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The circadian rhythm of calcium and bone homeostasis in Maasai

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Abstract

Objectives: Ethnic groups differ in prevalence of calcium-related diseases. Differences in the physiology and the endogenous circadian rhythm (CR) of calcium and bone homeostasis may play a role. Thus, we aimed to investigate details of CR pattern in calcium and bone homeostasis in East African Maasai.

Methods: Ten clinically healthy adult Maasai men and women from Tanzania were examined. Blood samples were collected every 2nd hour for 24 h. Serum levels of total calcium, albumin, parathyroid hormone (PTH), 25(OH)D, creatinine, C-terminal telopeptide (CTX), bone-specific alkaline phosphatase (BSAP), procollagen type 1 N-terminal propeptide (P1NP), and osteocalcin were measured. Circadian patterns were derived from graphic curves of medians, and rhythmicity was assessed with Fourier analysis.

Results: PTH-levels varied over the 24 h exhibiting a bimodal pattern. Nadir level corresponded to 65% of total 24-h mean. CTX and P1NP showed 24-h variations with a morning nadir and nocturnal peak with nadir levels corresponding to 23% and 79% of the 24-h mean, respectively. Albumin-corrected calcium level was held in a narrow range and alterations were corresponding to alterations in PTH. There was no distinct pattern in 24-h variations of 25(OH)D, creatinine, osteocalcin, or BSAP.

Conclusions: All participants showed pronounced 24-h variations in PTH and bone turnover markers CTX and P1NP. These findings support that Maasai participants included in this study have typical patterns of CR in calcium and bone homeostasis consistent with findings from other ethnic populations.

Dirk Lund Christensen and Peter Schwarz shared senior authorship.

1 | INTRODUCTION

Calcium and bone metabolism are closely interlinked with high interdependency (Ahmad et al., 2003; Song, 2017). The two systems are regulated by serum levels of different substances such as hormones and minerals, including among others parathyroid hormone (PTH) and calcium, respectively.

Several studies have shown that hormones, minerals, and bone turnover markers (BTMs) work in a circadian pattern which provide important insights to the dynamics of bone remodeling (Ahmad et al., 2003; Calvo et al., 1991; Fraser et al., 2004; Song et al., 2018).

Numerous physiological processes of mammals are regulated by the circadian clock system, which is an endogenous, timekeeping pacemaker that operates with a period close to 24 h in order to coordinate rhythms in activity, metabolism, hormonal secretion and cell cycle (Chaix et al., 2016). Various bone turnover markers and bone metabolism-regulating hormones such as melatonin and PTH display diurnal rhythmicity (Song et al., 2018).

According to previous research, disruption of the circadian clock due to shift work, sleep restriction, or clock gene knockout is associated with osteoporosis or other abnormal bone metabolism, showing the importance of the circadian clock system for maintaining homeostasis of bone metabolism. Moreover, common causes of osteoporosis, including postmenopausal status and aging, are associated with changes in the circadian clock. Thus, osteoporosis interventions at different time periods across the adult life cycle can provide varying degrees of bone protection, showing the importance of accounting for circadian rhythms (CRs) for optimal curative effects in clinical treatment of osteoporosis. For a review of the above-mentioned circadian rhythm description and daily patterns of BTMs, please see (Song et al., 2018).

Accordingly, research in the CRs of mineral and bone metabolism or homeostasis can help us to better understand the pathogenesis of disturbances in the two systems. Among other diseases, osteoporosis (Fraser et al., 1998) and secondary hyperparathyroidism (Logue et al., 1990) are associated with different CRs of bone turnover and level of PTH compared to healthy controls, which support the important role of CRs on disease manifestation (Ahmad et al., 2003; Fraser et al., 1994; Redmond et al., 2016).

Previous studies have shown that prevalence of calcium and bone related diseases is different between ethnic groups all over the world (Bayray et al., 2012; Demeke et al., 2015; Mazocco & Chagas, 2017). In different ethnic groups, variability of BTMs are affected by modifiable and non-modifiable factors; for modifiable factors these include CR, seasonal variation, fasting and food intake, physical activity, and menstrual variation, and for non-modifiable factors they include age, sex, and menopausal state among others (Szulc et al., 2013). As especially the modifiable factors may vary between ethnic groups/population groups daily patterns of BTMs may differ potentially contributing differently to bone diseases.

Despite the large range of studies exploring and describing the CR of calcium homeostasis, rate of bone turnover, and bone mineral density in Caucasians (Ahmad et al., 2003; Fraser et al., 1994; Song, 2017), there is only sparse knowledge when it comes to black populations in sub-Saharan Africa (Cauley et al., 2014; Redmond et al., 2014;2015; 2016). Previous research describing 24-h variations of calcium and bone homeostasis include a cross-sectional study examining diurnal rhythms of PTH and BTMs in 30 Gambian, 30 white British, and 30 Chinese men and women (Redmond et al., 2015; 2016), and a US-based study comparing diurnal rhythms in 7 white and 7 premenopausal African American women (Bell et al., 2001). The multi-ethnic studies showed that Africans have higher renal calcium conservation than their British counterparts, and PTH, BTMs and 1,25-dihydroxyvitamin D were higher in Africans compared to British and Chinese counterparts, while the US-based study showed a higher conservation in the kidneys of calcium in the African American group.

These studies highlight that complex interactions between genetics, lifestyle and environmental factors, hormone status, and hormone secretion patterns are presumably some of the factors causing ethnic differences in basic bone physiology. These differences may have an impact on development of diseases such as osteoporosis, and manifestation of a disease such as bone fractures. Therefore, investigating different ethnic groups is likely to enhance our understanding of calcium homeostasis, bone homeostasis, and calcium-related diseases (Finkelstein et al., 2002; Kleerekoper et al., 1994; Paik et al., 2012) given that exposure factors such as dietary intake, physical activity, and seasonal variation are taken into account.

