Title Page

Title: Serum calcium levels, chronic inflammation and glucose metabolism: A cross- sectional analysis in Andhra Pradesh Parents and Children Study (APCaPS)

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Running Title

Calcium and inflammation in glucose metabolism

Abbreviations:

fasting plasma glucose (FPG); fasting insulin (FI); inflammatory score (IScore); noncommunicable diseases (NCDs); cardiovascular disease (CVD); chronic obstructive respiratory disease (COPD); disability-adjusted life-years (DALYs); parathyroid hormone (PTH); Hyderabad-Nutrition-Trial (HNT); Integrated Child Development Services (ICDS); Physical Activity Level (PAL); metabolic equivalent task (MET) ; electro chemiluminescence immune assay (ECLIA); integrated energy index (IEI); Genetics and Biochemistry Laboratory (GBL); South Asia Network for Chronic Disease, Public Health Foundation of India (SANCD, PHFI); National Institute of Nutrition (NIN), the Indian Council of Medical Research (ICMR); London School of Hygiene and Tropical Medicine (LSHTM); analysis of variance (ANOVA); log-likelihood ratio test (LRT); coronary artery calcium (CAC).

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Conflict of Interest

Authors have no potential conflicts of interest.

ABSTRACT

BACKGROUND:

Evidence suggests a role for elevated serum calcium in dysregulated glucose metabolism, linked through low-level chronic inflammation.

OBJECTIVE:

We investigated the association of elevated serum calcium levels (corrected-for-albumin) with markers of dysregulated glucose metabolism and type-II diabetes and tested if these associations were accounted for by chronic inflammation in a rural Indian population.

DESIGN:

A cross-sectional analysis of participants aged 40-84 years was conducted from the 'Andhra Pradesh Children and Parents Study [N=2699; 52.2% women]. Comprehensive information on household, sociodemographics and lifestyle factors; medical and family history; physical measurements; blood measurements including fasting plasma glucose (FPG), fasting insulin (FI), serum calcium, albumin, phosphorous, vitamin-D (in a sub-set) and creatinine were analysed. Additionally, on a random sample of healthy participants (N=1000), inflammatory bio-markers (IL-6, IL-18, sICAM-1, adiponectin, hsCRP) were measured and an inflammatory score (IScore) calculated.

RESULTS:

After adjustments for sociodemographics, lifestyle factors and anthropometry the highest calcium quartile (Q4 *vs*.Q1) was associated with FI [β=1.4 µU/ml; 95%CI:1.2,1.5µU/ml; p-trend:<0.001], 'homeostasis model assessment' for insulin resistance (HOMA-IR) [β=1.4; 95%CI:1.2,1.5; p-trend:<0.001] and modestly associated with FPG [β=2.1 mg/dL; 95%CI:-0.9, 5.2mg/dL; p-trend:0.058] and prevalent type-II diabetes [OR=1.6; 95%CI:1.0, 2.6; p-trend:0.020]. In the healthy sub-group, the association of the highest calcium quartile was similar for FI and HOMA-IR. Additional adjustment with IScore did not alter the associations. Further, in a subset, all these associations were independent of endogenous regulators of calcium metabolism (serum vitamin-D, phosphorus and creatinine). Independently, after accounting for potential confounders, the highest Iscore quartile (Q4 vs.Q1) was positively associated with FPG, FI, HOMA-IR and prevalent prediabetes and also with serum calcium levels in men.

CONCLUSIONS:

Elevated serum calcium was positively associated with markers of dysregulated glucose metabolism and prevalent type-II diabetes in a rural Indian population. Chronic inflammation did not mediate this association but was independently associated with markers of dysregulated glucose metabolism. Inflammation might be responsible for elevated serum calcium levels in men.

KEY WORDS

Calcium, glucose metabolism, insulin resistance, type-II diabetes, prediabetes, chronic inflammation, India, APCaPS.

Word Count: 313

INTRODUCTION

Type-II diabetes is one of the leading causes of morbidity and mortality due to noncommunicable diseases (NCDs) globally (1-3). Over the past decade, while major NCDs including cardiovascular disease (CVD) and chronic obstructive respiratory disease either increased modestly or notably declined in some geographical regions, diabetes has increased globally [relative increase from 2006 to 2016: 24·4% (95% CI: 22·7–26·2) in disability-adjusted life-years (DALYs) and 31.1% (95% CI: 28·9–33·4) in absolute deaths] (1-3). The estimated 1.4 million deaths and over 570 million DALYs due to diabetes in 2016 are also likely underestimates (1-2). Asia, with the most populous countries of the world (i.e., China and India), is at the epicentre of this burden with a different pattern of disease compared to their Western counterparts (3). Although demographic and epidemiological transitions may partly explain this burden, novel aetiopathological explorations are imperative for planning effective preventive and therapeutic strategies (3-4).

Calcium modulates enzymatic functions in the liver for the production of glucose and break-down of glycogen. Calcium levels in blood and tissues are vital for secretion of insulin by the beta-cells of the pancreas (4). Thus, glucose production and beta-cell function are calcium dependent and calcium homeostasis in blood and tissues is important for controlled glucose metabolism (4). Elevated serum calcium levels have been associated with an increased risk of type-II diabetes in longitudinal studies (5-8) independent of potential confounders including adiposity and endogenous regulators of calcium metabolism such as vitamin-D, phosphorus, parathyroid hormone (PTH) or renal function (9). Further, cross-sectional studies (10-13) have shown positive associations for elevated serum calcium levels with markers of dysregulated

glucose metabolism such as increased levels of fasting glucose, glucose intolerance, insulin resistance and defective β-cell function as well as type-II diabetes.

