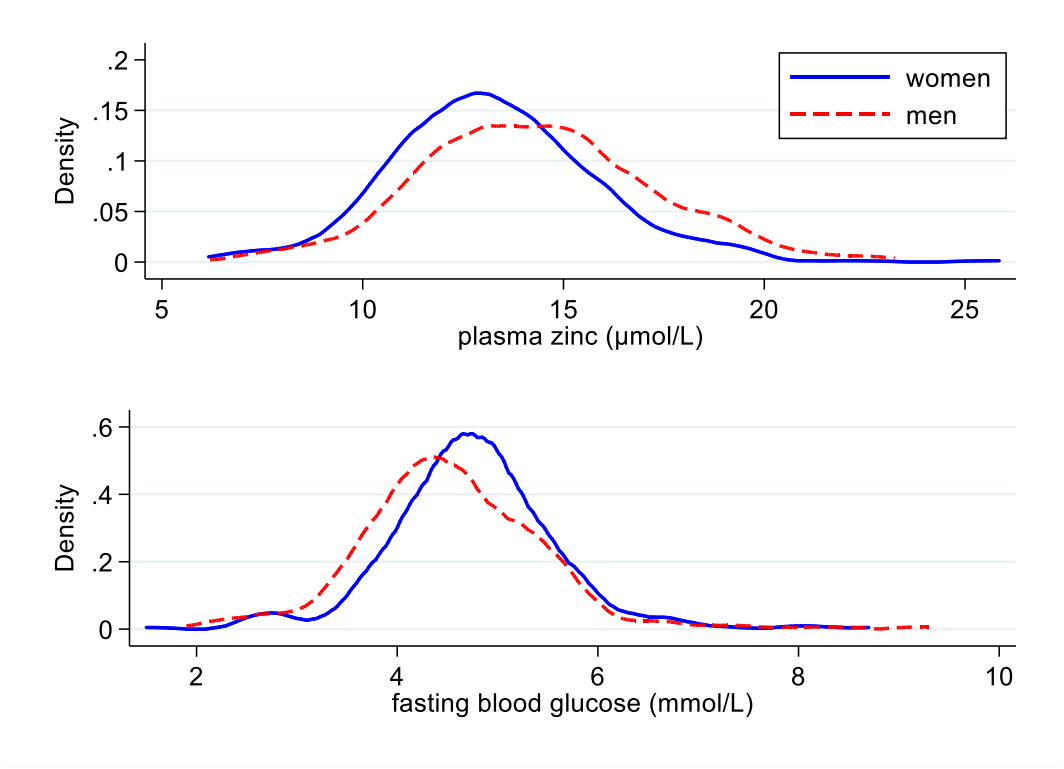

Figure 6.1 : Distribution of plasma zinc and fasting blood glucose by sex (Cameroon study, n=587)

6.6.2 Factors affecting plasma zinc concentration

Level of education and smoking status were positively associated with plasma zinc concentration while female sex, physical activity, self-reported vegetable intake and 2-h glucose were all inversely associated (Table 6.3 and 6.4). There was no evidence of a linear trend in fasting glucose and HOMA-IR across increasing tertiles of plasma zinc.

After adjusting for age, and sex, positive correlates of plasma zinc concentration were male sex (adjusted for age only) and measures of adiposity (Table 6.5). Physical activity and self-reported intake of fruits and vegetables were negatively correlated with plasma zinc concentration. Rural/urban residential site was not associated with plasma zinc concentrations.

Table 6.3 : Socio-demographic and behavioural characteristics by thirds of plasma zinc concentration (Cameroon study, n=596)

Characteristics	Plasma zinc categorised by tertiles			p for linear trend
	1st	2nd	3rd	
Zinc (µmol/L), range	6.2 – 12.4	12.5 – 14.5	14.6 – 25.8	
Age (years)	38.7 ± 8.1	38.9 ± 8.9	37.3 ± 8.8	0.09
Sex, n(%)				
Women	146(72.6)	127(64.1)	104(52.8)	< 0.001
Education (completed), n(%)				
< primary school	36(18.0)	37(18.7)	30(15.2)	
Primary school	100(50.0)	83(41.9)	81(41.1)	0.04
Secondary school	41(20.5)	53(26.8)	56(28.4)	
University	23(11.5)	25(12.6)	30(15.2)	
Smoking status, n(%)				
Never	165(82.1)	155(78.3)	144(73.1)	
Past	26(12.9)	26(13.1)	27(13.7)	0.02
Current smoker	10(5.0)	17(8.6)	26(13.2)	
Alcohol intake, n(%)				
Never	21(10.4)	17(8.6)	27(13.7)	
Past	14(7)	22(11.1)	22(11.2)	0.07
Current	166(82.6)	159(80.3)	148(75.1)	
Residence, n(%)				
Rural	100(49.7)	77(38.9)	98(49.7)	0.99
PAEE (KJ/Kg/day)	51.5 ± 23.6	51.3 ± 22.2	48.7 ± 23.7	0.26
Sedentary time (min/day)	960.9 ± 154.1	937.0 ± 148.8	968.9 ± 154.7	0.63
LPA time (min/day)	356.2± 103.7	381.2 ± 105.2	362.7 ± 108.8	0.57
MVPA time (min/day)	122.9 ± 90.3	121.7 ± 84.3	108.4 ± 85.3	0.13
GPAQ PAEE (KJ/Kg/day)	48.6 (6.7 - 146.9)	20.3 (3.3 - 113.7)	7.6 (3.2 - 71.2)	< 0.001
GPAQ work (MET-min/week)	3840(0 - 12480)	0(0 - 10080)	0(0 - 5760)	< 0.001
GPAQ leisure (MET - min/week)	0(0-0)	0(0-0)	0(0-0)	
GPAQ travel (MET - min/week)	1440(560-3360)	840(280-3360)	840(280-1680)	0.002
Fruit (times/week)	2(1 - 6)	3(1 - 5)	2(1 - 3)	0.09
Vegetable (times/week)	6(1 - 8)	4(2 - 8)	3(2 - 5)	< 0.001