1.1 | Maasai lifeways and health

The Maasai are an ethnic group living across East Africa, mainly inhabiting southern Kenya and northern Tanzania with a total population size of approximately 1 500 000 (Kenya National Bureau of Statistics, 2019; Maasai, 2018). They are agro-pastoralists herding domesticated cattle, goats, sheep and donkeys, grow maize, practice circumcision in men and women, and they are polygamous with families residing in an

engan'g (cluster of homes for men, women and children (Amin et al., 1987)). The traditional Maasai diet to a large degree consists of milk and milk products with some studies suggesting that these account for ~20% of the daily energy intake (Hansen et al., 2011). As with other populations who rely heavily on dairy products as staples, genetic polymorphisms associated with lactase persistence have also been identified for the Maasai (Wagh et al., 2012). Other studies have shown that the Maasai have similar or higher average physical activity level compared to other rural populations (Luo and Kamba from Kenya), and European populations (Christensen et al., 2012; InterAct et al., 2012), respectively. However, a recent study in Maasai men indicated that physical activity is declining (Christensen et al., 2021).

It has been reported that Maasai and other traditionally living East African people have high vitamin D-levels (Luxwolda et al., 2012), despite their dark skin color with a high melanin level, which inhibits vitamin D production (Kagotho et al., 2018; Luxwolda et al., 2012; Luxwolda et al., 2013; Webb et al., 2018). Thus, a suitable vitamin D cut-off value for people with dark skin tones does not exist, and it has not been determined whether a standard interpretation of low vitamin D level influences African people's health from a clinical perspective. This is especially relevant for equatorial populations in Africa living in open savanna or semi-arid areas in East and West Africa where exposure to sunlight is high even if skin pigmentation is highly variable in equatorial Africa (Lasisi & Shriver, 2018). In geographical regions in higher latitudes, seasonal changes in bone homeostasis primarily depend on exposure to sunlight, which during wintertime is too low to maintain a sufficiently high vitamin D level (Webb et al., 2018).

In East Africa, seasonal changes are divided into two dry and two wet seasons (Amin et al., 1987). Since the Maasai live close to the equator and spend a large part of their time in the sun, they experience abundant sun exposure all year. Instead, the difference between dry and wet seasons affects the availability of food in different rural African populations such as the Maasai in East Africa (Galvin & Waweru, 1987), and pre-pubertal boys in the Gambia in West Africa (Munday et al., 2006). This may lead to seasonal changes in intake of different nutrients including dairy products and thereby calcium.

We aimed to investigate CR in calcium homeostasis and bone homeostasis in East African Maasai, a population traditionally living on a calcium-rich diet, with high physical activity, and high annual sun exposure in order to shed light on 24-h serum levels of calcium, PTH, vitamin D, and BTMs.

2 | METHODS

2.1 | Study Population

Ten volunteers of Maasai descent without diseases affecting calcium or bone homeostasis, including five women and five men with an age range of 30–57 years, were studied. Three of the female participants are likely to have been at peri- or post-menopausal stage (age 48, 50, and 57 years), while none of the female participants were lactating. None of the participants were biologically related to one another. The participants were semi-urban individuals residing on the outskirts of Monduli Town in Arusha region of Tanzania, where all data were collected in July 2018 in a makeshift laboratory in Monduli Town.

2.2 | Selection procedure

Inclusion criteria were Maasai descent (self-reported), age ≥ 18 years, and no known severe illness. All participants were presented with a standard statement by a local coordinator, describing the current study as a bone survey including a general health check. All participants were given a written and oral account of the study, and all information was translated from English to Kiswahili. Upon enrollment into the study, all participants gave a verbal and a fingerprint-signed informed consent and were given a unique identification number. Eighteen individuals were initially enrolled in the study following 12 h of overnight fasting. Information on medical history and dietary intake was obtained using questionnaires. Height, weight, waist and hip circumferences were measured according to World Health Organization standard procedures (WHO, 2017), and body mass index (BMI, kg/m^2) as well as waist-to-hip ratio were calculated. Blood pressure while seated was measured according to standard procedures including also heart rate and calculating pulse pressure. Hemoglobin and fasting blood glucose were analyzed using portable equipment (Hemocue AB, Ängelholm, Sweden) based on a capillary blood test from a finger, while C-reactive protein, insulin and a standard lipid profile were analyzed based on a venous blood test using standard assays following centrifuging and initially being kept in −20 °C and then −80 °C before shipment to Denmark for analyses. Eventually, ten participants were selected to join the study based on representativeness of normal health conditions without diseases affecting calcium or bone homeostasis. Ethical approval was obtained from the National Institute of Medical Research in Tanzania (ref. no.: NIMR/HQ/R.8a./Vol IX/2806).

2.3 | Study protocol

During the study, the participants were given accommodation in hotel rooms in the makeshift laboratory study site. The participants were restricted to stay on the laboratory site area, and were primarily spending their time sitting down. Since endurance exercise is known to alter calcium homeostasis, the study participants were asked to abstain from exertive exercise during the study period, for a review see (Maimoun & Sultan, 2009). Participants went to bed according to their normal routines and were only woken lightly during the night when the blood samples were collected, allowing them to follow their normal 24-h rhythm as much as possible. All participants had a venous catheter placed in a forearm vein and venous blood was drawn every second hour. During the 24-h study period, complete dietary and fluid intake were registered. The research team had 8-h shifts for drawing venous blood in order to avoid unnecessary mistakes due to lack of sleep among the investigator team (AS, KK and JS + assistants). Accumulated urine was collected in individual glass bottles containing thymol crystals, which has been shown to be effective in maintaining metabolite stability in urine most likely caused by an inhibitory effect of thymol on urine microbiota (Wang et al., 2019). At the end of study, the 24-h-urine bottles were shaken, and three ml of mixed urine were put in cryo-tubes.

2.4 | Dietary registration procedure

All participants received food as close to their typical daily diet as possible. Three main meals were served and dietary intake was registered. For each participant, the ingested amount of food and drinks was precisely measured on an electronic scale (Sonashi Digital Food Scale: model SKS-006, Dubai, U.A.E) to the nearest gram to know the exact amount of dietary calcium. Participants exclusively used their own plate and cup of known weight. Amount of calcium in the food was derived from the Tanzania Food Composition Table (Lukmanji et al., 2008). When thirsty, the participants were offered 0.5 L of mineral water with no content of calcium.

