Fasting ketonuria is inversely associated with coronary artery calcification in non-diabetic individuals

OPEN ACCESS

Fasting ketonuria is inversely associated with coronary artery calcification in non-diabetic individuals

In Young Cho, MD,[a] Yoosoo Chang, MD, PhD,[b, c, d,*,1] Eunju Sung, MD, PhD,[a,b] Yejin Kim, MHS,[b] Jae-Heon Kang, MD, MPHD, PhD,[a] Hocheol Shin, MD, MPH, PhD,[a, b] Sarah H. Wild, MB BChir, PhD,[e] Christopher D. Byrne, MB BCh, PhD,[f, g] and Seungho Ryu, MD, PhD [b, c, d,*,1]

[a] Department of Family Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, South Korea

[b] Center for Cohort Studies, Total Healthcare Center, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, South Korea

[c] Department of Occupational and Environmental Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, South Korea

[d] Department of Clinical Research Design and Evaluation, SAIHST, Sungkyunkwan University, Seoul, South Korea

[e] Usher Institute of Population Health Sciences and informatics, University of Edinburgh, Edinburgh, UK

[f] Nutrition and Metabolism, Faculty of Medicine, University of Southampton, Southampton, UK

[g] National Institute for Health Research Southampton Biomedical Research Centre, University Hospital Southampton, Southampton, UK

*Corresponding author.

Seungho Ryu

Department of Occupational and Environmental Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Samsung Main Building B2, 250 Taepyung-ro 2ga, Jung-gu, Seoul 04514, Republic of Korea

Tel: +82-2-2001-5137, Fax: +82-2-757-0436, E-mail: sh703.yoo@gmail.com

and

Yoosoo Chang

Department of Occupational and Environmental Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Samsung Main Building B2, 250 Taepyung-ro 2ga, Jung-gu, Seoul 04514, Republic of Korea

Tel: +82-2-2001-5137, Fax: +82-2-757-0436, E-mail: yoosoo.chang@gmail.com

[1]**Drs. Chang and Ryu are the corresponding authors.**

Number of tables and figures: Tables 4, Figures 1

Abstract

Background and Aims: Increased levels of ketone bodies, an alternative fuel when glucose availability is low, may exert beneficial effects on cardiovascular disease (CVD) risk factors. Whether increased ketone bodies are associated with coronary artery calcium (CAC), a recognized and strong cardiovascular risk factor, remains unknown. We investigated the association of fasting ketonuria with CAC and its progression.

Methods: Cross-sectional and longitudinal studies were conducted in adults without diabetes or CVD. Subjects underwent routine health examinations including cardiac computed tomography estimations of CAC scores. Logistic regression models were performed to compute the odds ratios (ORs), 95% confidence intervals (CIs), for prevalent CAC scores >0 according to fasting ketonuria categories (0, 1, and ≥2). Linear mixed models with random intercepts and random slopes were used to estimate CAC progression.

Results: Of 144,346 subjects, 12.3% had CAC scores >0 at baseline. Overall, higher fasting ketonuria was associated with decreased prevalence of coronary calcification than no ketonuria. Multivariable-adjusted ORs (95% CIs) for prevalent CAC by comparing ketonuria categories 1 and ≥ 2 with no ketonuria, were 0.94 (0.84–1.06) and 0.82 (0.71–0.95), respectively. The associations did not differ according to clinically relevant subgroups. Ketonuria was associated with lower CAC progression over time; the multivariable adjusted ratio of progression rates comparing ketonuria ≥ 2 versus no ketonuria was 0.976 (0.965–0.995).

Conclusions: We found an inverse association between fasting ketonuria and subclinical coronary atherosclerosis, in both prevalence and progression. The potentially protective role of increased ketone body formation in CVD requires further investigation.

1. Introduction

Cardiovascular disease (CVD) remains the leading cause of death with an estimated 18.6 million deaths in 2019, and a predicted global increase of 18% by 2030.[1, 2] Though medications such as statins have markedly improved cardiovascular outcomes, patients who achieve the recommended low-density lipoprotein cholesterol (LDL-C) levels through intense statin therapy still have a significant residual CVD risk,[3, 4] with coronary artery calcium (CAC) as an important predictor of residual risk.[5] Furthermore, 20% of individuals with CVD have no established conventional risk factors.[6] Thus, it is important to assess new and novel risk factors for CVD, as it may be possible to treat these new risk factors and further lower the CVD risk.

Recently, both experimental and clinical studies have suggested the protective role of ketone bodies in CVD.[7] Ketone bodies are synthesized in the liver mainly through fatty acid oxidation and undergo oxidation for energy metabolism in the extrahepatic tissues during physiological states such as limited carbohydrate intake, strenuous exercise, and prolonged fasting.[8] Circulating ketone body concentrations can increase from < 0.3 mM during normal state to 1–3 mM after fasting, prolonged exercise,[9] or nutritional ketosis[10] in healthy adults. This is very different from the pathologic states such as diabetic ketoacidosis, in which ketone concentrations can increase to over 20 mM.[9] The metabolism and signaling roles and the epigenetic effects of ketone bodies at low to medium concentrations[11] have a beneficial effect on the vascular endothelium,[12] in protecting against inflammation and injury,[9] and in improving metabolic and inflammatory markers such as lipid profiles, insulin levels, hemoglobin A1c, fasting glucose, and high-sensitivity C-reactive protein (hsCRP).[13]

Ketonuria is an easily measured and relatively cost-effective indicator of ketosis that is often used as a common measure of adherence to the ketogenic diet.[14] Urinary ketone levels correlate well with quantitative serum ketone levels,[15] and urinary ketone testing is an

essential tool for monitoring patients, especially type 1 diabetic patients on insulin.[16] Recently, fasting ketonuria has been associated with decreased prevalence of obesity and metabolic syndrome[17] and with a lower risk of incident diabetes,[18] all of which may have a preventive effect against CVD. However, the clinical significance of fasting ketonuria in assessing coronary atherosclerosis remains unknown. CAC scores (CACS) obtained through computed tomography (CT) are useful for identifying subclinical coronary atherosclerosis[19] and for refining the estimated risk of prospective cardiovascular events.[20, 21] Therefore, our aim was to analyse the association between fasting ketonuria and subclinical coronary atherosclerosis assessed through CACS in a large sample of nondiabetic healthy adults who underwent routine health screening examinations. In this study, we investigated the association between fasting ketonuria and CAC using a cross sectional analysis, and the association between fasting ketonuria and CAC progression in a longitudinal analysis.

2. Patients and Methods

2.1. Study subjects

Study subjects belonged to the Kangbuk Samsung Health Study, which comprises a cohort of Koreans aged ≥ 18 years who receive comprehensive health examinations at one of the Kangbuk Samsung Hospital Total Healthcare Centres in either Seoul or Suwon, South Korea, on an annual or biennial basis.[22, 23] We included a subsample of the subjects who underwent a cardiac CT, allowing for CACS calculations, during their health examinations between 2011 and 2019. If a participant visited more than once with CACS measures, the first visit was chosen for the cross-sectional study (N = 164,048).

A total of 19,702 subjects were excluded due to (*Figure 1*): missing data on urinary ketone or body mass index (BMI) (n = 4,183), diabetes (defined as having a fasting serum glucose ≥ 126 mg/dL [7.0 mmol/L], glycated hemoglobin ≥ 6.5% [48 mmol/mol], or self-reported

4

insulin or medication for diabetes) (n = 9,884), malignancy history (n = 4,653), and self-report of previous CVD history (n = 2,179). Since some subjects had more than one exclusion criterion, 144,346 non-diabetic participants without clinically evident CVD were included for the analysis. For the longitudinal analysis, we analysed a subset of study subjects who had a baseline and at least one follow-up cardiac CT (n = 40,695). This study complied with the Declaration of Helsinki, and was granted approval by the Institutional Review Board of the Kangbuk Samsung Hospital (IRB no. KBSMC 2021-01-039), which waived the need for informed consent because we analysed de-identified retrospective data obtained from routine health screenings.