Results are presented as arithmetic mean ± SD [or median (25^{th}-75^{th} percentile) for non-normally distributed variables] or n (%). p-values for trend are from a chi-squared test for trend for categorical variables or from a linear regression model for continuous variables including thirds of plasma zinc as a continuous exposure. n=596 except for PAEE where n=543

PAEE, physical activity energy expenditure; LPA, light physical activity; MVPA, moderate to vigorous physical activity

Table 6.4 : Metabolic characteristics of the population by thirds of plasma zinc concentration (Cameroon study, n=596)

Characteristics	Plasma zinc categorised by tertiles			p for linear trend
	1st	2nd	3rd	
Waist circumference (cm)	88.0 ± 12.2	89.2 ± 12.2	88.6 ± 12.0	0.63
BMI (kg/m^2)	25.9 ± 5.3	26.4 ± 5.2	25.9 ± 5.2	0.97
Body fat (%)	28.8 ± 11.1	28.9 ± 10.7	26.7 ± 11.4	0.05
Systolic blood pressure (mmHg)	120.7 ± 20.5	122.6 ± 22.1	124.6 ±19.6	0.07
Diastolic blood pressure (mmHg)	75.2 ± 12.8	76.8 ± 13.9	77.3± 13.4	0.11
Fasting glucose (mmol/L)	4.82 ± 1.06	4.79 ± 1.31	4.71 ± 1.61	0.40
2-hour glucose (mmol/L)	6.68 ± 1.85	6.20 ± 1.78	5.99 ± 1.99	0.0003
Fasting insulin (pmol/L)	21.7(11.5 - 37.5)	18.8(11.0 - 34.8)	22.7(12.4 - 33.4)	0.63
HOMA-IR index	0.75(0.37 - 1.32)	0.66(0.34 - 1.23)	0.76(0.39 - 1.21)	0.48
Total cholesterol (mmol/L)	3.78 ± 0.97	3.85 ± 0.99	3.91 ± 0.97	0.18
HDL cholesterol (mmol/L)	1.20 ± 0.35	1.23 ± 0.34	1.24 ± 0.30	0.29
LDL cholesterol (mmol/L)	2.18 ± 0.84	2.25 ± 0.84	2.30 ± 0.84	0.17
Triglycerides (mmol/L)	0.75(0.61 - 0.93)	0.73(0.58 - 0.92)	0.74(0.57 - 0.98)	0.59
CRP (mg/L)	3.97(2.44 - 7.37)	5.28(2.69 - 8.39)	5.43(2.47-10.12)	0.44

(n= 596, except for 2-h glycaemia where, n=587 and HOMA-IR, n=591)

Results are presented as arithmetic mean ± SD [or median (25th-75th percentile) for non-normally distributed variables] or n (%). p-values for trend are from a chi-squared test for trend for categorical variables or from a linear regression model for continuous variables including thirds of plasma zinc as a continuous exposure.

BMI, body mass index; HOMA-IR, Homeostatic model assessment for insulin resistance; CRP, C-reactive protein

Table 6.5 : Factors affecting plasma zinc concentration (Cameroon study: n=596)