2.5 | Blood samples

The first blood samples were drawn following 12 h of overnight fasting (between 9 am and 9.30 am) and thereafter, every 2 h (± 10 minutes) for 24 h. Each participant had blood drawn thirteen times. The venous catheter was flushed with 5–10 ml of normal saline after each blood withdrawal to avoid clotting of the catheter. Blood samples were collected by using luer adapters connecting to a three-way-stop-cock monitored on the catheter. A new luer adapter was used for each blood withdrawal.

After discarding 1–2 ml of blood, the blood samples were drawn through the i.v. canula directly into a 5 ml no-additive serum tube and were kept at room temperature for maximum 20–30 minutes until coagulation was ensured. The coagulated blood was centrifuged for ten minutes. Serum was separated and immediately stored on ice until the end of the test day. They were moved to a − 20 °C freezer in the local district hospital in Monduli town. Subsequently, the blood samples were transported in liquid nitrogen to Kilimanjaro Clinical Research Institute in Moshi and kept in a − 80 °C freezer until they were shipped to Denmark for analysis.

2.6 | Biochemical analysis

All laboratory analyses were performed at Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark. In short, calcium, albumin, and creatinine were determined using colorimetric technique (Vitros 5, 1 FS/5600 Ortho Clinical Diagnostics [OCD]; Raritan, NJ). Serum parathyroid hormone 1–84 (PTH) was measured with immunometric technique (Vitros 3600/5600 Ortho Clinical Diagnostics [OCD]). Serum 25-hydroxyvitamin D (25(OH)D) and Fibroblast Growth Factor 23 (FGF-23) were measured by competitive electrochemiluminescence technique (Liaison XL, Diasorin, Saluggia, IT). Serum cross-linked C-telopeptide of type I collagen (CTX). The amino-terminal propeptide of type 1 procollagen (P1NP), and osteocalcin were measured using cheminoluminescence technique (IDS-iSYS, Immunodiagnostic Systems Plc, Tyne and Wear, UK). Alkaline phosphatase was measured using spectrophotometric technique (IDS-iSYS). The serum level of the fasting blood samples was determined using standard automated laboratory methods. Albumin-adjusted serum calcium level was calculated by the formula: calcium +16.6 × (39.9 - albumin)/1000.

2.7 | Statistical analyses

For normally distributed data, results are reported as means ± standard error of the mean (SEM). For non-normally distributed data, results are reported as medians and inter-quartile range. $P < 0.05$ was considered significant. Median, first quartile (Q1) and third quartile (Q3) were calculated for each time-point for the 24-h blood samples. Differences in serum levels during the day were quantified in percentages of medians from mean values and maximum peak median values.

TABLE 1 Baseline for standard health characteristics

	Women $N = 5$	Men $N = 5$	All $N = 10$
Demographic characteristics			
Age (years)	48 (35–57)	48 (30–55)	48 (30–57)
Height (cm)	158.9 (144.2–165.6)	174.9 (170.0–175.7)	167.8 (144.2–175.7)
Weight (kg)	60.7 (45.7–67.7)	65.4 (58.7–82.0)	64.9 (45.7–82.0)
BMI (kg/m^2)	22.6 (19.0–32.6)	22.0 (19.0–26.8)	22.2 (19.0–32.6)
Waist (cm)	85.6 (65.2–103.3)	84.3 (73.0–104.5)	85.0 (65.2–104.5)
Hip (cm)	100.7 (86.7–110.8)	99.1 (90.9–104.5)	100.0 (86.7–110.8)
Waist-hip ratio	0.88 (0.75–0.93)	0.85 (0.80–1.0)	0.86 (0.75–1.0)
MUAC (cm)	28.5 (26.3–38.8)	29.6 (28.5–33.8)	29.6 (26.3–38.8)
BP systolic (mm Hg)	123 (112–135)	119 (113–146)	123.0 (112–146)
BP diastolic (mm Hg)	89 (76–91)	87 (81–106)	87 (76–106)
HR (bpm)	65 (56–91)	61 (52–76)	64 (52–91)
Pulse pressure (mm Hg)	38 (35–44)	34 (30–48)	37 (30–48)
Blood samples			
Hemoglobin (mmol/L)*	9.0 ± 0.6	9.7 ± 0.6	9.4 ± 0.7
Fasting plasma glucose (mmol/L)*	5.4 ± 0.7	5.0 ± 0.3	5.2 ± 0.6
CRP (mmol/L)	0.8 (0–12.9)	1.2 (0.2–5.3)	0.9 (0–12.9)
Insulin (pmol/L)	57.3 (26.4–71.6)	45.0 (21.4–48.8)	47.5 (21.4–71.6)
Total cholesterol (mmol/L)	4.4 (3.2–6.6)	4.5 (4.0–6.0)	4.5 (3.2-6.6)
HDL (mmol/L)	1.0 (0.9–1.7)	1.1 (0.9–1.2)	1.1 (0.9–1.7)
LDL (mmol/L)	3.1 (2.2–4.5)	3.0 (2.5–3.9)	3.1 (2.2–4.5)
Triglyceride (mmol/L)	1.1 (0.6–1.5)	0.9 (0.7–2.6)	1.1 (0.6–2.6)

Note: Values are shown as medians and ranges. Values with an asterisk are shown as means ± standard error of the men (SEM).
Abbreviations: BMI, body mass index (kg/m^2); BP, blood pressure; CRP, C-reactive protein; HDL, high-density lipoprotein; HR, heart rate; LDL, low-density lipoprotein; MUAC, mid-upper arm circumference; Pulse pressure, Systolic–diastolic BP.

To investigate the frequency content of the time series, we performed least squares Fourier regression. On fine grid of values of period, τ, and phase, φ, we fitted a harmonic component of the form $m + a\cos(2\pi t/\tau + \varphi)$ to the data as a whole and stratified by sex. The results from these fits were illustrated as heatmaps colored by the value of the mean squared residuals. A regression model with two harmonic components and sex-dependent amplitudes of the form $(t) = m_{gender} + a_{1,gender}\cos(2\pi t/\tau_1 + \varphi_1) + a_{2,gender}\cos(2\pi t/\tau_2 + \varphi_2)$ was then fitted to each biomarker using non-linear least squares where m_{gender} denotes a sex-specific overall mean and $a_{1,gender}$ and $a_{2,gender}$ the sex-specific amplitudes of two harmonic components with periods $\tau_1$and τ_2 and phases φ_1 and φ_2.