2.2. Measurements

Data on physical measurements, and serum biochemical measurements were retrieved from the routine health screening programs. Information on the subjects' demographics, lifestyle behaviours, and medical history was obtained using standardized, self-report questionnaires.[22, 23] Current alcohol use was determined based on the frequency of alcohol consumption per week and the amount consumed per occasion. Physical activity levels were estimated using the Korean International Physical Activity Questionnaire-Short Form, which was previously validated.[24] Health-enhancing physical activity (HEPA) was assessed as physical activity that met the following criteria: (1) vigorous activity $\geq$ 3 days/week, for a total of $\geq$ 1,500 metabolic equivalent -min/week or (2) walking or performing moderate/vigorous intensity activities for 7 days, for a total of $\geq$ 3,000 metabolic equivalent -min/week.[24] Information on dietary patterns was collected through a 103-item food frequency questionnaire (FFQ). Participants were first asked whether their diet had changed markedly during the past year compared with a previously maintained diet; if they answered "No", they proceeded with the FFQ and if they answered "Yes," they were instructed to answer

according to their usual diet.

Nurses were trained to measure the blood pressure (BP), height, weight, and waist circumference of the study subjects. Hypertension was determined based on systolic BP ≥ 140 mmHg, diastolic BP ≥ 90 mmHg, or current use of any antihypertensive medication.

Blood and urine samples were obtained after a minimum of 10 hours of fasting. The blood sample measurements included those of total cholesterol, LDL-C, high-density lipoprotein cholesterol (HDL-C), triglycerides, glucose, and hsCRP. Insulin resistance was based on the homeostatic model assessment of insulin resistance (HOMA-IR): fasting serum insulin (mU/mL) × fasting serum glucose (mmol/L) / 22.5. For semi-quantitative measurement of urine ketone levels, urine dipstick was used (URiSCAN urine test strips; YD Diagnostics, Yongin-si, Republic of Korea). This urine dipstick-based ketonuria and serum β-hydroxybutyrate (βHB) levels had been compared previously, and serum βHB levels gradually increased with increasing ketonuria levels (p for trend <0.001).[18] The levels of urine ketone bodies were reported as absent, trace (50 mg/L [0.49 mmol/L]), 1+ (100 mg/L [0.98 mmol/L]), 2+ (500 mg/L [4.9 mmol/L]), and 3+ (1,000 mg/L [9.8 mmol/L]). For analysis of fasting ketonuria as the key exposure, results were categorized as 1) absent or trace, 2) 1+, and 3) ≥ 2+.

The abdominal ultrasound findings of fatty liver were interpreted by an experienced radiologist using standard criteria, which included diffuse increase in fine echoes of the liver relative to that observed in the spleen or kidney, deep beam attenuation, and brightening of the vasculature.[25]

2.3. CAC measurement

CAC was detected using a LightSpeed VCT XTe-64 slice multidetector computed tomography scanner (GE Healthcare, Tokyo, Japan) with the same standard scanning

protocol[22] of 400-ms rotation time, 2.5-mm thickness, 120-kV voltage, and 124-mAS (310 mA x 0.4 s) tube current under electrocardiogram-gated dose modulation in both the Seoul and Suwon centres. The calcium score was analysed using semi-automatic methodology with GE Smartscore software (GE Healthcare), and confirmed by experienced technicians and radiologists. CACS were calculated using the method proposed by Agatston et al.[26] The inter-observer and intra-observer reliabilities for CACS were excellent (intraclass correlation coefficient = 0.99), as previously reported.[22]

2.4. Statistical analysis

The study subjects' characteristics were examined through descriptive statistics, according to the key exposure ketonuria categories (0, 1, and ≥2). For the association between CACS and ketonuria categories, we performed logistic regression analyses to compute the odds ratios (ORs) with 95% confidence intervals (CIs) for prevalent CACS > 0. We also performed a sensitivity analysis with CACS ≥ 10 as the outcome. Additionally, we presumed that CACS were distributed log-normally, with left-censored data at 0 Agatston units (i.e., non-detectable scores). Hence, as secondary analyses evaluating the association between ketonuria categories and CACS as a continuous variable with left censoring, Tobit regression analysis was performed with natural log (CACS + 1) as the outcome, using Huber–White estimation of standard errors.[27, 28] We used Tobit models to compute the ratios and 95% CIs of CACS + 1, and compared ketonuria categories with the no ketonuria category.

We used two models for adjustment of covariates: Model 1 was adjusted for the subjects' age and sex; Model 2 was additionally adjusted for the centre (Seoul or Suwon), year of the health screening exam, smoking (never, past, current, or unknown), average alcohol consumption (0, <20, ≥20 g/day, or unknown), highest level of education achieved (≤high school graduate, college or university graduate, ≥graduate school, or unknown), total energy

intake (quintiles, or unknown), medication for hyperlipidaemia, history of hypertension, SBP, and LDL-C. Confounding variables were selected according to the following criteria: 1) causally associated with the outcome (CACS) and 2) non-causally or causally associated with the exposure (ketonuria) and 3) is not an intermediate variable in the pathway between the exposure (ketonuria) and outcome (CACS).[29]

Predefined subgroup analyses were conducted for age in years (<50 vs. ≥50), sex (male vs. female), current smoking (non-current smokers vs. current smokers), average alcohol consumption (<20 vs. ≥20 g/day), performance of HEPA (no vs. yes), BMI (<18 kg/m^2, 18.5–22.9 kg/m^2, 23–24.9 kg/m^2, 25–29.9 kg/m^2, and ≥30 kg/m^2, in accordance with Asian-specific BMI criteria[30]), glucose (<100 mg/dL vs. ≥100 mg/dL), HOMA-IR (<2.5 vs. ≥2.5), hsCRP (<1.0 vs. ≥1.0 mg/L), and fatty liver (no vs. yes). We examined the interactions between fasting ketonuria categories and each subgroup by performing likelihood ratio tests and comparing models with and without multiplicative interaction terms.

We also analysed the prospective association between fasting ketonuria and CAC progression. We performed linear mixed models with random intercept and random slopes[31] for estimating progression of CACS over time, with adjustment for potential confounders. We transformed CACS as natural log(CACS+1) for the outcome, because CACS were right-skewed. The annual CAC progression rates with 95% CI were calculated, with 2 models for adjustment of covariates: Model 1 was adjusted for age and sex, and Model 2 was further adjusted for centre, year of screening exam, smoking, alcohol intake, educational level, total energy intake, medication for hyperlipidaemia, history of hypertension, SBP, and LDL-C. We also performed a sensitivity analysis by setting a higher threshold for CAC and calculated the hazard ratios for incident CACS ≥10 among participants with CACS = 0 at baseline.

Statistical analyses were performed using STATA version 16.0 (StataCorp LP, College Station, TX, USA). Two-sided *p*-values < 0.05 were determined as significant.

3. Results

3.1. Characteristics of study participants

Table 1 shows the characteristics of the 144,346 participants without known CVD according to the fasting ketonuria category. The presence of fasting ketonuria was positively associated with HEPA and HDL-C, and was inversely associated with male sex, alcohol intake, current smoking, hypertension, obesity, BP, glucose, LDL-C, triglycerides, HOMA-IR, and total energy intake. Participants in the ketonuria category ≥2 showed lower total energy intake, and slightly lower carbohydrate and slightly higher fat intake than participants in the no ketonuria category. Although lipid-lowering agent use appeared to be higher in the no ketonuria category than categories 1 and ≥2, there was no difference after adjustment for age, sex, and BMI (*p* for trend = 0.333, **Supplementary Table 1**).