Correlates	β (95% CI)	p-value
Age (years)	-0.02(-0.04 to 0.01)	0.15
Men (vs women)	1.08(0.63 to 1.53)	<0.001
Education level (< Primary education (ref))		
Primary education	-0.21(-0.83 to 0.41)	
Secondary school	-0.38(-0.31 to 1.06)	0.52
University	-0.24(-0.57 to 1.05)	0.28
		0.56
Smoking status (never smoked (ref))		
Former	-0.29(-0.97 to 0.38)	0.39
Current	-0.60(-0.25 to 1.44)	0.17
Alcohol intake (never (ref))		
Former	-0.21(-0.74 to 1.16)	0.66
Current	-0.39(-1.09 to 0.31)	0.28
Residential area (rural (ref))		
Urban	0.21(-0.22 to 0.64)	0.34
Marital status (single (ref))		
Married	-0.08(-0.66 to 0.50)	0.79
Divorced	-0.26(-1.50 to 0.99)	0.69
Widowed	-0.63(-1.69 to 0.43)	0.24
Family size (< 3 (ref))		
3-5	0.31(-0.21 to 0.84)	0.24
>5	0.86(0.21 to 1.51)	0.01
Fruit intake (times/week)	-0.11(-0.17 to -0.04)	0.001
Vegetable intake (times/week)	-0.14(-0.19 to -0.09)	<0.001
PAEE (KJ/kg/day)	-0.01(-0.02 to -0.005)	0.004
Objective sedentary time (h/day)	0.08(-0.01 to 0.17)	0.09
Objective LPA (h/day)	-0.02(-0.15 to 0.11)	0.74
Objective MVPA (h/day)	-0.20(-0.36 to -0.04)	0.01
GPAQ PAEE (KJ/Kg/day)	-0.006(-0.009 to -0.004)	<0.001
GPAQ work (MET-h/week)	-0.004(-0.005 to -0.002)	<0.001
GPAQ leisure (MET-h/week)	0.01(0.002 to 0.02)	0.02
GPAQ travel (MET-h/week)	-0.01(-0.02 to -0.007)	<0.001
BMI (kg/m^2)	0.05(0.009 to 0.09)	0.02
Body fat (%)	0.04(0.01 to 0.07)	0.006
Waist circumference (cm)	0.02(0.004 to 0.04)	0.02
CRP (mg/L)	-0.001(-0.01 to 0.01)	0.93

β-coefficient represents the difference in plasma zinc in µmol/L per unit difference in the predictor. Estimates are adjusted for age and sex (except for age adjusted for sex only and sex adjusted for age only)

PAEE, physical activity energy expenditure; LPA, Light physical activity; MVPA, moderate to vigorous physical activity; GPAQ, Global physical activity questionnaire; BMI, body mass index

6.6.3 Plasma zinc concentration and glycaemic markers

Table 6.6 shows the results of multiple linear regression analyses between plasma zinc concentration and glycaemic markers. 2-hour glucose was lower by -0.63 (95 % CI -1.02 to -0.23) mmol/L among those in the highest tertile of plasma zinc compared with those in the lowest tertile (p-value for linear trend 0.002) in unadjusted analysis. This remained significant in model 3 adjusted for age, sex, smoking status, alcohol intake, education level, area of residence, adiposity and objectively measured physical activity (β: -0.43(95 % CI -0.82 to -0.04) mmol/L, p-value for linear trend = 0.03.

Similar inverse associations were observed between plasma zinc concentration and fasting glucose and HOMA-IR after adjusting for potential confounders. Compared with participants in the lowest tertile of plasma zinc concentration, being in the highest tertile was associated with lower fasting glucose (-0.25 (-0.48 to -0.01) mmol/L) and lower HOMA-IR (-0.23(-0.44 to -0.03)), both p-value for linear trend < 0.05 in a multivariable model adjusted for socio-demographic characteristics and health-related behaviours (model 3). Results were unchanged in sensitivity analyses further adjusting for self-reported fruit and vegetables intake or BMI replaced by body fat in model 3 and when using multiple imputation to investigate the impact of missing data.

There was no evidence of a non-linear association between plasma zinc and any of the outcomes using restricted cubic splines. The test for interaction between sex, rural/urban area of residence or BMI categories and plasma zinc concentrations on any of the glycaemic markers was not significant.

Table 6.6 : Associations between plasma zinc concentration and glycaemic markers (Cameroon study)

	β-coefficient (95% confidence interval)			p-value for linear trend
	Thirds (T) of plasma zinc			
	T 1	T 2	T 3	
Fasting glucose (mmol/L) (n=543)				
Model 1	1.0 (ref)	-0.04(-0.29 to 0.21)	-0.20(-0.45 to 0.04)	0.10
Model 2	1.0 (ref)	-0.03(-0.27 to 0.20)	-0.18(-0.41 to 0.06)	0.14
Model 3	1.0 (ref)	-0.06(-0.29 to 0.17)	-0.25(-0.48 to -0.01)	0.04
2-h glucose (mmol/L) (n=536)				
Model 1	1.0 (ref)	-0.49(-0.86 to -0.12)	-0.63(-1.02 to -0.23)	0.002
Model 2	1.0 (ref)	-0.47(-0.83 to -0.11)	-0.54(-0.94 to -0.15)	0.007
Model 3	1.0 (ref)	-0.42(-0.77 to -0.07)	-0.43(-0.82 to -0.04)	0.03
HOMA-IR (n=540)				
Model 1	1.0 (ref)	-0.07(-0.27 to 0.14)	-0.02(-0.24 to 0.19)	0.81
Model 2	1.0 (ref)	-0.02(-0.22 to 0.18)	0.07(-0.14 to 0.28)	0.53
Model 3	1.0 (ref)	-0.13(-0.30 to 0.05)	-0.23(-0.44 to -0.03)	0.02