Chronic low-levels of inflammation triggered by energy excess is an important biological mechanism which may cause elevated serum calcium levels (4) and mediate its association with dysregulated glucose metabolism (4, 14). However, given the complexity of endogenous (vitamin-D, renal function, hormones) and exogenous regulators (dietary intake, supplements, medications such as diuretics) of calcium metabolism (15), testing these associations in different settings and populations (9) and understanding the potential biological mechanism underlying the associations are vital (15). Data from rural Indian adults, with a different pattern of type-II diabetes (3, 16) and distinctive risk exposures such as tobacco chewing, regional dietary patterns, possible infectious history and genetics, provide the opportunity to assess the relationship between calcium, inflammation and glucose metabolism within a different structure of confounders compared to populations more often studied. Andhra Pradesh Children and Parents' Study (APCaPS) is a prospective birth cohort study with data collected from rural Indian adolescents and adults at three time points (2003-05; 2009-10; 2011-12) (17). We evaluated the association of serum calcium levels and levels of fasting plasma glucose (FPG), fasting insulin (FI), 'homeostasis model assessment' for insulin resistance (HOMA-IR) and prevalence of prediabetes (FPG 100-125 mg/dL) and type-II diabetes (doctor-diagnosed plus FPG $\geq$ 126 mg/dL) in rural Indian adults aged 40+ years using data from the 2011-12 collection round. We included additional inflammatory data in a sub-set of healthy participants to test if these associations were partly or fully accounted for by chronic inflammation after excluding participants with self-reported history of any chronic disease including hypertension, diabetes, CVD, chronic obstructive respiratory disease and cancer.

METHODS

The analyses in this study used data from the third follow-up of APCaPS (2010-12), a prospective cohort study established through long-term follow-up of the Hyderabad Nutrition Trial (1987-1990). Brief details of the study design are included as supplemental material along with a participant flow chart. A total of 6,944 out of a potential of 10,213 individuals participated during this phase. This included adults born between 1987 and 1990, their parents and household members. Our analysis included men and women aged 40+ years (N=2699) from the third follow-up phase. Comprehensive information on household, socio-demographic, socio-economic, life-style factors, medical and family history as well as physical measurements (anthropometrics and blood pressure) were collected. Fasting blood samples (8-hr) were collected and bio-chemical levels of FPG, FI, and serum levels of nutrients including calcium, phosphorus as well as vitamin-D in a sub-set (N=1,713) along with full blood count, and kidney function tests (e.g., serum creatinine, albumin) were measured. Vitamin-D data was available for adults born between 1987 and 1990 and their parents (N=1713/2699) and was not available for other members of the household or community. Inflammatory bio-markers IL-6, IL-18, sICAM-1, adiponectin and hsCRP were measured in a sub-set of randomly selected healthy participants aged 40+ years (N=1000) excluding participants with self-reported history of any chronic disease including hypertension, diabetes, CVD, chronic obstructive respiratory disease and cancer.

Data collection (between January 2010 and December 2012)

Questionnaire data: Structured interviewer-administered questionnaires were used to collect data from all participants. Comprehensive data on socio-demographics, socio-economics and life-style factors were collected. The study participants lived in rural locations with gradual improvements in existing facilities and infrastructure during the entire study period (2003-2012).

This degree of urbanization was calculated using a composite urbanisation score and the participants were classified into tertiles. A subset of questions (14/29) from the National Health Family Survey-2, (18) was used to estimate socioeconomic position based on information on the quality of house, toilet facilities, source of lighting, drinking water, household articles and agricultural land. A standard of Living Index (SLI) was derived based on these estimates.

Clinical assessments: Weight was measured to the nearest 0.1kg with a digital balance (SECA 899) and standing height measured to the nearest 1 mm with a plastic stadiometer (Leicester height measure; supplied by Chasmors, London). Measurements were taken twice and the average of two values was used in the analysis. Body mass index (BMI) was calculated as weight (kg) / height (m^2). Systolic and diastolic blood pressure was measured with a validated oscillometric device (Omron M5-I, Matsusaka Co., Japan) in the supine position, with appropriate cuff sizes. Each measure was taken three times and the average used. Physical Activity Level (PAL) was assessed based on Metabolic Equivalent Tasks (METs) (19) (for details please see supplementary material). Hypertension included doctor-diagnosed disease and/or a systolic BP ≥140 mm Hg or a diastolic BP ≥90 mm Hg at the time of the interview. Diabetes included doctor-diagnosed disease and/or a FPG criterion of > 126 mg/dL at the time of the interview. Prediabetes included a FPG criterion of 100-125 mg/dL at the time of the interview.

Bio-chemical measurements: Participants attended morning clinics and were asked to fast overnight. Venous blood samples (20mL) were drawn in plain and fluoride vacutainers (Becton & Dickinson, USA) by a trained phlebotomist, centrifuged and transferred to the laboratory on ice. All assays, except for FPG and vitamin-D (measured at National Institute of Nutrition, Hyderabad, India), were performed in the Genetics and Biochemistry Laboratory of the South

Asia Network for Chronic Disease, Public Health Foundation of India, New Delhi. FPG was analysed by enzymatic method using glucose oxidase/peroxidase-4- aminophenazone-phenol (GOD-PAP) method on the same day and the remaining serum samples were stored at -80^{0}C for further analysis; FI was analysed on 411 auto-analyser using reagents from Roche Diagnostics GmbH, Sandhofer Strasse, Mannheim, which is electro chemiluminescence immune assay based on sandwich principle (ECLIA); *Albumin* by Bromocresol green method and *creatinine* by Jaffe's method, rate blanked and compensated.. Serum calcium was estimated with 0-cresolphthalein complex-one under alkaline conditions. Total serum calcium was corrected for albumin using the formula: (serum calcium mg/dL) + (0.8 * (4 – serum albumin g/dL)). *Inorganic phosphate* was measured by forming ammonium phosphomolybdenum complex; *vitamin-D* using quantitative high-pressure liquid chromatography and detected at 265 nm by using an ultraviolet detector (coefficient of variation (CV) 7%). Hs-CRP by particle enhanced immunoturbidimetry method, *IL-6, IL-18, sICAM-1* were assayed using enzyme-linked immunoassay (ELISA; Krishgen BioSystems) *and adiponectin* was assayed by sandwich-assay using kits from Mediagnost, Diagnostika GmbH, Germany . The intra-assay and inter-assay CV were at <3% and <5 % respectively.

Homoeostasis Model Assessment for insulin resistance (HOMA-IR) was calculated using the formula [(fasting glucose mg/dL* fasting insulin μU/ml)/405] if fasting insulin was not equal to -1 μU/ml (20). Chronic inflammation was assessed through calculation of a standardised composite inflammatory score (Iscore) by summing the z-scores of markers as follows: [(z-score IL-6) + (z-score hsCRP) + (z-score IL-18) + (z-score sICAM-1)] – [z-score adiponectin] as previously described in Tabung et al in 2016 (21) with the exception of TNF-α (a pro-inflammatory marker) which was not available. Participants were categorized as anti- or pro-

inflammatory based on negative and positive Iscores respectively as described in Tabung et al in 2016 (21).