3.2. Association between fasting ketonuria and CAC

Table 2 shows the prevalence of CAC based on the fasting ketonuria category. Of the 144,346 participants, 17,762 (12.3%) had detectable CACS > 0. Overall, a higher fasting ketonuria level was associated with lower prevalence of coronary calcification than no ketonuria. The age- and sex-adjusted ORs (95% CIs) for prevalent CACS comparing fasting ketonuria categories 1 and ≥ 2 with no ketonuria (reference) were 0.91 (0.82–1.02) and 0.77 (0.67–0.88), respectively. After adjustment for age, sex, year and centre of screening examination, smoking, alcohol consumption, education, total energy intake, medication for hyperlipidaemia, history of hypertension, SBP, and LDL-C, the multivariable-adjusted ORs (95% CIs) for prevalent CACS comparing fasting ketonuria categories 1 and ≥ 2 to no ketonuria were 0.94 (0.84–1.06) and 0.82 (0.71–0.95), respectively. The observed association between ketonuria categories and CACS was similar when analyses were performed with

9

CACS ≥ 10 as the outcome, with a multivariable-adjusted OR (95% CI) of 0.74 (0.62–0.88) for ketonuria ≥ 2 vs. no ketonuria (**Supplementary Table 2**).

Similarly, in the Tobit regression analysis (**Table 3**), the multivariable-adjusted CACS ratios which compared fasting ketonuria categories 1 and ≥ 2 with the no ketonuria category were 0.91 (0.72–1.15) and 0.66 (0.49–0.88), respectively.

The associations observed above did not differ among clinically relevant subgroups in terms of age, sex, smoking, average alcohol consumption, performance of HEPA, BMI, glucose, HOMA-IR, hsCRP, and presence of fatty liver (**Supplementary Table 3**).

Finally, we evaluated prospective association between fasting ketonuria and progression of CACS over time among those with a follow-up CAC CT (n = 40,695). The median duration of follow-up was 4.0 years (interquartile range 2.6-5.7, maximum 8.7). The annual rates of CAC progression for no ketonuria, ketonuria category 1, and category ≥ 2 were 8.7%, 7.9%, and 6.2%, respectively (**Table 4**). The multivariable adjusted ratios (95% CI) of annual progression rates comparing fasting ketonuria categories 1 and ≥ 2 vs. no ketonuria were 0.994 (0.980–1.009) and 0.976 (0.965–0.995), respectively. For incident CACS ≥10 as the outcome, among participants with CACS = 0 at baseline, the multivariable-adjusted hazard ratios (95% CI) in ketonuria categories 1 and ≥2 vs. no ketonuria were 1.11 (0.83–1.49) and 0.80 (0.53–1.18), respectively (**Supplementary Table 4**).

4. Discussion

Our study is the first to show an inverse association between fasting ketonuria and CAC using a large sample of non-diabetic Korean adults. Adults with higher fasting ketonuria levels showed a lower prevalence of CAC compared to those without ketonuria. This association remained even after adjusting for predictors of coronary atherosclerosis (age, current smoking, alcohol consumption, hypertension, medication for hyperlipidaemia, SBP,

and LDL-C) and did not differ in the subgroup analyses, including in the presence or absence of fatty liver. Among participants with a follow-up CAC test, ketonuria of ≥ 2 was also associated with lower CAC progression over time.

In our study, subjects with fasting ketonuria showed a lower prevalence of hypertension and more favourable lipid profiles than those without ketonuria, which are possibly related to the healthier lifestyle behaviours observed in this group such as higher physical activity, lower alcohol consumption, and less smoking. Likewise, low-carbohydrate ketogenic diets are associated with weight loss, improved lipid profiles and glucose levels,[12, 32] as well as the improvement of non-alcoholic fatty liver disease.[33] Though this association is thought to be attributable to ketosis, it may also be related to reduced energy intake in participants consuming low-carbohydrate ketogenic diets.[34] In our study, participants with fasting ketonuria also had lower total energy intake than those without ketonuria. However, adjusting for lifestyle behaviours, total energy intake, hypertension, medication for hyperlipidaemia, SBP, and LDL-C did not completely attenuate the association between fasting ketonuria and CACS.

Nonetheless, clearly favourable metabolic profiles in individuals with fasting ketonuria may be accountable for the reduction in the prevalence of CAC and its progression. Previous studies have shown that sporadic or fasting ketonuria is associated with favourable metabolic profiles[17] and a lower risk of incident diabetes.[18] A previous study that confirmed the presence of nutritional ketosis through blood measurements found that inflammatory responses measured through hsCRP and white blood cell count, triglycerides, small dense LDL particles, and the 10-year risk of atherosclerotic CVD decreased with ketogenic diets.[35] However, studies analysing the effect of ketogenic diets on carotid atherosclerosis in diabetic patients showed that there were no differences between those with induced nutritional ketosis and those under usual care.[35, 36] Indeed, individuals with manifest diabetes or features of the

metabolic syndrome may be resistant to ketonuria or less likely to benefit from ketosis.[37] In previous studies of individuals either practicing Ramadan fasting[38] or following a ketogenic diet,[39] obese subjects did not show ketosis, while non-obese subjects showed elevated urine ketone levels. However, in our study the association between fasting ketonuria and CAC did not differ in subgroup analysis according to BMI, glucose, HOMA-IR, and presence of fatty liver. Interestingly, the inverse association between ketonuria and CAC was not observed in the underweight group (<18.5 kg/m^2); however, the inconclusive result for the underweight group can be explained by the small number included in this category, which may have been insufficient to establish a relationship and may have led to imprecise estimates.

Fasting ketonuria was present in 5.7% of our study population, showing that this condition is not an uncommon phenomenon in non-diabetic individuals. In previous studies including relatively healthy populations, fasting ketonuria was present in 2.2%[18] and 8.8%[17] of the study population. Non-diabetic individuals with fasting ketonuria may have a greater capacity for ketogenesis and oxidation of fat than those without fasting ketonuria under certain conditions such as fasting or low-carbohydrate ketogenic diets. The beneficial effects of ketogenic diets are attributed to the metabolic shift to ketones over glucose as an energy source,[13] and low-carbohydrate ketogenic diets have been explored for their associations with favourable metabolic profiles, weight loss, and reduced cardiovascular risk, but have not always shown consistent results.[34] The ability to regulate ketogenesis and an individual's metabolic or genetic background as well as the difference in the composition of ketogenic diets may underlie the discrepancies in previous studies.[40] The rate of hepatic ketogenesis is proportional to total fat oxidation,[9] possibly indicating that ketonuria might be reflective of high fat oxidation ability. The circulating ketone body levels vary across healthy adult populations even after controlling for age and fasting hours;[16] hence, future studies including markers of ketosis and genetic variability may help clarify the metabolic and cardiovascular

effects of ketosis. In addition, the 10 hours of fasting in our study participants may have been a relatively short time window for inducing ketosis, since most intermittent fasting regimens recommend longer periods of fasting, such as 16 hours of fasting between 8 hours of eating.[41, 42] Studies with longer periods of fasting may provide more insight into the association between fasting ketonuria and CAC.

The mechanism responsible for the association between fasting ketonuria and CAC in relatively healthy adults without diabetes is unclear. Ketone bodies have a pleiotropic effect as signaling molecules and epigenetic modifiers, as well as metabolic intermediates.[12] The ketone body β-hydroxybutyrate inhibits histone deacetylase activity, which is known to extend lifespan in model organisms.[11] While high ketone body levels in patients with diabetic ketoacidosis are detrimental to the vascular system, recent studies have shown that ketone bodies at low levels may have beneficial effects on the endothelium through modulation of the inflammatory status,[9] senescence, and metabolism of the endothelial cells.[12] Inflammation is a well-known atherogenic factor, and the anti-inflammatory effect of low ketone body concentrations[9, 12] may be beneficial for coronary atherosclerosis.[10] Nutritional ketosis through restriction of carbohydrate intake reduces insulin levels,[13] and insulin at low levels is beneficial for the vasculature through its association with nitric oxide production, vasodilation, decreased monocyte adhesion, and reduced inflammation and oxidative stress.[43] Nevertheless, further studies controlling for the method of inducing ketosis are warranted to explain the associations found in our study.