HOMA-IR, Homeostatic model assessment for insulin resistance

Model 1: Unadjusted

Model 2: Adjusted for age and sex,

Model 3: model 2 + smoking status, alcohol intake, education level, residential site (4 sites), BMI (continuous) and PAEE (continuous)

6.7 Discussion

In this population-based cross-sectional study of 596 participants in Cameroon, I observed that plasma zinc concentration was inversely associated with glycaemic markers (2-h glucose, fasting glucose and HOMA-IR). The inverse associations between plasma zinc concentration and fasting glucose and HOMA-IR became significant only after adjusting for sociodemographic characteristics and health-related behaviours. This suggests the role of zinc in glucose homeostasis in this population.

There are limited data from representative surveys on the distribution of plasma zinc concentration from Africa in part because of the financial and technical resources required to analyse plasma zinc. The mean plasma zinc concentration in our study was comparable to those reported in studies in the US (286) and Europe (178), (287) but higher than in previous studies in Africa (284), (288), (289), (290). This could be because the previous studies in Africa were conducted mostly in children (< 5 years) or women of reproductive age. These population sub-groups are known to have higher zinc turnover (280). In addition, blood samples in some of the studies were collected in non-fasting participants and the afternoons. Plasma zinc concentrations follow a diurnal variation and are higher in the mornings and fasting participants (274).

Self-reported fruit and vegetables intake and physical activity were inversely associated with plasma zinc concentration. In this study, fruit and vegetables intake may be a proxy for a diet low in meat or high in plant-rich diets, which are high in phytates that bind to zinc to form an insoluble complex, thereby inhibiting zinc absorption in the intestines (266). The inverse association between plasma zinc concentration and physical activity is consistent with previous studies suggesting that physical activity promotes higher zinc excretion in sweat and urine (291).

Previous epidemiological studies on the association between dietary zinc intake and diabetes have been limited by measurement error of dietary zinc assessment and show inconclusive results (271), (273), (272), (292). The quantification of plasma zinc concentration offers the advantage of being an objective marker of both dietary zinc intake and body stores but has not been widely applied to test diet-disease association probably owing to the high cost of the plasma zinc analysis. As a result, evidence from previous observational studies using plasma zinc concentration is limited, with the majority of the studies coming from China (275), (293), (294), (295), (296). Some of these studies did not account for health-related behaviours that

confound the relationship between plasma zinc concentration and glycaemia. Although previous observational studies reported either a weak or absent association between dietary zinc intake and plasma zinc concentrations this could be attributed to measurement error of the dietary assessment and variability of bioavailability of zinc from foods (281). Food composition tables are sometimes unavailable for local food items to calculate zinc and phytate intakes accurately, which is a drawback as the zinc content of plant-based foods may be influenced by soil zinc concentrations and phytates inhibit dietary zinc absorption (280).

A previous case-control study in 1796 participants in China examining the relationship between zinc status and diabetes reported that higher plasma zinc concentration was associated with lower odds of type 2 diabetes which is consistent with our results (277). Similar findings of an inverse association between plasma zinc concentration and type 2 diabetes have been reported in other small case-control studies in Pakistan and Russia (294), (295). In contrast, a cross-sectional study in the US in 5153 adults reported that higher serum zinc concentration was associated with higher odds of pre-diabetes and diabetes (275). A similar positive association between serum zinc concentration and risk of type 2 diabetes was reported in a 20-year prospective study of middle-aged and older men in Finland (276). In these studies showing a positive association between serum zinc concentration and type 2 diabetes, the prevalence of low blood zinc concentration was low and it is has been suggested that excessive bioavailability of zinc may lead to overactive β-cells and eventually β -cell failure due to prolonged overactivity of the β –cell (297).

Plasma zinc concentrations respond to zinc supplementation (281), (288). To date, published studies of zinc supplementation trials for diabetes prevention and management are mostly of small sample sizes and short duration. A meta-analysis of 32 RCTs reported a reduction in fasting glucose, postprandial glucose, glycated haemoglobin, fasting insulin and HOMA-IR estimates with zinc supplementation compared with controls (298). Notably, over 80% of the studies included in this meta-analysis were of short duration (< 6 months) and from Asia, where inadequate zinc exposure from low dietary zinc and high phytate intakes are prevalent (268). A Mendelian randomisation study reported no causal association between blood zinc and risk of type 2 diabetes (299). However, uncertainty remains due to the small sample size and only two single nucleotide polymorphisms (SNPs) included in the analyses.