Ethical approval:

The study received approvals from the ethics committees of the National Institute of Nutrition (NIN), the Indian Council of Medical Research (ICMR), Hyderabad, India (latest approval 03/CR/2011/IV dated:23rd Dec 2011) and London School of Hygiene and Tropical Medicine, London, United Kingdom (latest approval LSHTM ethics ref: 6471, dated 29th July 2013). Approval was also sought from the village heads and their committees in each of the 29 villages. The participants provided written informed consent, or a witnessed thumbprint if illiterate, before their inclusion in the study. This analysis received ethical approval from London School of Hygiene and Tropical Medicine, London, United Kingdom (LSHTM MSc Ethics Ref**:** 12242, dated 16th June 2017) and Public Health Foundation of India, Gurugram, Haryana, India (TRC-IEC-349/17 dated 10th July 2017).

Statistical methods:

All data are presented as mean (±SD), median (IQR) or as numbers (%). The levels of outcome variables (FPG, FI, HOMA-IR and Iscore) were continuous. Corrected serum calcium levels were grouped in quartiles. Bivariate comparisons for differences in means, proportions or medians of socio-demographics, lifestyle factors, anthropometrics and biochemical parameters based on gender (men vs. women) and different quartiles of calcium levels (Q1 to Q4) were done using appropriate tests of significance (t-test, analysis of variance (ANOVA), Chi-square test, Wilcoxon Rank-sum test and Kruskal-Wallis ANOVA as per data type). As the data were correlated at household level, multilevel random-effects regression models (linear or logistic) accounting for household clustering were conducted to investigate the association of calcium

quartiles with FPG, FI, HOMA-IR, prevalent prediabetes and type-II diabetes after adjusting for socio-demographics [age (years), gender (men/women), degree of urbanization (tertiles)], SLI tertiles, life-style factors [tobacco (never/ever), alcohol (never or daily-weekly-monthly or on special occasions), physical activity (categories based on physical activity levels as extremely inactive, sedentary, moderately active and vigorously active), anthropometrics [BMI (kg/m^2), waist circumference (cm)] as well as endogenous regulators of serum calcium levels such as serum phosphorus (mg/dL), serum creatinine (mg/dL) and vitamin-D (ng/ml) in a sub-set. Evidence of statistical interactions were assessed by log-likelihood ratio test (LRT) of models with and without the interaction terms. We also conducted sensitivity analysis by excluding participants who used any anti-hypertensive medications. Missing data for different variables were less than 5% and were excluded from the analysis. To assess if observed effects might be mediated through chronic inflammation, we employed the *Baron and Kenny approach* (1986) (22, 23) This was done in the random sample of healthy sub-group of participants. Multilevel random-effects model for the associations of corrected serum calcium levels with FPG, FI, HOMA-IR and prevalent prediabetes were conducted with and without adjustments for Iscore. Further, independent associations of Iscore with corrected serum calcium levels, FPG, FI, HOMA-IR and prevalent prediabetes were also tested. All analyses were performed using STATA Software, version 10.1 (Stata Corp, College Station, Texas) and the multi-level sampling scheme was reflected in all of the analyses.

RESULTS

(a) Associations of serum calcium levels with markers of dysregulated glucose metabolism and prevalent type-II diabetes

The analysis included 2,699 APCaPS participants aged (mean ± SD) 50.3± 7.0 years (Range: 40-84 years) with 52.2% women. For the whole population, 86.1% were village dwellers, most with no formal education (85.7%). One-fourth of the participants were current tobacco users (24.9%); 21.5% consumed alcohol regularly; 98.4% ate a non-vegetarian diet and 50.9% reported sedentary life-style. Median (IQR) corrected serum calcium was 9.3 (8.9, 9.7) mg/dL, mean (± SD) phosphorus was 4.1 ± 0.8 mg/dL and vitamin-D was 20.0 ±9.1 ng/mL [data available for a sub-set of participants; N=1713]. These characteristics stratified by sex are shown in **Table 1**. Although serum calcium levels were not different between men and women, there were significant differences in serum levels of phosphorus, vitamin-D, creatinine as well as fasting insulin. Baseline characteristics stratified by quartiles of corrected serum calcium levels are presented in **Table 2**. Overall, participants in the highest quartile tended to be living in locations with higher urbanization score and had higher serum phosphorus, creatinine, fasting glucose, fasting insulin, insulin resistance as well as prevalent type-II diabetes.

Multivariable adjusted results for the association of corrected serum calcium quartiles with markers of glucose metabolism and prevalent prediabetes and type-II diabetes are presented in **Table 3**. After adjustments for demographic, lifestyle and anthropometric characteristics, participants in the highest calcium quartile were more likely to have higher levels of FI and HOMA-IR with a modest increase in FPG and prevalence of type-II diabetes than those in the lowest quartile (Q4 vs. Q1) (FI: β =1.4, 95% CI: 1.2, 1.5 µU/ml, p-trend: <0.001; HOMA-IR: β =1.4, 95% CI: 1.2, 1.5, p-trend: <0.001; FPG β = 2.1, 95% CI: -0.9, 5.2 mg/dL, p-trend: 0.058

and prevalent type-II diabetes OR=1.6 95% CI: 1.0, 2.6, p-trend: 0.020]. There was no association of serum calcium with prevalent prediabetes and there were no significant interactions in associations between serum calcium and the outcomes by sex. We conducted two sensitivity analyses. First, in the sub-set with vitamin-D data (N = 1713) a similar pattern but with slightly stronger associations was found, including when adjusting for serum levels of vitamin-D, phosphorus and creatinine **(Supplemental Table 1).** The vitamin D sub-set comprised younger men and women in lower SLI compared to participants who did not have vitamin-D data (data not shown). In the second sensitivity analyses we excluded participants who regularly used any hypertensive medication (N=188). Again, the results were similar, although slightly attenuated **(Supplemental Table 1)**.