4.1. Clinical implications

Several methods such as low-carbohydrate ketogenic diets and intermittent fasting are available for inducing ketosis. Exercise may be recommended as an adjunct for improving coronary vascular health, as it facilitates β-oxidation and ketogenesis through depletion of

13

glycogen stores and by increasing the energy demand, and may enhance the signaling induced by ketosis.[10] Ketosis can also be induced by ingestion of exogenous ketones in the form of ketone salts or ketone esters, or by ingesting precursors such as medium-chain triglycerides or 1,3-butanediol.[7] Nevertheless, low-carbohydrate diets that are high in fat may exacerbate or induce the occurrence of hypercholesterolemia in patients with genetic predisposition to hypercholesterolemia, and precautionary measures such as monitoring of lipid levels and ketosis may be required when recommending ketogenic diets.[44] Although LDL-C appeared to decrease with increasing ketonuria category in our study participants, after adjustment for age, sex, and BMI, LDL-C was lowest in the no ketonuria category. Furthermore, it is unclear whether any predisposing factors for ketonuria played a role in our study population, as information on the reason for ketonuria, such as genetic predisposition, longer fasting duration, recent dietary characteristics, or adherence to intermittent fasting, was not available. Nonetheless, our results indicate that ketosis may have an underestimated impact on the primary prevention of coronary heart disease and progression of coronary atherosclerosis. Further studies are needed to confirm whether these methods for inducing ketosis are beneficial for coronary atherosclerosis.

4.2. Limitations

Despite the strengths of our study, including the large population of relatively healthy individuals without diabetes, laboratory measurements, and lifestyle factors, some limitations should be noted. First, semi-quantitative urine tests were used to assess fasting ketonuria as a surrogate marker of ketosis. That said, urinary ketone levels correlate well with serum ketone levels[15] and remain an essential tool for monitoring patients.[16] Second, because this was an observational study, the reasons underlying the differences in fasting ketonuria between the study subjects remain unclear. Future studies controlling for methods that induce ketosis may

14

help clarify the association between ketosis and coronary atherosclerosis. Third, information on fasting time, recent dietary habits and intermittent fasting, which may affect ketonuria levels, was not available. Dietary information was collected through self-administered FFQs, which reflect usual food intake throughout the previous year, and may not reflect recent diet compositions.[45] The FFQ is also limited in assessing macronutrient composition and may underestimate fat and cholesterol intake compared with dietary records, because it does not include seasonings and oils, which are used in pre-seasoned dishes typical in South Korean diets.[45] Fourth, information on ketonuria was collected once at baseline; thus, the changing status of ketonuria was not incorporated into the analysis. Finally, our study included relatively healthy, young, middle-aged Koreans, thus limiting the generalizability of our results to populations of other ethnicities or with other comorbidities.

4.3. Conclusions

Our study showed that fasting ketonuria, especially at higher levels, was associated with a decreased CAC prevalence and lower CAC progression in non-diabetic individuals. Ketosis may have a beneficial effect on coronary atherosclerosis, but further studies are warranted to confirm this association.

CRediT authorship contribution statement

In Young Cho: Writing - original draft, Writing - review & editing

Yoosoo Chang: Conceptualization, Methodology, Data curation, Writing - original draft, Writing - review & editing

Eunju Sung: Writing - review & editing

Yejin Kim: Writing - review & editing

Jae-Heon Kang: Supervision, Project administration

Hocheol Shin: Supervision, Project administration

Sarah H. Wild: Writing - review & editing

Christopher D. Byrne: Writing - review & editing

Seungho Ryu: Conceptualization, Methodology, Data curation, Formal analysis, Writing - review & editing

REFERENCES

[1]   Roth, GA, Mensah, GA, Johnson, CO, et al., Global Burden of Cardiovascular Diseases and Risk Factors, 1990-2019: Update From the GBD 2019 Study, J Am Coll Cardiol, 2020;76:2982-3021.

[2]   Timmis, A, Townsend, N, Gale, CP, et al., European Society of Cardiology: Cardiovascular Disease Statistics 2019, Eur Heart J, 2020;41:12-85.

[3]   Sampson, UK, Fazio, S and Linton, MF, Residual cardiovascular risk despite optimal LDL cholesterol reduction with statins: the evidence, etiology, and therapeutic challenges, Curr Atheroscler Rep, 2012;14:1-10.

[4]   Mach, F, Baigent, C, Catapano, AL, et al., 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk, Eur Heart J, 2020;41:111-188.

[5]   Wong, ND, Zhao, Y, Quek, RGW, et al., Residual atherosclerotic cardiovascular disease risk in statin-treated adults: The Multi-Ethnic Study of Atherosclerosis, J Clin Lipidol, 2017;11:1223-1233.

[6]   Khot, UN, Khot, MB, Bajzer, CT, et al., Prevalence of conventional risk factors in patients with coronary heart disease, JAMA, 2003;290:898-904.

[7]   Yurista, SR, Chong, CR, Badimon, JJ, et al., Therapeutic Potential of Ketone Bodies for Patients With Cardiovascular Disease: JACC Focus Seminar, J Am Coll Cardiol, 2021.

[8]   Cotter, DG, Schugar, RC and Crawford, PA, Ketone body metabolism and cardiovascular disease, Am J Physiol Heart Circ Physiol, 2013;304:H1060-1076.

[9]   Puchalska, P and Crawford, PA, Multi-dimensional Roles of Ketone Bodies in Fuel Metabolism, Signaling, and Therapeutics, Cell Metab, 2017;25:262-284.

[10]   Miller, VJ, Villamena, FA and Volek, JS, Nutritional Ketosis and Mitohormesis: Potential Implications for Mitochondrial Function and Human Health, J Nutr Metab, 2018;2018:5157645.

[11]   Newman, JC and Verdin, E, Ketone bodies as signaling metabolites, Trends Endocrinol Metab, 2014;25:42-52.

[12]   Nasser, S, Vialichka, V, Biesiekierska, M, et al., Effects of ketogenic diet and ketone bodies on the cardiovascular system: Concentration matters, World J Diabetes, 2020;11:584-595.

[13]   Gershuni, VM, Yan, SL and Medici, V, Nutritional Ketosis for Weight Management and Reversal of Metabolic Syndrome, Curr Nutr Rep, 2018;7:97-106.

[14]   Hartman, AL and Vining, EP, Clinical aspects of the ketogenic diet, Epilepsia, 2007;48:31-42.

[15]   Coleman, MD and Nickols-Richardson, SM, Urinary ketones reflect serum ketone concentration but do not relate to weight loss in overweight premenopausal women following a low-carbohydrate/high-protein diet, J Am Diet Assoc, 2005;105:608-611.

[16]   Laffel, L, Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes, Diabetes Metab Res Rev, 1999;15:412-426.

[17]   Joo, NS, Lee, DJ, Kim, KM, et al., Ketonuria after fasting may be related to the metabolic superiority, J Korean Med Sci, 2010;25:1771-1776.

[18]   Kim, G, Lee, SG, Lee, BW, et al., Spontaneous ketonuria and risk of incident diabetes: a 12 year prospective study, Diabetologia, 2019;62:779-788.

[19]   Budoff, MJ, Achenbach, S, Blumenthal, RS, et al., Assessment of coronary artery disease by cardiac computed tomography: a scientific statement from the American Heart Association Committee on Cardiovascular Imaging and Intervention, Council on Cardiovascular Radiology and Intervention, and Committee on Cardiac Imaging, Council on Clinical Cardiology, Circulation, 2006;114:1761-1791.

[20]   Pletcher, MJ, Tice, JA, Pignone, M, et al., Using the Coronary Artery Calcium Score to Predict Coronary Heart Disease Events: A Systematic Review and Meta-analysis, Archives of Internal Medicine, 2004;164:1285-1292.

[21]   Budoff, MJ, Young, R, Burke, G, et al., Ten-year association of coronary artery calcium with atherosclerotic cardiovascular disease (ASCVD) events: the multi-ethnic study of atherosclerosis (MESA), Eur Heart J, 2018;39:2401-2408.

[22]   Chang, Y, Kim, BK, Yun, KE, et al., Metabolically-healthy obesity and coronary artery calcification, J Am Coll Cardiol, 2014;63:2679-2686.