Mechanistic evidence of the potential role of zinc in the pathogenesis of type 2 diabetes comes from animal and human studies (270), (300), (301). Zinc has a positive effect on insulin

signalling in the skeletal muscles by stimulating the tyrosine phosphorylation of insulin receptors, thus promoting glucose uptake (300). Moreover, zinc is found in abundance in the pancreatic β-cells and is essential for the synthesis of zinc-insulin crystals (insulin crystallisation), the form in which insulin is stored in the pancreas. It has been suggested that the type, size and morphology of the zinc-insulin crystals regulate the conversion of pro-insulin to insulin (301). Zinc also appears to be an insulin-mimetic with the potential to modulate insulin storage, secretion and receptor signal transduction (270), (300). Some of the anti-inflammatory effects of zinc could explain its beneficial role in diabetes. Finally, zinc also acts as a co-factor for superoxide dismutase and other enzymes against oxidative stress (302).

I did not find evidence of a non-linear association between plasma zinc concentration and any of the glycaemic markers. However, a recent large cohort study in China showed a U-shaped relationship between dietary zinc intake and type 2 diabetes risk, with an inflection point at 9.1 mg/day (292). Future prospective studies are needed to confirm these findings of an inverse association between plasma zinc concentration and diabetes. If evidence of a beneficial effect of zinc in diabetes is shown in intervention studies, public health strategies to increase dietary zinc intake may offer a cheap and complementary primary prevention approach for diabetes.

6.8 Strengths and limitations

The strength of this study lies in the use of plasma zinc concentration measured using ICP-MS to characterise zinc status objectively. Inadequate zinc status may result from insufficient dietary zinc intake, but also poor dietary zinc absorption (e.g. high dietary phytate intake that inhibits zinc absorption.). Thus in low and middle-income settings where dietary phytate intake is high, plasma zinc concentration may be a better indicator of zinc exposure than estimated dietary intake of zinc (269), (303).

This study also has limitations. In addition to the cross-sectional design of the study which limits the possibility of causal inference, the blood samples in this study were not collected in trace element-free tubes and the resulting plasma zinc concentrations may have been affected by contamination from environmental zinc, including zinc from the tubes in which the samples were stored or even the long-term storage. However, plasma zinc appears to be relatively stable after long-term storage and the contamination from tubes has been shown to be minimal (304). Even if plasma zinc concentrations were affected by contamination or long-term storage, this is unlikely to affect the observed associations as the effect of storage or contamination was likely to be random.

6.8.1 Conclusion

This population-based study in rural and urban Cameroon shows that plasma zinc concentration was independently inversely associated with fasting glucose, 2-h glucose and HOMA-IR. This suggests a role of zinc in glucose metabolism possibly involving both insulin secretion and insulin resistance. Given that plasma zinc is a biomarker that is elevated by intake of protein-rich foods, further work is required to disentangle the specific effects of zinc on diabetes from the effects of the food groups that influence zinc status. Additionally, the current cross-sectional findings should be investigated in prospective study designs.

Chapter 7 : Conclusion

7.1 Summary

Many countries in SSA are undergoing rapid urbanisation, which is characterised by nutritional transitions and shifts in physical activity behaviours. Yet, there is limited evidence on the relationship between dietary factors and diabetes and related metabolic disorders from SSA, a continent with the highest rate of predicted increase in the number of people with diabetes. Understanding the role of diet in the development of diabetes and related metabolic disorders is crucial for the design of contextually tailored and effective interventions for the prevention of these disorders. The overall aim of this PhD was to contribute toward the understanding of the relationship between diet and cardiometabolic risk factors in SSA. To achieve this, I used a wide range of objectively measured nutritional biomarkers to test diet-diabetes associations in a sub-Saharan African setting.

In Chapter 2, I aimed to describe the differences in the characteristics of rural and urban participants in the Cameroon study. The results showed that there are rural-urban differences in the behavioural determinants of diabetes and the metabolic profile. Participants living in the rural settings were more physically active, self-reported a higher frequency of consumption of fruits and vegetables and had a better metabolic profile than the urban residents had.

In Chapter 3, I aimed to describe the determinants of vitamin D status in participants in rural and urban Cameroon and examine the association between serum 25(OH)D the best marker of vitamin D status and a clustered score of the metabolic syndrome and glycaemic markers. I observed that rural residents had higher levels of serum 25(OH)D than urban residents. Factors positively associated with serum 25(OH)D concentrations were the male sex and physical activity levels, while level of education and measures of adiposity were inversely associated with serum 25(OH)D concentration. Regarding the association between serum 25(OH)D concentration and the outcomes, I observed that higher serum 25(OH)D concentration was associated with lower metabolic syndrome score, although this was confounded by socio-demographic and behavioural characteristics. The inverse association between serum 25(OH)D concentration and fasting glucose was independent of the potential confounders adjusted for.