(b) Mediation of chronic inflammation in associations between serum calcium and markers of dysregulated glucose metabolism in a healthy sub-set of participants

Characteristics of the healthy sub-set of participants (N=1,000) with inflammatory markers assessed were similar to the whole population and are shown in **Supplementary Table 2** stratified by sex. Men had a higher inflammatory score (Iscore) than women (mean Iscore: 0.2 (± 2.4) vs. -0.3 (± 2.2) in women). Men had higher IL-18 but lower IL-6 and adiponectin compared to women ($p<0.001$). While there were no differences in serum calcium levels between men and women, levels of serum phosphorus, vitamin-D, fasting insulin and insulin resistence differed (**Supplementary Table 2**). Within the healthy subset, after adjustment for demographic characteristics, lifestyle factors and anthropometrics the highest corrected serum calcium quartile was positively associated with FI [$\beta = 1.3$, 95% CI: 1.2, 1.5, p-trend <0.001 and HOMA-IR [$\beta = 1.3$, 95% CI: 1.1, 1.5, p-trend <0.001] (**Table 4**, model 1). On further adjustment with Iscore, the associations did not change (**Table 4**, model 2). The associations were not materially

different after adjustments for either individual inflammatory markers or the combined Iscore (data not shown). Similar patterns, but with slightly stronger associations were found in the sub-set with vitamin-D data (N=687) **(Supplemental Table 3)**. We then assessed if inflammatory score was independently associated with the glucose dysregulation outcomes FPG, FI, HOMA-IR and prevalent diabetes and observed significant associations (Q4 vs. Q1) for all outcomes **(Table 5)**. Finally, we assessed if inflammatory score was independently associated with corrected serum calcium levels **(Table 6)** and observed a significant interaction between Iscore and sex revealing a positive association only in men**.**

DISCUSSION

In this study, elevated corrected serum calcium levels were significantly associated with markers of dysregulated glucose metabolism (increased FI and HOMA-IR as well as FPG), and prevalent type-II diabetes but not prevalent prediabetes in a rural Indian population after adjustment for all measured potential confounders. When investigated in a sub-set with available data, these associations were observed to be independent of endogenous regulators of calcium metabolism including serum phosphorus, creatinine and vitamin-D. The findings were consistent, but slightly attenuated, after excluding participants who used regular medication for hypertension that could have altered calcium metabolism. Furthermore, we observed similar patterns of associations in the sub-set of participants who had no self-reported history of chronic disease and in whom inflammatory markers were assessed. Inclusion of IScore in this sub-group as a measure of chronic inflammation did not alter the observed measures of association between serum corrected calcium and glucose dysregulation. However, Iscore was independently associated with markers of dysregulated glucose metabolism in both sexes and with elevated serum calcium levels, but only in men.

Our results are consistent with previous cross-sectional studies in other populations. In South Korean adults aged 40+ years, elevated serum calcium was positively associated with type-II diabetes and metabolic syndrome (10). Although we did not find any evidence of sex specific associations for serum calcium and glucse dysregulation in our study population, these have been observed in other populations but not consistently. In a healthy Canadian population significantly higher levels of FBG and HOMA-IR were observed in the high-calcium group compared to low-calcium group which was more evident in women than men (12). Conversely, in Japanese type-II diabetics significant positive correlations between serum calcium and FPG and HOMA-IR were observed in men (11) but not in women. A study on oral glucose intolerance in UK participants aged 40-65 years, found 2-hour plasma glucose to be positively associated with total serum calcium and parathyroid hormone (PTH) both among men and women. Cohort studies (5-9) conducted so far have mostly confirmed temporal associations between elevated serum calcium and incident type-II diabetes. These associations reported across different populations were also unaffected by endogenous regulators of calcium metabolism including vitamin-D, phosphorus, magnesium, serum creatinine or glomerular filtration rate) as well as PTH. However, a recent prospective study among South East Asians reported no association between serum calcium and incident type-II diabetes and on the contrary reported higher dietary calcium to be associated with a reduced risk of type-II diabetes development. (24).

Vitamin-D and PTH may also be independently involved in diabetes risk (8, 25, 26). We did not have information on PTH, which could have limited our interpretations in terms of some residual confounding. However, the dose-response association (Q4 vs. Q1) was consistent in the overall study population as well as across several sub-sets within the study population (i.e., a healthy sub-set, a sub-set excluding participants with regular hypertensive medication use as well as a

sub-set with vitamin-D data). The associations with markers of dysregulated glucose metabolism and prevalent type-II diabetes were strengthened in the sub-set with vitamin-D data. This sub-set included young men and women living in locations with higher urbanization score and low SLI. This might have been a chance finding due to relatively smaller sample size but could possibly be indicating high-risk individuals within this population.

The probable causal role of elevated serum calcium for type-II diabetes is becoming established through cohort studies and consistent cross-sectional findings across different populations worldwide (4-13, 27). Randomised clinical trials report prevention and/or stable control of type-II diabetes using calcium-channel blockers or other medications compared to diuretics in patients with other disease conditions including hypertension (14, 28).

The proposed mechanisms for the associations of elevated serum calcium on glucose metabolism dysregulation include the role of calcium in reducing the function of glucose transporters on adipocytes, (15) altering beta-cell function and increasing insulin resistance. Serum calcium levels could be elevated by underlying chronic low levels of inflammation (4, 27). Inflammation and elevated calcium could synergistically contribute to organelle dysfunction and damage leading to dysregulated glucose metabolism (4, 27). In our study, chronic inflammation did not appear to mediate associations between serum calcium and dysregulated glucose metabolism. Inadequate measurement of inflammation could have influenced these associations. However, although we did not have information on TNF-α and IL-10, important pro- and anti-inflammatory markers respectively, (29) the use of a composite inflammatory score has been shown previously to adequately characterize the overall inflammatory status of the participants (21). The role of chronic inflammation in the pathogenesis of type-II diabetes is well established, (4, 27). We found independent positive associations of chronic inflammation with markers of

dysregulated glucose metabolism in the healthy sub-set of participants consistent with the existing evidence.