[23]   Chang, Y, Ryu, S, Sung, KC, et al., Alcoholic and non-alcoholic fatty liver disease and associations with coronary artery calcification: evidence from the Kangbuk Samsung Health Study, Gut, 2019;68:1667-1675.

[24]   Craig, CL, Marshall, AL, Sjostrom, M, et al., International physical activity questionnaire: 12-country reliability and validity, Med Sci Sports Exerc, 2003;35:1381-1395.

[25]   Mathiesen, UL, Franzen, LE, Aselius, H, et al., Increased liver echogenicity at ultrasound examination reflects degree of steatosis but not of fibrosis in asymptomatic patients with mild/moderate abnormalities of liver transaminases, Dig Liver Dis, 2002;34:516-522.

[26]   Agatston, AS, Janowitz, WR, Hildner, FJ, et al., Quantification of coronary artery calcium using ultrafast computed tomography, J Am Coll Cardiol, 1990;15:827-832.

[27]   Reilly, MP, Wolfe, ML, Localio, AR, et al., Coronary artery calcification and cardiovascular risk factors: impact of the analytic approach, Atherosclerosis, 2004;173:69-78.

[28]   Qasim, A, Mehta, NN, Tadesse, MG, et al., Adipokines, insulin resistance, and coronary artery calcification, J Am Coll Cardiol, 2008;52:231-236.

[29]   Szklo, M and Nieto, J, Epidemiology: Beyond the Basics, Jones & Bartlett Learning, 2007.

[30]   World Health Organization and Regional Office for the Western Pacific, The Asia-Pacific perspective: redefining obesity and its treatment, Sydney, Health Communications Australia, 2000.

[31]   Gassett, AJ, Sheppard, L, McClelland, RL, et al., Risk Factors for Long-Term Coronary Artery Calcium Progression in the Multi-Ethnic Study of Atherosclerosis, J Am Heart Assoc,

2015;4:e001726.

[32]   Yancy, WS, Jr., Olsen, MK, Guyton, JR, et al., A low-carbohydrate, ketogenic diet versus a low-fat diet to treat obesity and hyperlipidemia: a randomized, controlled trial, Ann Intern Med, 2004;140:769-777.

[33]   Tendler, D, Lin, S, Yancy, WS, Jr., et al., The effect of a low-carbohydrate, ketogenic diet on nonalcoholic fatty liver disease: a pilot study, Dig Dis Sci, 2007;52:589-593.

[34]   Kosinski, C and Jornayvaz, FR, Effects of Ketogenic Diets on Cardiovascular Risk Factors: Evidence from Animal and Human Studies, Nutrients, 2017;9.

[35]   Bhanpuri, NH, Hallberg, SJ, Williams, PT, et al., Cardiovascular disease risk factor responses to a type 2 diabetes care model including nutritional ketosis induced by sustained carbohydrate restriction at 1 year: an open label, non-randomized, controlled study, Cardiovasc Diabetol, 2018;17:56.

[36]   Athinarayanan, SJ, Hallberg, SJ, McKenzie, AL, et al., Impact of a 2-year trial of nutritional ketosis on indices of cardiovascular disease risk in patients with type 2 diabetes, Cardiovasc Diabetol, 2020;19:208.

[37]   Kim, BR, Seo, JW, Kim, SM, et al., The Presence of Urinary Ketones according to Metabolic Status and Obesity, Journal of Korean medical science, 2020;35:e273-e273.

[38]   Mohammadiha, H, Resistance to ketonuria and ketosis in obese subjects, Am J Clin Nutr, 1974;27:1212-1213.

[39]   Kekwick, A, Pawan, GL and Chalmers, TM, Resistance to ketosis in obese subjects, Lancet, 1959;2:1157-1159.

[40]   Caprio, M, Infante, M, Moriconi, E, et al., Very-low-calorie ketogenic diet (VLCKD) in the management of metabolic diseases: systematic review and consensus statement from the Italian Society of Endocrinology (SIE), J Endocrinol Invest, 2019;42:1365-1386.

[41]   Anton, SD, Moehl, K, Donahoo, WT, et al., Flipping the Metabolic Switch: Understanding and Applying the Health Benefits of Fasting, Obesity (Silver Spring, Md.),

2018;26:254-268.

[42]   Mattson, MP, Longo, VD and Harvie, M, Impact of intermittent fasting on health and disease processes, Ageing Res Rev, 2017;39:46-58.

[43]   O'Neill, B and Raggi, P, The ketogenic diet: Pros and cons, Atherosclerosis, 2020;292:119-126.

[44]   Goldberg, IJ, Ibrahim, N, Bredefeld, C, et al., Ketogenic diets, not for everyone, J Clin Lipidol, 2021;15:61-67.

[45]   Ahn, Y, Kwon, E, Shim, JE, et al., Validation and reproducibility of food frequency questionnaire for Korean genome epidemiologic study, Eur J Clin Nutr, 2007;61:1435-1441.

Graphical Abstract

144,346 non-diabetic participants without a history of cardiovascular disease			
Ketonuria status	**No ketonuria**	**Ketonuria 1+**	**Ketonuria ≥ 2+**
Prevalent CAC — aOR (95%CIs)	1 (reference)	0.94 (0.84-1.06)	0.82 (0.71-0.95)
Prevalent CAC score — CAC score ratios (95%CIs)	1 (reference)	0.91 (0.72-1.15)	0.66 (0.49-0.88)

40,695 participants with at least one follow-up CAC CT			
Median follow-up of 4.0 years			
CAC progression over time — Ratios of progression rate (95%CIs)	1 (reference)	0.994 (0.980–1.009)	0.976 (0.965–0.995)

22

Figure legends

Figure 1 Study participants selection process.

Participants who underwent a cardiac tomography examination as part of a comprehensive health examination at Kangbuk Samsung Hospital in South Korea from 2011 to 2019 (n = 164,048)

Participants excluded (some met multiple exclusion criteria) (n = 19,702)
- Presence of diabetes defined as fasting serum glucose ≥126 mg/dL (7.0 mmol/L), glycated hemoglobin ≥6.5% (48 mmol/mol), or self-reported use of insulin or antidiabetic medications (n = 9,884)
- History of malignancy (n = 4,653)
- History of cardiovascular disease (n = 2,179)
- Missing data on urinary ketone or body mass index (n = 4,183)

Participants included in the cross-sectional analysis (n = 144,346)

Participants excluded due to no follow-up CAC CT (n = 103,651)

Participants included in the analysis on CAC progression (n = 40,695)

Table 1 Baseline characteristics by fasting ketonuria category

Characteristics	Overall	Fasting ketonuria category			*p* for trend
		0	1	≥2	
Number of participants	144,346	136,159	4,486	3,701	
Age (years)	41.3 (8.4)	41.3 (8.4)	40.5 (8.9)	39.4 (8.4)	<0.001
Men (%)	75.8	76.6	65.1	61.2	<0.001
Seoul centre (%)	48.8	48.6	56.3	49.0	<0.001
Alcohol intake[a] (%)	44.4	44.8	39.3	36.5	<0.001
Current smoker (%)	22.1	22.5	16.8	13.0	<0.001
HEPA (%)	19.0	16.4	16.2	18.5	<0.001
High education level[b] (%)	83.4	83.4	83.9	83.9	0.231
Hypertension (%)	14.7	14.9	12.3	10.5	<0.001
Family history of CVD (%)	12.0	12.0	12.9	12.0	0.357
Lipid lowering agent (%)	3.5	3.6	2.6	2.8	<0.001
Fatty liver (%)	38.9	39.7	27.4	24.8	<0.001
BMI category					