3 | RESULTS

Mean age was 48.0 (range: 30–57) years, median BMI was 22.2 (range: 19.0–32.6) kg/m^2, median systolic and diastolic blood pressure were 123 (112–146) and 87 (76–106) mm Hg, respectively, and mean hemoglobin was 9.4 (0.7) mmol/L for all participants. Baseline characteristics are presented in Table 1.

3.1 | Calcium homeostasis

Table 2 shows the baseline results of calcium homeostasis related regulatory factors and BTMs. The participants had no vitamin D-deficiency, had phosphate metabolism within standard reference range based on standard FGF-23, PTH, and 25(OH)D ranges, as well as metabolism within standard range of calcium and bone based on standard albumin-corrected calcium, PTH, 25(OH)D, and BTMs. Our data indicated that all participants were without kidney disease.

The amount of calcium intake for each meal are shown in Table 3, and CRs of albumin-corrected

TABLE 2 Baseline specific for calcium and bone homeostasis in adult Maasai

	Women ($N = 5$)	Men ($N = 5$)	All ($N = 10$)
Calcium homeostasis, fasting morning blood samples			
Calcium (mmol/L)	2.45 (2.37–2.52)	2.33 (2.24–2.56)	2.43 (2.24–2.56)
Albumin (g/L)	47 (43–50)	46 (39–53)	47 (39–53)
Albumin corrected calcium (mmol/L)	2.32 (2.28–2.38)	2.31 (2.19–2.40)	2.32 (2.19–2.40)
PTH (pmol/L)	3.79 (3.60–8.30)	6.14 (5.01–7.29)	5.58 (3.60–8.30)
25(OH)D (pg/mL)	72.1 (49.5–92.1)	76.4 (41.2–83.8)	74.8 (41.2–92.1)
Creatinine (μmol/L)	52 (43–70)	85 (75–91)	73 (43–91)
FGF-23 (pg/ml)	30.30 (15.43–31.16)	27.00 (20.86–36.50)	28.34 (15.43–36.50)
Urine sample			
Calcium/creatinine ratio*	0.0055 ± 0.00097	0.0042 ± 0.0011	0.0048 ± 0.00073
Bone turnover markers, morning fasting blood samples			
BSAP (μg/L)	21.8 (14.5–31.4)	24.0 (14.9–27.6)	22.9 (14.5–31.4)
CTX (ng/L)	414.0 (230.6–663.2)	761.5 (392.1–956.5)	524.4 (230.6–956.5)
P1NP (μg/L)	67.9 (41.7–82.5)	107.0 (35.3–146.4)	71.0 (35.3–146.4)
Osteocalcin (μg/L)	13.9 (7.7–24.2)	20.2 (10.0–27.4)	16.6 (7.7–27.4)

Note: Baseline blood samples specific for calcium and bone homeostasis. Values are shown as medians and ranges. Values with an asterisk are shown as means ± standard error of the men (SEM).
Abbreviations: BSAP, bone-specific alkaline phosphatase; CTX, C-terminal telopeptide; P1NP, procollagen type 1N-terminal propeptide; PTH, parathyroid hormone.

TABLE 3 Calcium intake from food and drinks in adult Maasai

Participant no.	Breakfast day 1 T0 (0900 h) Calcium (mg)	Lunch T4 (1300 h) Calcium (mg)	Dinner T9 (1800 h) Calcium (mg)	Total day 1 Calcium (mg)	Breakfast day 2 T23 (0800 h) Calcium (mg)
Men					
1001	155	53	191	351	103
1002	82	45	175	265	35
1003	480	49	224	710	287
1004	157	58	180	347	95
1005	146	54	181	336	93
Women					
1006	245	52	150	398	209
1007	59	209	177	445	91
1008	133	65	174	312	95
1009	109	43	175	288	48
1010	134	47	207	347	99
Mean					
Men	155 (82–480)	53 (45–58)	181 (175–224)	347 (265–710)	95 (35–287)
Women	133 (59–245)	52 (43–209)	175 (150–207)	347 (288–445)	95 (48–209)
Total	140 (59–480)	53 (43–209)	179 (150–224)	347 (265–710)	95 (35–287)

Note: Calcium intake from food and drinks. Median values are shown as medians and ranges. *T* refers to time-points. Calcium content from the meals are derived from Tanzania Food Composition Tables.