In Chapters 4 and 5, I aimed to examine the associations of biomarkers of intake of fruits and vegetables (plasma carotenoids, tocopherol and serum folate) and serum holoTC, a biomarker of intake of animal-sourced foods with diabetes and related metabolic disorders. The results showed that plasma total carotenoids, α- and β-carotene were positively correlated with both self-reported intakes of fruit and vegetables, but there was no correlation between plasma

tocopherol, serum folate and intake of fruits and vegetables. In aetiological analyses, I observed independent inverse associations between plasma total carotenoids and fasting glucose and between serum folate and the metabolic syndrome score. HoloTC was positively associated with the metabolic syndrome score but this association was confounded by sociodemographic and behavioural characteristics.

In Chapter 6, my objective was to identify the factors that affect plasma zinc concentration and to examine the association between plasma zinc concentration and glycaemic markers. The results showed that fruit and vegetables intake and physical activity were inversely associated with plasma zinc while the male sex and BMI were positively associated with plasma zinc concentrations. Plasma zinc concentration was independently inversely associated with fasting and 2-h glucose and HOMA-IR.

Therefore, my studies on folate and carotenoids suggest that diets rich in fruits and vegetables may be beneficial for the prevention of cardiometabolic diseases in Cameroon. Given that zinc is a biomarker that is elevated by intake of protein-rich foods, further work may be required to disentangle the specific effects of zinc from the effects of the food groups that it is most influenced by.

7.2 Validity of results

When appraising the validity of a study, 2 types of validity need to be considered: internal validity and external validity. Internal validity is the extent to which a study measures what it purports to measure within a studied population and external validity is the extent to which the study is valid within the general population (generalisability). Bias and confounding are the major threats to the internal validity of a study.

Selection bias, specifically sampling bias may have been introduced in this study as a result of non-probability sampling used. Sampling was done using a study-established sampling frame of individuals aged 25-55 years rather than complete population registers. Even with the study-established sampling frame, the absence of fixed houses and telephone numbers made it difficult to use a random sampling technique. While sampling bias may not affect the internal validity of this study, it may limit our ability to generalise our results to the target population if our sample was systematically different from the target population. However, participants' characteristics such as level of education, BMI and blood pressure were similar to those from larger previously conducted population-based studies in Cameroon (68), (75), (76).

The presence of information bias in this study may also affect the validity of our results. The use of self-reported instruments to collect data on fruit and vegetable intake, physical activity, smoking status and alcohol intake is subject to recall bias and social desirability bias, which may result in misclassification. Social desirability bias is particularly common in nutritional epidemiology. In this study, respondents were asked to report their intakes of fruits and vegetables, which may have resulted in overestimated self-reported intakes given that these foods as perceived as healthy. This social desirability bias in fruit and vegetable assessment has been reported previously (44). However, for the exposure of interest (fruit and vegetable intake) and important covariates (such as physical activity) in these studies, I used objectively measured nutritional biomarkers and physical activity to reduce the measurement error and recall bias associated with the self-reports.

The observed associations in these studies may also be the result of residual confounding and unmeasured confounding eg by diet. In our studies, the lack of dietary data other than fruit and vegetable intake was a major limitation. For example, plasma zinc is elevated by the intake of protein-rich foods. However, in these analyses, I was not able to adjust for the effect of foods that affect plasma zinc status to disentangle the specific effects of zinc on diabetes from the effects of the food groups that influence zinc status. To limit the potential of residual confounding, I adjusted for objective measures of the covariates wherever possible (such as objective measures of physical activity, adiposity assessed using BMI and bioimpedance analyses, etc).

I also considered whether the effect of the nutritional biomarkers on the outcomes varied depending on the level of a third variable (effect modification) which may affect the external validity. In all the analyses, I investigated the potential effect modification by age, sex, rural-urban residential site and BMI and presented results stratified by these factors if the interaction term was significant. In chapter 4, I additionally investigated the effect of smoking status on the associations of carotenoids tocopherol and fasting glucose given that these biomarkers are known anti-oxidants. Smoking is a potent oxidative stressor and it has been postulated that smoking may neutralise the anti-oxidative activity of the carotenoids and tocopherol. A prospective study in young adults in the US showed that higher circulating carotenoids were associated with lower insulin resistance and diabetes risk in non-smokers but not in smokers (166). Finally, this study was conducted in healthy adults aged 25-55 years and our findings may not be generalisable outside of this age range and beyond the geographical location in which the study was conducted.

7.3 Causal inference

Inferring causality is the aim of aetiological analyses. The most commonly used method to assess causality from epidemiological studies is by applying Bradford Hill's criteria. Sir Bradford Hill in 1965 suggested nine viewpoints that could be used to argue whether an observed association is more or less likely to be causal. These criteria include consistency, strength, specificity, temporality, biological plausibility, coherence, experimental evidence, analogy and dose-response relationship. Even when these criteria are fulfilled, we are left with the issue of confounding and reverse causation. I will apply these criteria to our findings from the different chapters to judge whether the observed associations were more or less likely to be causal.