<18.5 kg/m^2	2.3	2.2	3.7	5.1	<0.001
18.5-22.9 kg/m^2	32.8	32.2	42.8	45.6	<0.001
23-24.9 kg/m^2	26.1	26.2	24.3	22.8	<0.001
25-29.9 kg/m^2	33.7	34.2	25.2	22.7	<0.001
≥30 kg/m^2	5.1	5.2	3.9	3.9	<0.001
BMI (kg/m^2)	24.3 (3.3)	24.4 (3.3)	23.5 (3.3)	23.3 (3.4)	<0.001
SBP (mmHg)	112.4 (12.4)	112.5 (12.4)	111 (12.6)	110 (12.7)	<0.001
DBP (mmHg)	72.7 (9.8)	72.8 (9.8)	71 (9.6)	69.8 (9.5)	<0.001
Glucose (mg/dL)	95.6 (8.4)	95.9 (8.2)	90.9 (8.5)	87.1 (10.2)	<0.001
Total cholesterol (mg/dL)	199 (34.1)	199.1 (34)	198.8 (35.3)	197 (36)	0.001
LDL-C (mg/dL)	130.1 (32.1)	130.2 (31.9)	129.2 (34.2)	127.7 (34.6)	<0.001
HDL-C (mg/dL)	64 (29.4)	63.8 (29.6)	67.9 (27.2)	68.4 (25.9)	<0.001
Triglycerides (mg/dL)	109.0 (76.0–158.0)	112.0 (79.0–162.0)	74.0 (56.0–104.0)	66.0 (52.0–89.0)	<0.001
hsCRP (mg/L)	0.5 (0.3–1.0)	0.5 (0.3–1.0)	0.5 (0.3–1.1)	0.5 (0.3–1.2)	0.003
HOMA-IR	1.45 (0.97–2.15)	1.49 (1.00–2.19)	0.93 (0.60–1.44)	0.78 (0.46–1.24)	<0.001

Total energy intake (kcal/d)[c]	1430 (1070–1826)	1432 (1074–1826)	1405 (1028–1825)	1363 (981–1808)	<0.001
Carbohydrate proportion (%)	68.1 (61.3–73.9)	68.2 (61.4–74.0)	67.2 (60.2–73.0)	67.1 (59.4–72.9)	<0.001
Fat proportion (%)	18.2 (13.8–23.3)	18.1 (13.7–23.3)	18.8 (14.3–24.1)	19.0 (14.7–24.9)	<0.001
Protein proportion (%)	13.6 (12.0–15.6)	13.6 (12.0–15.6)	13.8 (12.0–15.9)	13.8 (12.1–15.9)	<0.001
Carbohydrate <50 g/day (%)	5.5	5.4	6.5	7.2	0.016
CACS category					
0 AU	87.7	87.5	89.9	92.8	<0.001
1-100 AU	10.2	10.4	8.1	6.1	<0.001
>100 AU	2.1	2.1	2.0	1.2	<0.001

Data are presented as mean (standard deviation), median (interquartile range), or percentages.

Abbreviations: HEPA, health-enhancing physical activity; CVD, cardiovascular disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein cholesterol; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; hsCRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; CACS, coronary artery calcium score; AU, Agatston unit.

[a] ≥20 g of ethanol per day.

[b] ≥College graduate.

[c] Among 87,482 participants with plausible estimated energy intake (within three standard deviations of the log-transformed mean energy intake).

Table 2 Odds ratios[a] (95% CIs) for coronary artery calcification by fasting ketonuria category in 144,346 health checkup examinees at Kangbuk Samsung Hospital between 2011 and 2019

	Fasting ketonuria category			p for trend
	0	1	≥2	
Number	136,159	4,486	3,701	
Cases (N) of CACS >0	17,041	453	268	
Age- and sex-adjusted prevalence[b] (95% CIs)	12.4 (12.2–12.5)	11.6 (10.7–12.5)	10.2 (9.1–11.2)	
Adjusted odds ratios [a] (95% CIs)				
Age- and sex-adjusted	1.00 (reference)	0.91 (0.82–1.02)	0.77 (0.67–0.88)	<0.001
Multivariable-adjusted	1.00 (reference)	0.94 (0.84–1.06)	0.82 (0.71–0.95)	0.004

Abbreviations: CACS, coronary artery calcium scores; CI, confidence interval.

[a] Estimated from binomial logistic regression models used with prevalent CACS >0 as the outcome

The multivariable model was adjusted for age, sex, centre, year of screening exam, smoking status, alcohol intake, educational level, total energy intake, medication for hyperlipidaemia, history of hypertension, SBP, and LDL-C.

[b] per 100 persons.

Table 3 Coronary artery calcium score (CACS) ratios[a] (95% CI) by fasting ketonuria category of 144,346 health checkup examinees at Kangbuk Samsung Hospital between 2011 and 2019

	Fasting ketonuria category			*p* for trend
	0	**1**	**≥2**	
Number	22,052	22,385	21,595	
Adjusted CACS ratios[a] (95% CIs)				
Age- and sex-adjusted	1.00 (reference)	0.85 (0.66–1.08)	0.56 (0.42–0.76)	<0.001
Multivariable-adjusted	1.00 (reference)	0.91 (0.72–1.15)	0.66 (0.49–0.88)	0.005

Abbreviations: CI, confidence interval.

[a] Estimated from robust Tobit regression models performed with natural log(CACS+1) as the outcome.

The multivariable model was adjusted for age, sex, centre, year of screening exam, smoking status, alcohol intake, educational level, total energy intake, medication for hyperlipidaemia, history of hypertension, SBP, and LDL-C.

Table 4 Ratio (95% CI) of annual progression rates of coronary artery calcium score by fasting ketonuria category at baseline (n = 40,695)

Ratio of annual progression rates[a]	Fasting ketonuria category		
	0	1	≥2
Number	38,558	1,201	936
Annual rate of CAC progression	1.087 (1.085–1.090)	1.079 (1.066–1.092)	1.062 (1.049–1.076)
Ratio of annual progression rates[a]			
Model 1	1.0 (reference)	0.992 (0.980–1.004)	0.976 (0.964–0.988)
Model 2	1.0 (reference)	0.994 (0.980–1.009)	0.976 (0.965–0.995)

Model 1 was adjusted for age and sex. Model 2 was adjusted for age, sex, centre, year of screening exam, smoking status, alcohol intake, educational level, total energy intake, medication for hyperlipidaemia, history of hypertension, SBP, and LDL-C.

Abbreviations: CAC, coronary artery calcification; CI, confidence interval.

[a] Estimated from linear mixed models with random intercept and random slopes used with natural log(CAC+1) as the outcome and inverse probability weighting.

CRediT authorship contribution statement

In Young Cho: Writing - original draft, Writing - review & editing

Yoosoo Chang: Conceptualization, Methodology, Data curation, Writing - original draft,
Writing - review & editing

Eunju Sung: Writing - review & editing

Yejin Kim: Writing - review & editing

Jae-Heon Kang: Supervision, Project administration

Hocheol Shin: Supervision, Project administration

Sarah H. Wild: Writing - review & editing

Christopher D. Byrne: Writing - review & editing

Seungho Ryu: Conceptualization, Methodology, Data curation, Formal analysis, Writing -
review & editing

- The article is not under consideration for publication elsewhere.
- Publication of the article is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out.
- If the article is accepted, it will not be published elsewhere by the authors, including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

<table>
<tr><td>

</td></tr>
</table>

Highlights

- The association between ketone bodies and coronary artery calcium remains unknown.

- Fasting ketonuria was inversely associated with prevalent coronary calcification.

- Fasting ketonuria was associated with lower progression of coronary calcification.

Cover letter

February 25, 2022

Arnold von Eckardstein

Editor-in-Chief

Atherosclerosis

Dear Dr. Eckardstein,

Thank you for the careful review of our manuscript titled "Fasting ketonuria is inversely associated with coronary artery calcification in non-diabetic individuals." We have revised the manuscript according to the reviewers' comments, and we believe that the manuscript has been improved substantially in this process. We are pleased to resubmit the revised version for publication in *Atherosclerosis*. We look forward to your final decision regarding our manuscript.

This paper is an original article that has not been published and has not been submitted for publication elsewhere. There are no conflicts of interest to declare, and all authors have participated in this work and have read and approved this manuscript. My colleagues and I greatly appreciate your consideration of our manuscript for publication in *Atherosclerosis*. Please contact us if you have any questions related to this study.