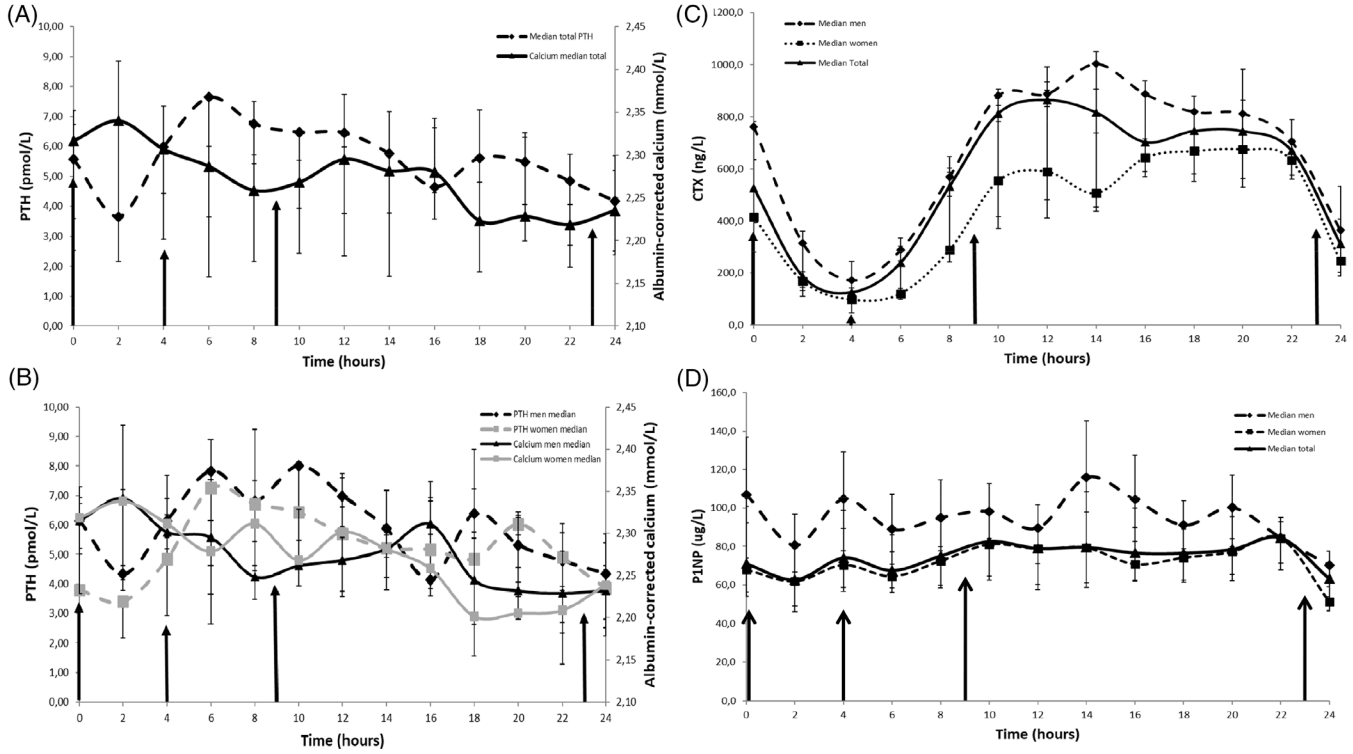

FIGURE 1 Circadian Rhythm of Albumin-corrected calcium and PTH and Circadian Rhythm of Markers of Bone Turnover. The 24-h rhythm of plasma PTH and calcium. (a) Total median concentration PTH (dashed line) and calcium (solid line). (b) Men's median PTH (dashed black line) and calcium (solid black line), women's median PTH (dashed gray line) and calcium (solid gray line). (c) Median concentration of CTX for the total group (solid line), men (dashed line), and women (dotted line). (d) Median concentration of P1NP for the total group (solid line), men (dashed line), and women (dotted line). Time 0 refers to the first blood sample for each person and correspond to a time between 9 am and 9.30 am and so forth with the other times. All participants had normal nocturnal sleep from about 2300–0700 h. The arrows indicate time for meal servings at 0900, 1300, 1800, and 0800 h. The results are shown as median values with belonging Q1 and Q3 on all figures.

calcium, PTH, CTX, and P1NP are presented in Figure 1 a-d. Figure 1a shows the 24-h profile of total serum calcium corrected for albumin and serum PTH in the total group of ten subjects. Calcium levels show alternately small fluctuations with the highest levels at day time and lowest at night time. PTH shows a bimodal pattern with a maximum peak in the afternoon and smaller peak at night. A nadir is seen in the morning (3.66 pmol/L, range 1.91–5.46) corresponding to 48% of the maximum peak level (7.64 pmol/L, range 4.81–11.81). An examination of the two profiles shows a tendency for a decrease in PTH when there is a decrease in calcium and vice versa.

Figure 1b shows the 24-h profile of total serum calcium corrected for albumin and PTH for the five men and five women separately. Both sexes exhibited small fluctuations with highest levels at day time. The level of PTH-secretion was generally higher in men and more stable in women.

No distinct pattern in the 24-h variation of vitamin D or creatinine was found (data not shown).

3.2 | Bone Turnover

Figure 1c shows the 24-h profile of serum CTX in the total group of ten, and men and women separately. All participants showed a continuous post-breakfast decrease in CTX-level reaching a nadir median value at 1300 h in day one (total group: 126.1 ng/L, range 39.5–381.9) corresponding to 23% of the 24-h mean value (total group: 543.5 ng/L ± 28.0). Both sexes generally showed a higher level at night with different timing of maximum peaks, with the men peaking at 11 pm (~1000 ng/L), and the women peaking at 5 am (~650 ng/L).

Figure 1d shows the mean 24-h profile of serum P1NP in the total group of ten, and men and women separately. All participants showed alternating small fluctuations during the day with a nadir median value at 1100 h (total group: 62.8 ug/L, range 32.9–119.9) corresponding to 79% of the 24-h mean value (total group: 79.9 ± 2.9 ng/L) and a more stable nocturnal level. No distinct pattern in the 24-h variation of BSAP or osteocalcin was found (data not shown).