There is a large body of preclinical data (*in vitro* and animal models) for the elevation of serum calcium levels by chronic inflammation (4, 27). We found positive associations of chronic inflammation (Iscore) with elevated corrected serum calcium among men, indicating a role for chronic inflammation in elevating serum calcium levels. We did not find similar associations among women suggesting that the factors for elevated serum calcium in women could be other than inflammation. Similar sex-specific differences have been observed with coronary artery calcium (CAC) deposition and inflammatory markers wherein adiponectin was found to be inversely associated (30) and hsCRP positively associated with CAC in men (31) but not among women. It is established that inflammatory responses and its influences on various chronic diseases including type-II diabetes are gender-specific due to sex hormones, genetic factors and differences in lifestyle risk exposures, (28) but further investigations are required to understand the mechanisms better. Inadequate measurement of inflammatory markers specific for women such as IFN-γ, IL-10 (28) could also have influenced these associations, however, this is less likely as chronic inflammation was associated with markers of dysregulated glucose metabolism.

Our results suggest that chronic inflammation may not be on the causal pathway between serum calcium and glucose dysregulation in our study population, but it remains possible that it may be involved in early elevation of serum calcium levels among men. In this population, serum calcium levels and chronic inflammation are associated with glucose metabolism but are acting independently of each other. Cautious interpretation of these results is required for the following reasons: (1) inherent limitations of a cross-sectional study design such as reverse causation and temporality; (2) potential measurement error of chronic inflammation, although the inflammatory

markers used in our study were among the most commonly used markers to examine disease endpoints, and have been validated for calculating inflammatory score in other populations (21) and previously associated with calcium and glucose metabolism (4,27,30,31); (3) the association between elevated serum calcium and prevalent prediabetes was not evident in this population.

Despite these limitations, this study possesses considerable merits. This was a rural Indian population-based study with adequate sample size to test dose-response. The dose-response results for the association of elevated serum calcium with markers of dysregulated glucose metabolism and type-II diabetes were stable across different subsets. Our results are in-line with scientific evidence available to date and are biologically plausible. Furthermore, we observed independent associations of chronic inflammation with dysregulated glucose metabolism and serum calcium levels. To the best of our knowledge, this is the first report of the associations of elevated serum calcium levels and chronic inflammation on markers of glucose metabolism dysregulation, evaluating them as independent risk factors, confounders and mediators in a representative rural Indian population.

CONCLUSIONS

Consistent with previous observational studies, elevated serum calcium is positively associated with markers of dysregulated glucose metabolism and prevalent type-II diabetes in a rural Indian population. This association was not mediated through chronic inflammation, but chronic inflammation was independently associated with glucose metabolism dysregulation. It remains possible that inflammation might be responsible for elevation of serum calcium early in the pathway, but only among men. Large scale population-based studies to validate the findings and clinical studies to address its clinical relevance and understand potential sex-specific effects are recommended.

Author Contributions:

KS, PKD, SEC designed research; SK, PKD, RG, SKL and DP conducted research; KS analyzed data; KS and SEC wrote the first draft of the paper; all authors had primary responsibility for final content. All authors read and approved the final manuscript.

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Inflammatory Obesity Phenotypes, Gender Effects, and Subclinical Atherosclerosis in African Americans: The Jackson Heart Study. Arterioscler Thromb Vasc Biol. 2016 Dec;36(12):2431-2438.

Table 1: Participant characteristics of rural Indian men and women aged 40-84 years (N=2699) in Andhra Pradesh Children and Parents Study (APCAPS, 2010-12).

		Men	Women	p-value*	Total
Number (%)		1291 (47.8)	1408 (52.2)		2699
Socio-demographic and socio-economic characteristics					
Age (years) [mean (± SD)]		53.6 (± 7.0)	47.3 (± 5.5)	<0.0001	50.3 (± 7.0)
Education [n (%)]					
No formal education		969 (75.1)	1342 (95.4)	<0.001	2311 (85.7)
Primary School (up to class IV)		228 (17.7)	52 (3.7)		280 (10.4)
Secondary School (class V to X/XII)		94 (7.3)	13 (0.9)		107 (4.0)
Occupation [n (%)]					
Unemployed		76 (5.9)	260 (18.5)	<0.001	336 (12.5)
Manual labour (skilled/unskilled)		1166 (90.4)	1139 (80.9)		2305 (85.5)
Skilled non-manual/semi-professional		48 (3.7)	8 (0.6)		56 (2.1)
Standard of living index** [mean (± SD)]		27.4 (± 8.1)	26.9 (± 8.4)	0.0966	27.1 (± 8.3)
Urbanization[#] (tertiles) [n (%)]	Low	467 (36.2)	513 (36.4)		980 (36.3)
	Medium	397 (30.7)	441 (31.3)	0.893	838 (31.0)
	High	427 (33.1)	454 (32.2)		881 (32.6)
Life-style characteristics [n (%)]					
Tobacco	Never	496 (38.4)	1048 (74.5)		1544 (57.2)
	Current	753 (58.3)	350 (24.9)	<0.001	1103 (40.9)
	Former	42 (3.2)	8 (0.6)		50 (1.8)
Alcohol	Never	437 (33.8)	1073 (76.4)		1510 (56.0)
	Daily/most days	98 (7.6)	5 (0.4)		103 (3.8)
	Weekends only	158 (12.2)	13 (0.9)	<0.001	171 (6.3)
	Monthly	249 (19.3)	59 (4.2)		308 (11.4)
	Special occasions	349 (27.0)	255 (18.1)		604 (22.4)
Physical Activity Level (PAL)					
Extremely inactive		177 (14.8)	68 (5.2)		245 (9.8)
Sedentary		486 (40.7)	538 (41.4)		1024 (41.1)
Moderately active		435 (36.5)	563 (43.3)	<0.001	998 (40.0)
Vigorously active		95 (8.0)	130 (10.0)		225 (9.0)
Physical characteristics [mean(±SD)]					
BMI (kg/m^2)		20.5 (± 3.7)	21.9 (± 4.2)	<0.0001	21.2 (± 4.0)
Waist circumference (cm)		76.8 (± 10.5)	73.4 (± 10.2)	<0.0001	75.0 (± 10.5)
Prevalent hypertension*** [n (%)]		529 (41.0)	396 (28.1)	<0.001	925 (34.3)