Sincerely,

Seungho Ryu and Yoosoo Chang

Seungho Ryu, Department of Occupational and Environmental Medicine, Kangbuk Samsung

Hospital, Sungkyunkwan University School of Medicine, Samsung Main Building B2, 250 Taepyung-ro 2ga, Jung-gu, Seoul 04514, South Korea

Tel: +82-2-2001-5137; Fax: +82-2-757-0436; Email: sh703.yoo@gmail.com

Yoosoo Chang, Department of Occupational and Environmental Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Samsung Main Building B2, 250 Taepyung-ro 2ga, Jung-gu, Seoul 04514, South Korea

Tel: +82-2-2001-5137; Fax: +82-2-757-0436; Email: yoosoo.chang@gmail.com

Reviewers' comments:

Reviewer #1: Summary:

Cho et al. study the association of ketonuria with CAC in non-diabetic South-Korean individuals. This is a very large study including >140.000 persons aged about 40 years, three quarters being men. Ketonuria categories were 0, 1 and 2. CAC prevalence, i.e. any CAC > 0, and CAC progression were target variables. A large set of CVD risk factors were assessed. Multivariable-adjusted ORs for prevalent CAC in ketonuria categories 1 and ≥ 2 vs no ketonuria were 0.94 and 0.82, respectively (both significant). Also, the authors observed an association with CAC progression.

Unfortunately, I could not find supplement information!

<u>Response</u>: Thank you for your appreciation of our study. Based on our understanding, the supplementary material was uploaded with the original submission; however, apparently there was a technical error. We apologize for any inconvenience this may have caused. We have uploaded the revised supplementary tables, along with the revised manuscript and main tables for your review.

Specific comments:

1) Methods: The authors describe 144.000 persons in 8 years. This is about 60-70 persons each day. How many sites were involved? How many scanners were used? Did all sites use the same scanning protocol and the same methodology in quantifying CVD risk factors?

<u>Response</u>: Thank you for your comment. The Kangbuk Samsung Hospital Total Healthcare Center (where study participants received health examinations) is composed of two centers, one in Seoul and another in Suwon, South Korea. Each center used the exact same machine

during the study period, a Lightspeed VCT XTe-64 slice multi-detected computed

tomography scanner (GE Healthcare, Tokyo, Japan), and testing was performed using

standard scanning protocols. Due to the huge demand, each center is open from 7 am to 5 pm

on weekdays and from 7 am to noon on Saturdays. We have added further details regarding

coronary artery calcium score (CACS) measurements as follows (page 6, last paragraph –

page 7, 1st paragraph):

> "CAC was detected using a Lightspeed VCT XTe-64 slice multi-detected computed
>
> tomography scanner (GE Healthcare, Tokyo, Japan) with the same standard scanning
>
> protocol[22] of 400-ms rotation time, 2.5-mm thickness, 120-kV tube voltage, and 124-
>
> mAS (310 mA × 0.4 s) tube current under electrocardiogram-gated dose modulation
>
> in both the Seoul and Suwon centers. The calcium score was analysed using semi-
>
> automatic methodology with GE Smartscore software (GE Healthcare), and confirmed
>
> by experienced technicians and radiologists. CACS were calculated using the method
>
> proposed by Agatston et al.[26] The inter-observer and intra-observer reliabilities for
>
> CACS were excellent (intra-class correlation coefficient = 0.99), as previously
>
> reported.[22]"

2) Methods: please describe specifically how CAC scores were determined. Were semi-automated algorithms used? Was a technician or a physician involved?

<u>**Response**</u>: Thank you for your comment. We have added detailed information to the Methods

section (please see the response above).

3) There is an issue with CAC categories: very low CAC scores, i.e. between 1 and 10,

may be noise and not indicate subclinical atherosclerosis. This effect should be negligible

in very large cohorts as this one. Yet, it is conceivable that very slim persons have more ketonuria and less noise on a CT image resulting in a pseudo-association. Please comment.

<u>Response</u>: Thank you for your comment. Previous studies have reported an increased risk of mortality, even in individuals with low CACS of 1–10; thus we did not treat the low-level category as "noise" or possible misclassification (Blaha, M, et al., 2009; Budoff, Mj, et al., 2007). We performed additional analysis using a CACS $\geq$ 10 as the outcome, and the inverse association between ketonuria $\geq$ 2 and prevalent CAC (defined as a score $\geq$ 10) was consistently observed (Supplementary Table 2). Specifically, the multivariable adjusted odds ratio (95% CI) for CACS $\geq$ 10 comparing the ketonuria category of $\geq$ 2 with no ketonuria was 0.74 (0.62–0.88). We have added this finding to the Results section narrative (page 7, 2nd paragraph; page 9, last paragraph – page 10, 1st paragraph) as well as Supplementary Table 2 in the revised manuscript as follows:

"We also performed a sensitivity analysis with CACS $\geq$ 10 as the outcome."

"The observed association between ketonuria categories and CACS was similar when analyses were performed with CACS $\geq$ 10 as the outcome, with a multivariable-adjusted OR (95% CI) of 0.74 (0.62–0.88) for ketonuria $\geq$ 2 vs. no ketonuria (**Supplementary Table 2**)."

References

Blaha, M, Budoff, MJ, Shaw, LJ, et al., Absence of coronary artery calcification and all-cause mortality, JACC Cardiovasc Imaging, 2009;2:692-700.

Budoff, MJ, Shaw, LJ, Liu, ST, et al., Long-term prognosis associated with coronary

calcification: observations from a registry of 25,253 patients, J Am Coll Cardiol, 2007;49:1860-1870.

4) It would be helpful to demonstrate a subgroup analysis for the association of CAC and ketonuria within narrow BMI-categories.

Response: Thank you for your comment. According to your suggestion, we have added the subgroup analysis for the association between CACS and ketonuria within the following categories based on Asian-specific body mass index (BMI) criteria (World Health Organization, 2000): underweight, BMI < 18.5 kg/m^2; normal weight, BMI of 18.5–22.9 kg/m^2; overweight, BMI of 23–24.9 kg/m^2; obese I, BMI of 25–29.9 kg/m^2; and obese II, BMI ≥ 30 kg/m^2 (Supplementary Table 3). The inverse association between ketonuria ≥ 2 and CAC was similarly observed, except in the underweight group, although there was no significant interaction among BMI categories. The underweight group (< 18.5 kg/m^2) might have been composed of unhealthy individuals with higher levels of ketone bodies, lower muscle mass and total calorie intake, and relatively higher CACS. However, the inconclusive results for the underweight group can be explained by the small number of patients in this category, which may have been insufficient to identify a relationship and possibly led to imprecise estimates. We described this analysis in the Methods (page 8, 2nd paragraph), Results (page 10, 3rd paragraph), and Discussion sections (page 12, 1st paragraph) of the revised manuscript as follows:

> "Predefined subgroup analyses were conducted for (…) BMI (<18 kg/m^2, 18.5–22.9 kg/m^2, 23–24.9 kg/m^2, 25–29.9 kg/m^2, and ≥30 kg/m^2, in accordance with Asian-specific BMI criteria)."

"The associations observed above did not differ among clinically relevant subgroups in terms of age, sex, smoking, average alcohol consumption, performance of HEPA, BMI, glucose, HOMA-IR, hsCRP, and presence of fatty liver (**Supplementary Table 3**)."

"Interestingly, the inverse association between ketonuria and CAC was not observed in the underweight group (<18.5 kg/m^2); however, the inconclusive result for the underweight group can be explained by the small number included in this category, which may have been insufficient to establish a relationship and may have led to imprecise estimates."

Reference

World Health Organization and Regional Office for the Western Pacific, The Asia-Pacific perspective: redefining obesity and its treatment, Sydney, Health Communications Australia, 2000.