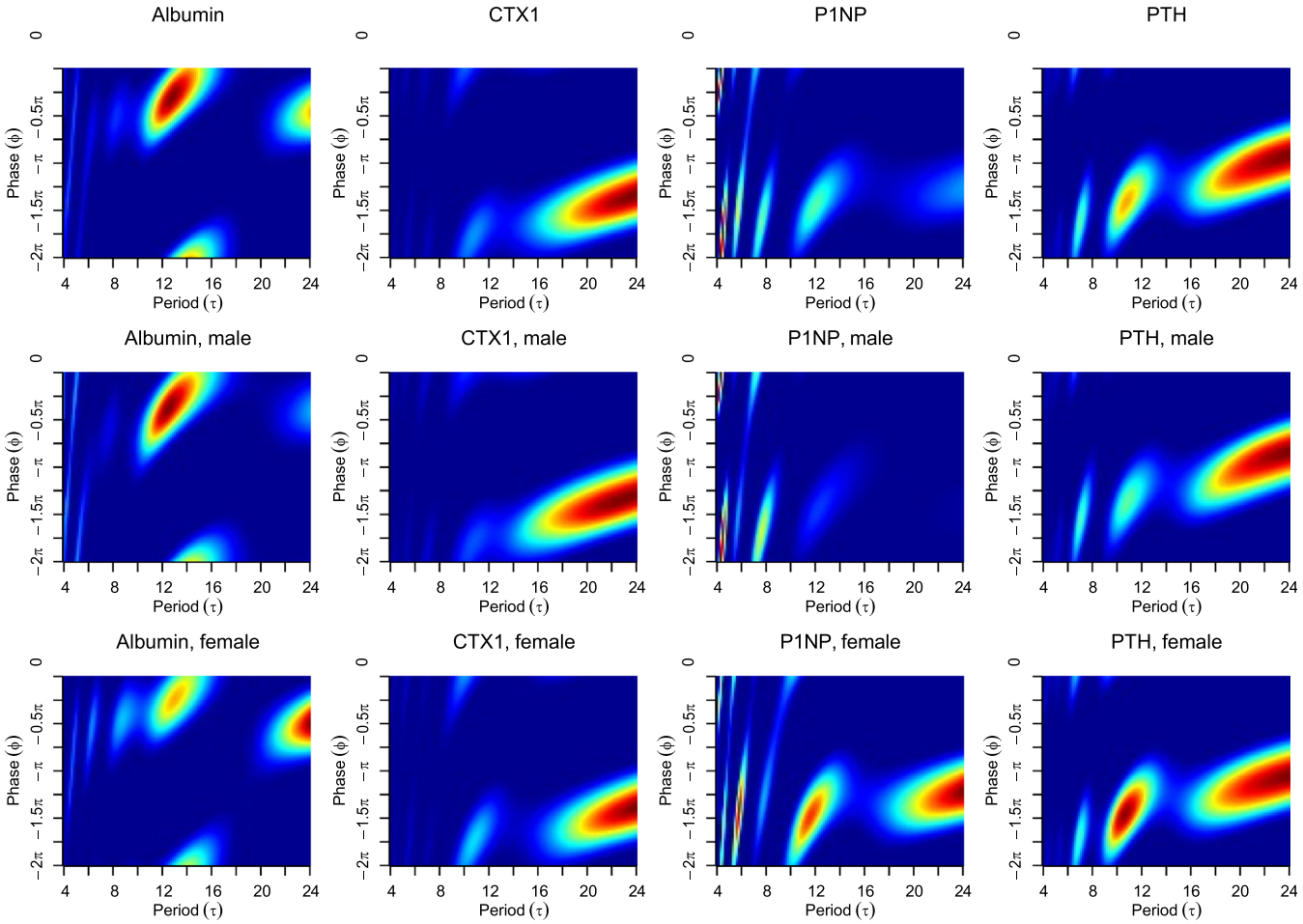

FIGURE 2 Heatmap showing the mean squared residuals from fitting a single harmonic component to the time series of Albumin-corrected Calcium, CTX, P1NP and PTH for both sexes (top row) and stratified by male (middle row) and female (bottom row). The coloring goes from blue to red in terms of decreasing mean squared residual. Red therefore shows a closed fit to the observed data.

3.3 | Harmonic analyses

Figure 2 shows the heatmaps from fitting a single harmonic component with different period and phase values to the biomarkers for all the participants and stratified by sex. The plots are colored by the value of the mean squared residuals in a gradient from blue (high) to red (low).

Regions with a red center therefore indicate combinations of period and phase with a good data fit. The plot shows that the frequency contest differs both between biomarkers and sex. For albumin-corrected calcium the circadian variation consists of a component with a period of around 12 h for both men and women, while data from the female participants also show a slower-varying component with a period of approximately a full day. For PTH the analyses show two components for men and women (ca. 24 and 10 h) where the component with the higher frequency is more pronounced for women. For the results are similar for men and women with a single harmonic component with a period of ca. 24 h and a faint component with a period of ca. 10 h.

Table 4 shows the estimated parameters from the two-component harmonic model. For all three biomarkers it is seen that the dominating component of the circadian variation in terms of amplitude is the slow-varying harmonic component with a period of approximately a day. The amplitude for this component was comparable between sexes for albumin-corrected calcium but larger for males compared to females for PTH and CTX. The faster component with a period of approximately half a day was comparable in amplitude for males and females for calcium-corrected albumin but larger for females compared to males for PTH and CTX. Figure 3 shows the fitted curves obtained from these parameter estimates.

4 | DISCUSSION

In this study, a tendency was demonstrated for Maasai participants to exhibit a 24-h variation in both calcium homeostasis and bone turnover. Our findings are

TABLE 4 Estimated parameters from a two-component harmonic model in adult Maasai.

	m_{male}	m_{female}	$a_{1,male}$	$a_{1,female}$	τ_1	φ_1	$a_{2,male}$	$a_{2,female}$	τ_2	φ_2
Calcium- corrected albumin	2.26	2.28	0.04	0.04	14.89	−0.18	0.05	0.05	24.00	−1.45
CTX	642.3 8	480.07	149.15	165.73	12.04	−4.99	315.55	283.88	21.74	−4.53
PTH	6.53	5.48	0.71	0.77	11.01	−4.49	1.18	0.63	22.76	−2.85