Regular medication for hypertension [n (%)]	108 (11.5)	80 (7.0)	<0.001	188 (9.0)
Prevalent prediabetes*** [n (%)]	287 (25.0)	291 (23.1)	0.274	578 (24.0)
Prevalent diabetes*** [n (%)]	122 (9.6)	103 (7.6)	0.060	225 (8.5)
Regular medication for diabetes [n (%)]	51 (4.0)	46 (3.4)	0.383	97 (3.7)
Biochemical parameters [mean (±SD)]				
Corrected serum calcium$ (mg/dL) [median (IQR)]	9.3 (8.9, 9.8)	9.3 (8.9, 9.7)	0.8051	9.3 (8.9, 9.7)
Serum calcium uncorrected (mg/dL) [median (IQR)]	9.8 (9.3, 10.3)	9.7 (9.3, 10.3)	0.1550	9.7 (9.3, 10.3)
Serum phosphorus (mg/dL)	4.0 (±0.8)	4.2 (±0.8)	<0.0001	4.1 (±0.8)
Serum vitamin-D^ (ng/ml)	19.2 (±10.1)	20.8 (±8.0)	0.0001	20.0 (±9.1)
Fasting plasma glucose (mg/dL)	99.1 (±27.8)	97.9 (±27.3)	0.2884	98.5 (±27.5)
Fasting insulin (µU/ml) [median (IQR)]	3.0 (1.6, 5.9)	4.8 (3.0, 7.8)	<0.0001	4.1 (2.3, 7.0)
Insulin resistance (HOMA-IR) [median (IQR)]	0.7 (0.4, 1.4)	1.1 (0.7, 1.9)	<0.0001	0.9 (0.5, 1.7)
Serum creatinine (mg/dL)	0.9 (±0.2)	0.7 (±0.1)	<0.0001	0.8 (±0.2)

* p-values are from t-test, Wilcoxon Rank-sum or chi-square test for difference in means, medians or proportions.

**standardized score based on household assets

based on composite score indicating degree of urbanization of the area

^ data were available for 63.4% of participants (N=1713/2699). Missing data for all other variables were <5% except for PAL (7.7%)

$ total serum calcium corrected-for-albumin using formula: (serum calcium mg/dL) + (0.8 * (4 – serum albumin g/dL))

*** Hypertension: doctor-diagnosed disease and/or a systolic BP ≥140 mm Hg or a diastolic BP ≥90 mm Hg at the time of the interview; diabetes: doctor-diagnosed disease and/or fasting blood glucose (FBG) > 126 mg/dL at the time of the interview; prediabetes: FBG 100-125 mg/dL at the time of the interview.

Table 2: Distribution of population characteristics by quartiles of corrected serum calcium levels† of rural Indian men and women aged 40-84 years (N=2699) in Andhra Pradesh Children and Parents Study (APCAPS, 2010-12).

	Quartiles of corrected serum calcium levels†				
	Q1	**Q2**	**Q3**	**Q4**	**p-value**
Median (IQR) (mg/dL)	8.7 (8.5, 8.8)	9.1 (9.0, 9.2)	9.5 (9.3, 9.6)	10.1 (9.9, 10.5)	
Number	654	650	651	651	
Socio-demographic and socio-economic characteristics					
Age (years) [mean (± SD)]	50.1 (7.0)	50.1 (7.1)	50.4 (7.0)	50.7 (7.0)	0.34
Sex [n (%)] Men	329 (50.3%)	302 (46.5%)	302 (46.4%)	327 (50.2%)	0.28
Education [n (%)] No formal education	555 (84.9%)	552 (84.9%)	561 (86.2%)	560 (86.2%)	0.50
Primary School (up to class IV)	72 (11.0)	63 (9.7)	70 (10.8)	66 (10.2)	
Secondary School (class V to X/XII)	27 (4.1)	35 (5.4)	20 (3.1)	24 (3.7)	
Occupation [n (%)] Unemployed	60 (9.2%)	89 (13.7%)	96 (14.7%)	76 (11.7%)	
Manual labour	579 (88.5)	550 (84.6)	542 (83.3)	552 (86.1)	
Skilled non-manual/semi-professional	15 (2.3)	11 (1.7)	13 (2.0)	14 (2.2)	0.078
Standard of living index** [mean (± SD)]	26.8 (7.9)	27.6 (8.3)	27.9 (8.1)	26.4 (8.7)	0.003
Urbanization[#] (tertiles) [n (%)] Low	198 (30.3)	208 (32.0)	231 (35.5)	308 (47.3)	<0.001
Medium	269 (41.1)	251 (38.6)	185 (28.4)	87 (13.4)	
High	187 (28.6)	197 (29.4)	235 (36.1)	256 (39.3)	
Life-style characteristics [n (%)]					
Tobacco Never	378 (57.9)	385 (59.2)	360 (55.3)	365 (56.2)	0.48
Ever	275 (42.1)	265 (40.8)	291 (44.7)	285 (43.8)	
Alcohol Never	346 (53.0)	389 (59.8)	353 (54.3)	357 (54.9)	0.12
Daily/weekly	70 (10.7)	50 (7.7)	71 (10.9)	74 (11.4)	
Monthly/Special Occasions	237 (36.3)	211 (32.5)	226 (34.8)	219 (33.7)	
Physical Activity Level (PAL)					
Extremely inactive	39 (7.1)	59 (10.1)	65 (10.3)	72 (11.3)	0.005
Sedentary	217 (39.7)	243 (41.4)	282 (44.8)	247 (38.6)	
Moderately active	249 (4.5)	235 (40.0)	232 (36.9)	244 (38.1)	
Vigorously active	42 (7.7)	50 (8.5)	50 (7.9)	77 (12.0)	
Physical characteristics [mean(±SD)]					
BMI (kg/m^2)	20.9 (3.7)	21.3 (3.9)	21.5 (4.3)	21.2 (4.1)	0.025
Waist circumference (cm)	74.2 (10.1)	75.2 (10.5)	75.8 (10.8)	75.1 (10.3)	0.044
Prevalent hypertension*** [n (%)]	200 (30.6)	216 (33.2)	240 (36.9)	234 (35.9)	0.071