Sir Bradford Hill suggested that if an association is consistently observed in different populations, places and times, then it is more likely to be causal. In Chapter 3, I showed an inverse association between serum 25(OH)D concentration and fasting glucose. Many cross-sectional and prospective studies have shown an inverse association between vitamin D status and glycaemic markers or diabetes risk. In a meta-analysis of prospective studies, 1 SD higher serum 25(OH)D concentration was associated with a 20% lower risk of type 2 diabetes (132). In Chapter 4, I observed that biomarkers reflecting intake of fruits and vegetables were inversely associated with fasting glucose, which is consistent with previous studies in different settings. A meta-analysis showed a non-linear association between individual blood carotenoids and type 2 diabetes risk, with greater effect sizes at lower concentrations of blood carotenoids (184).

The criterion of specificity requires that the exposure leads to a single effect. This criterion is hardly fulfilled as exposures may have multiple effects and the lack of specificity of an association does not exclude the possibility of a causal relationship. Vitamin D deficiency is primarily known to cause rickets in children and osteomalacia in adults. However, accumulating evidence suggests that vitamin D is associated with several non-musculoskeletal outcomes (108). There is convincing evidence from RCTs that folic acid supplementation reduces the risk of neural tube defects in the fetus. Zinc is a trace element and zinc deficiency that has been associated with stunting in children. Therefore, the criterion of specificity is not met for any of the biomarkers included in this study.

Sir Bradford Hill suggested that stronger associations were more likely to be causal than weaker associations. This criterion may be misleading as even small effects may add up and

some strong associations have shown to be non-causal. The criterion of temporality was not met given the cross-sectional design of our study. For example, for the inverse association between serum 25(OH)D and fasting glucose, it is possible that poor metabolic health could have led to dietary changes, reduced sun exposure, physical activity or increased inflammation, all of which could have lowered serum 25(OH)D concentrations. Regarding the criterion of dose-response relationship, I showed that there was a linear association between the biomarkers (serum 25(OH)D concentrations, carotenoids and zinc) and fasting glucose. However, even non-linear associations can be causal.

Our observed associations between the nutritional biomarkers and the outcomes are all biologically plausible with evidence coming from basic science research as described in the different chapters. Despite this, one needs to be careful about using biological plausibility to argue causality as biological plausibility can always be demonstrated. Sometimes epidemiological studies have not confirmed an association that had shown a strong biological plausibility in animal studies. The criterion of coherence is similar to biological plausibility and largely depends on the extent of knowledge at the time of the study. Experimental evidence could refer to evidence from experiments in basic research or experimental studies in humans. In some situations, an experimental study in humans is not feasible due to ethical concerns. In Chapters 3, 4 and 5, I discussed the tendency towards a null association of results from RCTs on the associations of vitamin D, carotenoids, tocopherol, and folate supplementation with diabetes outcomes.

Moreover, results from mendelian randomisation studies (which are conceptually equivalent to naturally occurring randomised trials due to the random assortment of alleles during gametogenesis) also suggest no association between the genetically predicted biomarkers concentrations and outcomes. However, the results from mendelian randomisation analyses and most RCTs have not taken into account the initial nutritional status and the effect of the biomarkers on the outcomes may vary depending on the initial nutritional status. For example, in a large RCT specifically designed to assess diabetes incidence as an endpoint, daily supplementation with 4000 IU/ day of vitamin D did not reduce diabetes risk in the overall sample (participants not selected for vitamin D insufficiency or deficiency) (84). However, in subgroup analyses, vitamin D supplementation lowered diabetes risk in people with obesity (pre-specified subgroup analysis) and people with vitamin D deficiency (post-hoc analysis). Therefore, although there is no evidence that genetically predicted serum 25(OH)D is associated with lower diabetes risk, and no overall effect of vitamin D supplementation from

RCTs, uncertainty remains about the benefits of vitamin D in people with vitamin D deficiency and obesity. Recent findings from non-linear mendelian randomisation analysis showed an L-shaped association between genetically predicted serum 25(OH)D concentration and CVD risk, where higher concentration of serum 25(OH)D lowered CVD risk and plateaued at ~ 50 nmol/L (305).

The analogy criterion implies that if there is evidence of a causal relationship between an exposure A and outcome B, then researchers should be more accepting of weaker evidence between an exposure similar to A and an outcome similar to B. Many epidemiologists have argued this criterion may just reflect the researcher's creativity and ability to find analogies.

7.4 Perspectives

This PhD project has laid foundations for the use of objectively measured nutritional biomarkers in SSA and contributed to the understanding of the potential role of diet in the prevention of diabetes and related metabolic disorders. I showed that there were important differences in the metabolic profile of rural and urban dwellers, which were partly related to the rural-urban differences in behavioural risk factors such as diet and physical activity resulting from urbanisation. Because of the small sample size of this study, I decided not to pre-specify subgroup analyses by rural-urban residential sites for the association between the nutritional biomarkers and the outcomes. Clearly, there is a need for larger population-based prospective studies which assess rural-urban population characteristics and are specifically designed to assess the relationship between dietary factors and risk of diabetes and related metabolic disorders.