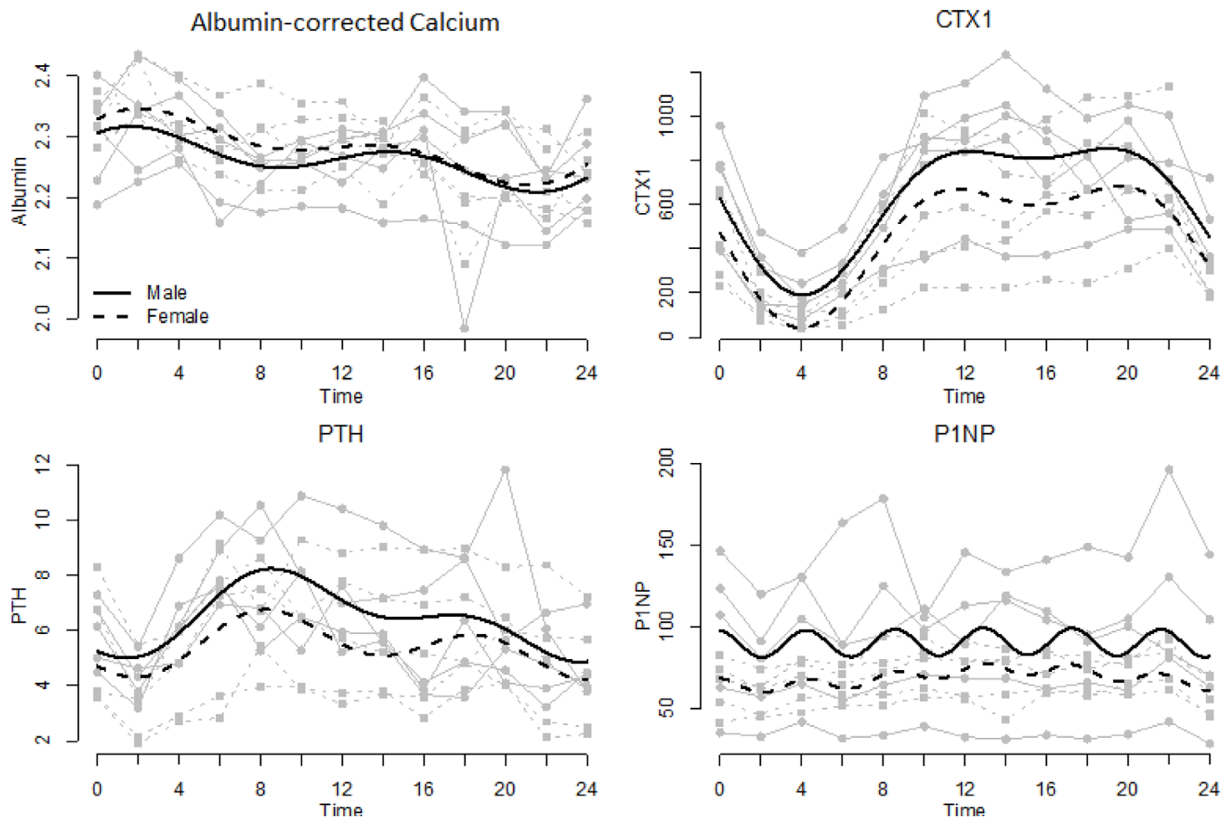

FIGURE 3 Estimated trajectories for the biomarkers based on the two-component harmonic model. The black solid figure lines present the men, the black dashed present the women, the gray dashed figure lines present the individual men, and the gray solid figure lines present the individual women.

consistent with other research that have reported diurnal rhythms of calcium, PTH, and BTMs across different ethnic groups (Bell et al., 2001; Redmond et al., 2014; 2016; Song et al., 2018). However, our results partly differ from earlier studies regarding the exact timing of peaks and troughs.

Most studies describing ethnic differences in calcium and bone metabolism are based on fasting morning samples. However, these studies include African Americans and not native African populations (Finkelstein et al., 2002; Kleerekoper et al., 1994; Paik et al., 2012). Moreover, fasting morning samples do not offer a reliable picture of calcium and bone metabolism because of the well-documented daily fluctuations of critical BTMs (Ahmad et al., 2003; Bell et al., 2001; Fraser et al., 1994; Redmond et al., 2016).

We observed a generally higher calcium level during the day and a lower nocturnal level in all participants. Meanwhile, PTH tends to exhibit an opposite pattern to calcium with lower levels during the day and higher levels during the night. Calcium levels showed only small fluctuations across the study time period, which emphasizes the presence of a normal calcium metabolism with serum calcium held tightly within a narrow range regulated through the kidneys and through bone (Riccardi & Brown, 2010).

The typical physiological response to hypocalcemia involves raising PTH-secretion minute to minute to restore normocalcemia (Bilezikian et al., 2019). This was consistent with our findings of decreases in PTH-level simultaneous with increases in calcium level and vice versa. The seemingly interdependent correlation between

calcium and PTH in our study supports the physiological pattern with serum calcium level as the primary regulator of PTH-secretion (Bilezikian et al., 2019).

PTH-secretion exhibited a bimodal pattern with a maximum peak level in the late afternoon and a relatively smaller nocturnal peak, which is consistent with several other studies (Fraser et al., 2004). In the US-based study by Bell et al. (2001), serum intact PTH increased at night only in the white women, while it was higher in the African American compared to white women throughout the day. However, urinary N-telopeptide of type I collagen and deoxypyridinoline, markers of bone resorption, increased at night in both groups. Increases in these markers occurred in the African American women in spite of little change in serum intact PTH. The results indicate that nocturnal increases in bone resorption in African American women may not be mediated by changes in the secretion of PTH.

Breakfast seems to influence the level of calcium and PTH-secretion (Budek et al., 2007), which corresponds to the relatively high amount of ingested calcium in the current study. In contrast, the lunch meal had the lowest calcium content and was not followed by an increase in calcium level nor a decrease in PTH-secretion. Since no food was served in the late evening or night, a postprandial response cannot account for the nocturnal fluctuations in calcium level and the second peak of PTH-secretion. This could point towards an endogenous response and a CR. The generally lower nocturnal calcium level, however, could be compatible with the absence of intake of calcium during the night and the corresponding generally higher level of nocturnal PTH-secretion to maintain normocalcemia. The two participants with the highest calcium intake showed larger fluctuations during the 24 h, and a more pronounced nocturnal nadir than the other participants (data not shown). The pronounced nocturnal nadir could be due to kidney excretion of calcium due to their calcium rich diet.

Baseline calcium level on day 1 is remarkably higher than the fasting morning blood sample on day 2. This could be due to the relatively low-dairy meals served in our study compared to the habitual, traditional diet of Maasai (Hansen et al., 2011), but another possible explanation is that participants consumed a dairy-rich diet in the days preceding the study, and it is possible that our results reflect a divergent 24- h rhythm (Fardellone et al., 2017; Fraser et al., 1994).

PTH regulates the serum calcium level minute by minute by increasing the renal tubular calcium reabsorption and the osteoclast activity. Furthermore, PTH also stimulate the hydroxylation of 25(OH)D in the kidney to the bioactive 1,25-dihydroxy vitamin D. 1,25-dihydroxyvitamin D increase thereafter stimulate the gastrointestinal calcium absorption and thereby support calcium absorption and normalisation of serum calcium (Blaine et al., 2015; DeLuca, 2004).