Prevalent prediabetes*** [n (%)]	137 (22.3)	136 (22.6)	164 (27.9)	132 (22.7)	0.071
Prevalent diabetes*** [n (%)]	40 (6.1)	48 (7.4)	61 (9.4)	69 (10.6)	0.016
Biochemical parameters [mean (±SD)]					
Serum phosphorus (mg/dL)	4.0 (1.2)	4.0 (0.6)	4.2 (0.6)	4.4 (0.6)	<0.001
Serum vitamin-D^ (ng/ml)	20.3 (10.2)	19.9 (8.4)	19.3 (8.4)	20.5 (9.3)	0.25
Fasting plasma glucose (mg/dL)	95.7 (20.0)	96.6 (24.3)	100.6 (29.2)	100.6 (33.1)	<0.001
Fasting insulin (µU/ml) [median (IQR)]	3.6 (2.1, 6.0)	3.8 (2.2, 6.8)	4.3 (2.5, 7.5)	4.5 (2.4, 7.8)	<0.001
Insulin resistance (HOMA score) [median (IQR)]	0.8 (0.5, 1.4)	0.9 (0.5, 1.6)	1.0 (0.6, 1.9)	1.1 (0.5, 1.9)	<0.001
Serum creatinine (mg/dL)	0.8 (0.2)	0.8 (0.2)	0.8 (0.2)	0.9 (0.2)	<0.001

*p-values are from ANOVA, Kruskal-Wallis ANOVA or chi-square test for difference in means, medians or proportions **standardized score based on household assets

based on composite score indicating degree of urbanization of the area

^ data were available for 63.4% of participants (N=1713/2699). Missing data for all other variables were <5% except for PAL (7.7%)

† total serum calcium corrected-for-albumin using formula: (calcium mg/dL) + ((0.8 * (4 – albumin g/dL))

*** Hypertension: doctor-diagnosed disease and/or a systolic BP ≥140 mm Hg or a diastolic BP ≥90 mm Hg at the time of the interview; Diabetes: doctor-diagnosed disease and/or fasing blood glucose (FBG) > 126 mg/dL at the time of the interview; Prediabetes: FBG 100-125 mg/dL at the time of the interview.

Table 3: Associations† of corrected serum calcium levels†† with fasting plasma glucose, fasting insulin, insulin resistance, prevalent prediabetes and diabetes in rural Indian men and women aged 40-84 years of Andhra Pradesh Children and Parents Study (APCAPS, 2010-12).

	Fasting plasma glucose (mg/dL) (β; 95% CI)	Fasting insulin* (μU/ml) (β; 95% CI)	Insulin resistance* (HOMA-IR) (β; 95% CI)	Prevalent prediabetes (OR; 95% CI)	Prevalent diabetes (OR; 95% CI)
Corrected serum calcium quartiles					
Model 1	N=2605	N=2604	N=2603	N=2385	N=2605
Q1	0.00 (ref)	0.00 (ref)	0.00 (ref)	1.00 (ref)	1.00 (ref)
Q2	0.7 (-2.2, 3.6)	1.1 (1.0, 1.2)	1.1 (1.0, 1.3)	1.0 (0.8, 1.4)	1.2 (0.8, 1.8)
Q3	4.6 (1.6, 7.5)	1.3 (1.1, 1.4)	1.3 (1.2, 1.5)	1.3 (1.0, 1.8)	1.6 (1.0, 2.4)
Q4	4.6 (1.6, 7.6)	1.4 (1.3, 1.6)	1.5 (1.3, 1.6)	0.9 (0.7, 1.3)	1.9 (1.2, 2.9)
p-trend	<0.001	<0.001	<0.001	0.749	0.001
Model 2	N=2394	N=2393	N=2392	N=2190	N=2394
Q1	0.00 (ref)	0.00 (ref)	0.00 (ref)	1.00 (ref)	1.00 (ref)
Q2	-1.4 (-4.4, 1.6)	1.1 (1.0, 1.2)	1.1 (1.0, 1.2)	0.9 (0.7, 1.2)	0.9 (0.5, 1.5)
Q3	1.3 (-1.6, 4.3)	1.2 (1.1, 1.3)	1.2 (1.1, 1.3)	1.0 (0.8, 1.4)	1.1 (0.7, 1.8)
Q4	2.1 (-0.9, 5.2)	1.4 (1.2, 1.5)	1.4 (1.2, 1.5)	0.7 (0.5, 1.1)	1.6 (1.0, 2.6)
p-trend	0.058	<0.001	<0.001	0.206	0.020
p-interaction by sex**	0.6589	0.3144	0.2690	0.8267	0.4047

Model 1: adjusted for age (years), sex, standard of living index (tertiles), urbanization score (tertiles)

Model 2: adjusted for age (years), sex, standard of living index (tertiles), urbanization score (tertiles) tobacco (never/ever), alcohol (categories), physical activity (categories), BMI (kg/m^2), waist circumference (cm)

†Multilevel random-effects linear (for FPG, FI and HOMA-IR) and logistic (for prevalent prediabetes and diabetes) regression model.

* Co-efficient and confidence-interval values were back-transformed (e^{β}) as outcome variables were natural log transformed before analyses

** likelihood-ratio test for interaction of sex with serum calcium quartiles.

†† Median (IQR) levels of serum calcium (mg/dL) corrected-for-albumin by quartiles: Q1 – 8.7 (8.5, 8.8); Q2 - 9.1 (9.0, 9.2); Q3-9.5 (9.3, 9.6); Q4 -10.1 (9.9, 10.5)

Table 4: Associations† of corrected serum calcium levels†† with fasting plasma glucose, fasting insulin, insulin resistance and prevalent prediabetes in a random sample of healthy rural Indian men and women aged 40-84 years (N=1000)[#] of Andhra Pradesh Children and Parents Study (APCAPS, 2010-12)