In Chapter 3, I showed that vitamin D was inversely associated with fasting glucose. I have also summarised above the totality of evidence on vitamin D and diabetes, which does not support a causal association between vitamin D and diabetes. However, uncertainty remains on the effect of vitamin D on diabetes in populations who are deficient/insufficient for vitamin D and people with obesity. In support of this, recent findings from non-linear mendelian randomisation analyses showed that genetically predicted serum 25(OH)D was associated with lower CVD risk only in patients with low serum 25(OH)D levels (305). Previous RCTs have mostly been conducted in the US (and a few in Europe) where the prevalence of low vitamin D status is low. Vitamin D deficiency causes osteomalacia in adults and rickets in children and randomising participants selected for vitamin D deficiency may be unethical. Our study together with previous studies suggests that Africa is a region with a high prevalence of vitamin

D insufficiency and deficiency and may represent a good site to conduct a RCT of vitamin D supplementation to assess diabetes endpoint.

In Chapters 4 and 5, I observed that the biomarkers reflecting intakes of fruits and vegetables were inversely associated with fasting glucose and metabolic syndrome score. One major limitation of this study is the lack of detailed dietary data to assess the correlation with the biomarkers. Previous studies have shown a weak to modest correlation between intakes of fruits and vegetables and the biomarkers (circulating carotenoids, α-tocopherol and folate), probably owing to the measurement error of self-reports. However, it is important to extend this work to understand what dietary changes can provide a sustainable change in the circulating levels of these biomarkers and if these changes have a positive effect on the reduction of metabolic risk in this population. Fruit and vegetables are rich sources of different biomarkers including carotenoids, vitamin C, tocopherol and folate. Therefore to ascertain the full range of intake of fruits and vegetables, there is a need for future studies to investigate whether a combination of the nutritional biomarkers (rather than in isolation), perhaps even in combination with other factors provides more reliable results.

In Chapter 6, I reported an inverse association between plasma zinc concentration and glycaemic markers, which is consistent with previous findings. Over 80% of RCTs assessing the effect of zinc supplementation on glycaemic markers have been of short duration (less than 6 months) and from Asia. Given the short duration, hard endpoints have not been assessed, therefore there is a need for RCTs of long duration to assess the effect of zinc supplementation on diabetes risk in other settings.

When reviewing the literature, I observed that most studies reporting an inverse association between zinc status and glycaemic markers were conducted in settings where the prevalence of low zinc deficiency is high, while studies reporting a null or positive association were conducted in settings where zinc deficiency is not an issue. We are testing the hypothesis (ongoing work) that the effect of zinc supplementation might be stronger in populations at higher risk of zinc deficiency. We are following up on a previously published meta-analysis of 36 RCTs, which showed that zinc supplementation lowered several glycaemic indicators (fasting glucose, 2-h glucose, HOMA-IR and glycated haemoglobin) in patients with and without diabetes (298). However, the effect of zinc supplementation on the glycaemic markers by baseline zinc status was not analysed. We used a meta-regression technique to test whether the effect estimates of zinc supplementation on glycaemic markers were correlated with

prevalence measures of country-level zinc deficiency. Country-level zinc deficiency estimates were obtained from a previously published paper (268). Preliminary results from the reanalysis of the published meta-analysis show that the effects of zinc supplementation on fasting glucose and glycated haemoglobin are greater in countries with a higher estimated prevalence of zinc deficiency. Ongoing work aims to update the meta-analysis, and extract data on the initial zinc status from all the studies included. So far we have identified 13 additional studies published since 2019.

7.5 Public health implications and conclusion

Results from this PhD can be used to inform future public health research and policy makers. Our results showed important differences in the metabolic profile of rural and urban participants, which may be attributed in part to differences in dietary, and physical activity factors. Rural and urban settings are at different levels of epidemiologic transition and our findings highlight the importance of including participants in studies from both rural and urban settings to generate evidence that is contextually relevant to improve metabolic health. The nutritional biomarkers used in this study can be used to evaluate the effectiveness of public health interventions aiming to change dietary behaviours or monitor adherence to dietary guidelines in populations such as fruits and vegetables, and meat intake. Despite the lack of a causal relationship, our results suggest the dietary factors studied in this PhD project may play a crucial role in the development of diabetes and related metabolic disorders in this population and are interesting targets for public health interventions.

In conclusion, my studies on circulating folate and carotenoids suggest that diets rich in fruits and vegetables may be beneficial in Cameroon for the prevention of cardiometabolic diseases in this population. Public health interventions to improve vitamin D and zinc status may improve glucose metabolism in this population and warrant further investigation in prospective studies.

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