Therefore, 25(OH)D is less likely to show a circadian pattern correlated to calcium. Studies exploring the CR in serum level of $1,25(OH)_2D$ have reported conflicting results with some studies confirming the presence of a CR (Rejnmark et al., 2002) while other studies have not found a significant CR (Prince et al., 1983).

One reason for the inconsistencies in study results is because of a lack of distinction between the active $1,25(OH)_2D$, which regulates calcium levels and 25(OH)D, and which is the best indicator for the body's vitamin D pool (Holick, 2007). Our results confirmed a 24-h pattern with changing serum levels of bone resorption marker, CTX, and bone formation marker, P1NP (Wheater et al., 2013). This supports that bone remodeling exhibits daily rhythmicity (Song et al., 2018) thought to be partly regulated by PTH (Silva & Bilezikian, 2015) and influenced by external factors such as food intake (Qvist et al., 2002). In vitro and experimental animal models have shown an independence of the diurnal rhythm of BTMs of environmental factors suggesting a CR (Zvonic et al., 2007).

CTX levels, in particular, showed substantial 24-h variance supporting previous reports from different ethnic groups (Bjarnason et al., 2002; Qvist et al., 2002; Redmond et al., 2016; Song et al., 2018); CTX level showed a drastic decrease after breakfast agreeing with earlier findings and studies showing a large decrease after ingesting food following overnight fasting (Clowes et al., 2002; Qvist et al., 2002). The CR of fasting CTX levels may be altered by fasting or eating (Henriksen et al., 2003), and was thought to explain the nocturnal peak in bone resorption. No postprandial decrease in CTX level was seen following lunch or dinner.

All ten participants exhibited a generally lower daytime CTX level and higher and steadier level in the evening and night. In three ethnic groups (White British, Gambian, and Chinese), a nadir has been reported at 1400 h in CTX level and a peak at 0500 h (Redmond et al., 2016). The participants from our study all exhibited a nadir at 1300 h. Four of the men had a maximum peak level at 2300 h while four of the women showed maximum peak levels at 0500 h or 0700 h. However, nine participants showed almost the same serum level at around 2300 h and early morning, respectively.

The timing of the nocturnal peaks were similar between participants from this study compared to previous reports from Gambian, White British and Chinese groups (Redmond et al., 2016), White males (Ahmad et al., 2003; Qvist et al., 2002; Wichers et al., 1999), and

premenopausal African and Caucasian American women (Bell et al., 2001). The similarity was seen despite differences in habitual diets and calcium intake, meal times, and other factors influencing fasting and overall 24-h mean concentrations of BTMs.

Similar to other studies, CTX showed a higher amplitude compared to the other BTMs measured (Redmond et al., 2014; Song, 2017). This could be due to CTX having a relatively short half-life of 1 h whereas P1NP and BSAP have 10 h and 1.7 days respectively and due to the larger feeding influence (Bjarnason et al., 2002; Clowes et al., 2002; Qvist et al., 2002). Our results show that decreases in PTH-secretion were followed by decreases in CTX level in all participants. This supports a bone resorption effect of PTH on osteoclasts, bone resorptive cells (Joseph et al., 2007; Silva & Bilezikian, 2015).

Both men and women showed a post-breakfast decrease in P1NP-level with a nadir on day 1 and another nadir on day 2. The nocturnal level of P1NP was stable and tends to be generally higher than during daytime. As the only BTM, almost all participants showed a small postprandial decrease in P1NP level following all four meals. The question is whether this is a matter of an external stimulus or an endogenous change. Other studies have reported that feeding has a much smaller effect on P1NP than CTX (Clowes et al., 2002; Walsh & Henriksen, 2010).

We acknowledge several limitations to this study. First, the number of participants was relatively small, which does not make firm conclusions possible in relation to the Maasai as a people. Furthermore, the ability to detect robust associations between men and women is limited. Second, serum ionized calcium, which is a powerful regulator of PTH synthesis, and serum $1,25(OH)_2D$, which is a more precise indicator of the active vitamin D regulating calcium levels, were not measured in this study. Third, seasonal changes in bone turnover level have been reported (Munday et al., 2006) due to availability, calorie content, and macro and micro nutrients of food (Hansen et al., 2011), and this study was performed during just one season (cold, dry season). Fourth, no data existed on normal or osteoporotic ranges of bone mineral density and the possibility that the current study included participants with subclinical or underdiagnosed abnormalities in bone metabolism cannot be excluded. Fifth, a more frequent blood sampling (every hour) would have enabled us to describe a more accurate trend in calcium and bone homeostasis. Finally, we did not ask about menopausal status, and thus caution has to be taken when interpreting the data of the female study participants.

5 | CONCLUSION

In this group of Maasai men and women, all participants showed pronounced 24-h variations in PTH and BTMs. These findings support that Maasai participants included in this study have typical patterns of CR in calcium and bone homeostasis consistent with what has been observed in other global ethnic populations. This suggests an endogenous component, and indicates that Maasai people have a "healthy" CR in calcium and bone homeostasis.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Anne Schou: Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); writing – original draft (equal). **Niklas Rye Jørgensen:** Formal analysis (equal); validation (equal); writing – review and editing (equal). **Venance Phillip Maro:** Project administration (equal); resources (equal); writing – review and editing (equal). **Kajiru Kilonzo:** Data curation (equal); investigation (equal); writing – review and editing (equal). **Kaushik L. Ramaiya:** Project administration (equal); resources (equal); writing – review and editing (equal). **Joseph Sironga:** Investigation (equal); project administration (equal); writing – review and editing (equal). **Andreas Kryger Jensen:** Formal analysis (equal); visualization (equal); writing – review and editing (equal). **Dirk Lund Christensen:** Conceptualization (equal); data curation (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); validation

(equal); writing – review and editing (equal). **Peter Schwarz:** Conceptualization (equal); data curation (equal); formal analysis (equal); methodology (equal); supervision (lead); validation (equal); writing – review and editing (equal).

DATA AVAILABILITY STATEMENT

Data have been collected in Tanzania, and thus belongs to the main institution of collaboration, which is Kilimanjaro Christian Medical University College. Please contact Prof. Venance P. Maro (venmaro@ymail.com) for further information.

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