	Fasting plasma glucose (mg/dL) (β; 95% CI)	Fasting insulin* (μU/ml) (β; 95% CI)	Insulin resistance* (HOMA-IR) (β; 95% CI)	Prevalent prediabetes (OR; 95% CI)
Corrected serum calcium quartiles				
Model 1	N=917	N=917	N=917	N=882
Q1	0.00 (Ref)	0.00 (Ref)	0.00 (Ref)	1.00 (Ref)
Q2	-1.6 (-5.2, 2.0)	1.0 (0.9, 1.2)	1.0 (0.9, 1.2)	0.8 (0.5, 1.4)
Q3	-0.13 (-3.8, 3.5)	1.1 (0.9, 1.2)	1.1 (0.9, 1.2)	1.0 (0.6, 1.7)
Q4	1.5 (-2.2, 5.2)	1.3 (1.2, 1.5)	1.3 (1.1, 1.5)	0.8 (0.5, 1.4)
p-trend	0.334	<0.001	<0.001	0.627
p-interaction by sex**	0.9972	0.0361	0.0634	0.7121
Model 2	N=914	N=914	N=914	N=879
Q1	0.00 (Ref)	0.00 (Ref)	0.00 (Ref)	1.00 (Ref)
Q2	-1.6 (-5.2, 2.0)	1.0 (0.9, 1.2)	1.0 (0.9, 1.2)	0.8 (0.5, 1.4)
Q3	-0.5 (-4.2, 3.1)	1.1 (0.9, 1.2)	1.1 (0.9, 1.2)	0.9 (0.5, 1.6)
Q4	0.7 (-3.0, 4.4)	1.3 (1.1, 1.5)	1.3 (1.1, 1.5)	0.7 (0.4, 1.3)
p-trend	0.613	<0.001	<0.001	0.368
p-interaction by sex**	0.9973	0.0467	0.0818	0.6351
p-interaction by Iscore[##]	0.6647	0.6499	0.7164	0.6182

Model 1: adjusted for age (years), sex, standard of living index (tertiles), urbanization score (tertiles), tobacco (never/ever), alcohol (categories), physical activity (categories), BMI (kg/m^2) and waist circumference (cm); Model 2: Model 1 plus overall inflammatory score;

† Multilevel random-effects linear (for FPG, FI and HOMA-IR) and logistic (for prevalent prediabetes) regression model

†† Median (IQR) levels of serum calcium (mg/dL) corrected for albumin by quartiles: Q1 – 8.7 (8.5, 8.8); Q2 - 9.1 (9.0, 9.2); Q3-9.5 (9.3, 9.6); Q4 -10.1 (9.9, 10.5)

* Co-efficient and confidence-interval values were back-transformed (e^{β}) as outcome variables were ln-transformed before analyses; ** likelihood-ratio test for interaction of sex and serum calcium quartiles

excluding participants with self-reported history of any chronic disease including hypertension, diabetes, cardiovascular disease, chronic obstructive respiratory disease and cancer.

likelihood-ratio test for interaction of Iscore and serum calcium quartiles (Iscore was categorised as anti-inflammatory score and pro-inflammatory score based on negative and positive values respectively (21))

SLI: Standard of living Index; PAL**:** Physical Activity Level; HOMA-IR: Homoeostasis-Model-Assessment for insulin resistance.

Table 5: Associations† of inflammatory score with fasting plasma glucose, fasting insulin, insulin resistance and prevalent prediabetes in a random sample of healthy# rural Indian men and women aged 40-84 years (N=1000) of Andhra Pradesh Children and Parents Study (APCaPS, 2010-12).

	Fasting plasma glucose (mg/dL) (β; 95% CI) (N=913)	Fasting insulin* (µU/ml) (β; 95% CI) (N=913)	Insulin resistance* (HOMA-IR) (β; 95% CI) (N=913)	Prevalent prediabetes (OR; 95% CI) (N=878)
Inflammatory score* Quartiles				
Q1	Ref	Ref	Ref	Ref
Q2	0.01 (-0.01, 0.04)	1.1 (1.01, 1.3)	1.1 (1.01, 1.3)	1.5 (0.9, 2.8)
Q3	0.02 (-0.006, 0.05)	1.08 (0.96, 1.2)	1.1 (0.96, 1.2)	1.2 (0.6, 2.1)
Q4	0.07 (0.04, 0.1)	1.3 (1.1, 1.5)	1.4 (1.2, 1.6)	2.8 (1.5, 5.4)
p-trend	<0.001	0.001	<0.001	0.003
p-interaction by sex**	0.2701	0.7967	0.7707	0.1256

†adjusted for age (years), sex, standard of living index (tertiles), urbanization score (tertiles), tobacco (never/ever), alcohol (categories), physical activity (categories), BMI (kg/m^2), waist circumference (cm), serum calcium (mg/dL), serum phosphorus (mg/dL) and serum creatinine (mg/dL) in random-effects linear/logistic multilevel model.

excluding participants with self-reported history of any chronic disease including hypertension, diabetes, cardiovascular disease, chronic obstructive respiratory disease and cancer.

*Inflammatory score was calculated summing the z-scores of markers to create a standardized overall inflammatory marker score for each participant as follows: [(z-score IL-6) + (z-score CRP) + (z-score IL-18) + (z-score sICAM1)] – [z-score adiponectin] (21).

** likelihood-ratio test for interaction of sex and inflammatory score quartiles

* Co-efficient and confidence-interval values were back-transformed (e^{β}) as outcome variables were ln-transformed before analyses

Table 6: Associations† of inflammatory score with corrected serum calcium levels in a random sample of healthy# rural Indian men and women aged 40-84 years (N=1000) of Andhra Pradesh Children and Parents Study (APCaPS, 2010-12).

	Corrected serum calcium levels (β; 95% CI)		
Inflammatory score* Quartiles			
	Overall (n=914)	Men (N=461)	Women (N=453)
Q1	Ref	Ref	Ref
Q2	0.09 (-0.02, 0.2)	0.3 (0.1, 0.5)	-0.07 (-0.2, 0.09)
Q3	0.1 (0.02, 0.2)	0.3 (0.2, 0.5)	-0.07 (-0.2, 0.1)
Q4	0.2 (0.1, 0.4)	0.4 (0.2, 0.6)	0.2 (-0.01, 0.3)
p-trend	<0.001	<0.001	0.113
p-interaction by sex**	0.0258	-	-

†adjusted for age (years), sex, standard of living index (tertiles), urbanization score (tertiles), tobacco (never/ever), alcohol (categories), physical activity (categories), BMI (kg/m^2), waist circumference (cm) using random-effects linear multilevel model.

excluding participants with self-reported history of any chronic disease including hypertension, diabetes, cardiovascular disease, chronic obstructive respiratory disease and cancer.

*Inflammatory score was calculated summing the z-scores of markers to create a standardized overall inflammatory marker score for each participant as follows: [(z-score IL-6) + (z-score CRP) + (z-score IL-18) + (z-score sICAM1)] – [z-score adiponectin] (21).

** likelihood-ratio test for interaction of sex and inflammatory score quartiles.

The associations followed a similar pattern when adjusted for serum levels of phosphorous, creatinine and vitamin D in a sub-set with vitamin D data (N=618).