5) Regarding progression: how did the authors handle the (frequent) scenario of CAC>0 in the first exam, and CAC=0 in the second exam? Was this considered regression or "no progression"? Which CAC increase was considered progression?
What happens to data if the threshold of "any CAC" is set to an Agatston score = 10? Obviously, the CAC prevalence decreases, but the data should be more robust against noise (see above). Please comment.
<u>Response</u>: Thank you for your comment. Regarding progression, we performed linear mixed models with random intercept and random slopes with natural log(CACS + 1) as the outcome for estimating progression of CACS over time. The beta coefficients in the models can be negative or positive. We did not treat the case where the CACS decreased over time as no

progression. The scenario of CAC >0 in the first exam and CAC =0 in the subsequent exam may have been attributed to measurement errors which might have attenuated true association between exposure and outcomes. And as suggested, we performed additional analyses by calculating the hazard ratios (HRs) for incident CACS ≥ 10 among participants with CACS = 0 at baseline (n = 33,774) (Supplementary Table 4). We found that the ketonuria category of ≥ 2 vs. no ketonuria tended to show lower HRs for decreased risk of incident CACS ≥ 10; the multivariable adjusted HR (95% confidence interval [CI]) for incident CACS ≥ 10 for ketonuria categories 1 and ≥ 2 vs. no ketonuria were 1.11 (0.83–1.49) and 0.80 (0.53–1.18), respectively. Although there was a similar direction of the association between ketonuria and incident CAC, the analysis for incident CACS ≥ 10 may have lacked statistical power to show a significant association between ketonuria and CAC. We have added a description of these analyses to the Methods (page 8, 3rd paragraph) and Results sections (page 10, 4th paragraph) of the revised manuscript as follows

"We also performed a sensitivity analysis by setting a higher threshold for CAC and calculated the hazard ratios for incident CACS ≥10 among participants with CACS = 0 at baseline."

"For incident CACS ≥10 as the outcome, among participants with CACS = 0 at baseline, the multivariable-adjusted hazard ratios (95% CI) in ketonuria categories 1 and ≥2 vs. no ketonuria were 1.11 (0.83–1.49) and 0.80 (0.53–1.18), respectively (**Supplementary Table 4**)."

Reviewer #2: In a large cohort of 144.346 young healthy Koreans (age, 41 yrs), the association of CACS 0, > 1 and > 100 AU at baseline and CACS progression with ketosis (category 0, 1, 2) was investigated. An inverse relationship was shown for both (higher ketosis => higher prev. of CACS 0 and less CACS progression). Diabetics were excluded. Data were collected from 2 centers.

The study addresses a timely and novel topic, which has not been reported yet. Overall, the impact of ketosis on coronary atherosclerosis not well investigated. Ketosis may be a very effective and non-invasive tool for primary prevention of CHD.

With regards to the potential impact on clinical management (prevention of CHD) my recommendation is:

Accept with minor revisions (comments below).

<u>Response</u>: We thank the reviewer for the supportive comments. We have addressed these comments and suggestions accordingly, and our responses are provided below.

Major comments.

1) *the cohort is rather young (41y) and the majority (94.3%) had no Ketonuria (Category 0.). The majority (87.7%) had CACS 0. Please explain which testing (statistical methods) were performed to ensure that analysis was not underpowered?

<u>Response</u>: Thank you for your comment. Power analysis was performed using the "power twoproportions" command in Stata (StataCorp LP, College Station, TX, USA). The total number of subjects analysed was 144,346. Of the participants in the no ketonuria category, 12% had detectable CACS > 0. The reference group (no ketonuria category) was compared to 2 categories (ketonuria categories 1 and ≥ 2); therefore, the allocation ratio was set to 2. As shown in the table and figure below, our analysis was not likely to be underpowered (as the power was approximately 100%).
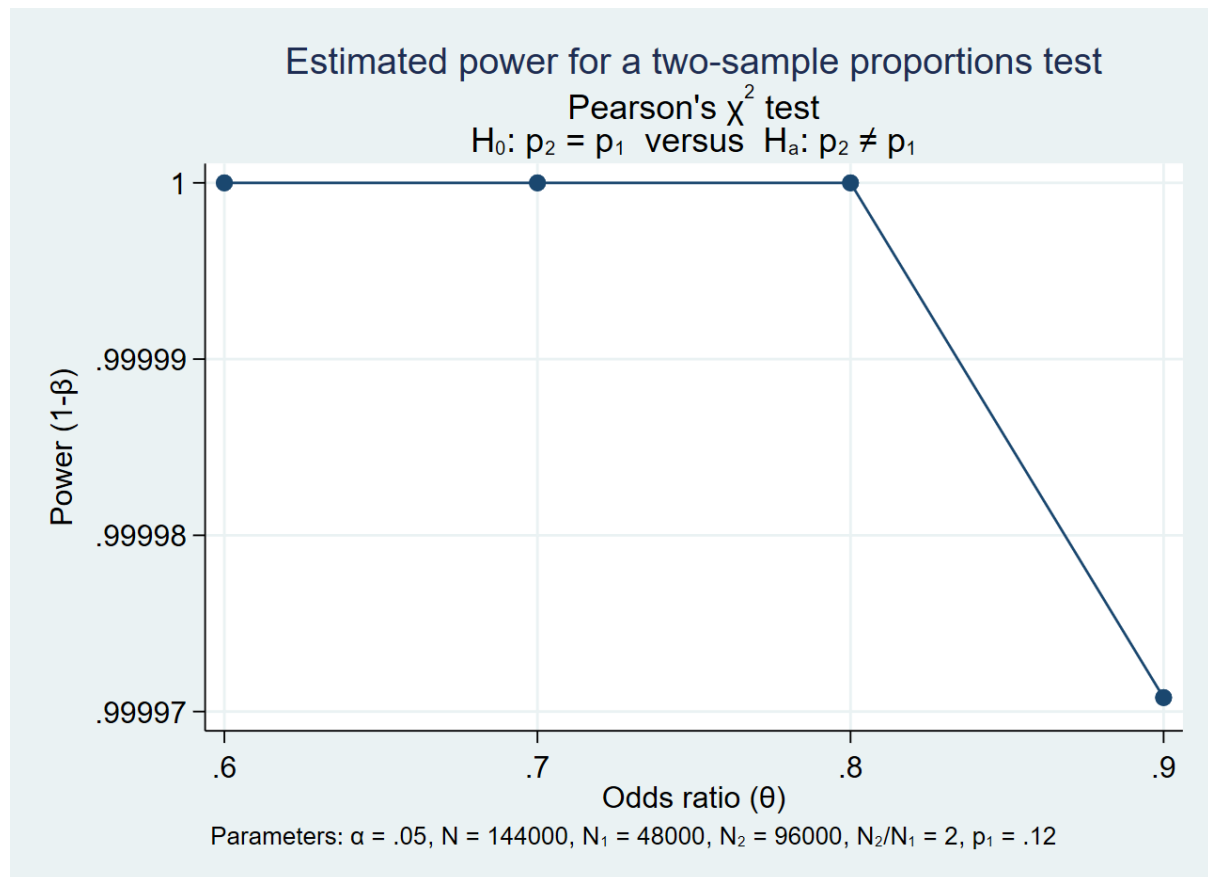
43

. power twoproportions 0.12, test(chi2) oratio(0.6 (0.1) 0.9) n(144000) nratio(2)
continuity

Estimated power for a two-sample proportions test
Pearson's chi-squared test
Ho: p2 = p1 versus Ha: p2 != p1

```
  +--------------------------------------------------------------------------+
  |    alpha    power        N        N1       N2   nratio   delta      p1        p2
oratio |
  |--------------------------------------------------------------------------|
  |     .05          1 1.4e+05    48000    96000
2      .6     .12  .07563       .6 |
  |     .05          1 1.4e+05    48000    96000
2      .7     .12  .08714       .7 |
  |     .05          1 1.4e+05    48000    96000
2      .8     .12  .09836       .8 |
  |     .05          1 1.4e+05    48000    96000
2      .9     .12   .1093       .9 |
  +--------------------------------------------------------------------------+
```


Estimated power for a two-sample proportions test
Pearson's χ^2 test
$H_0: p_2 = p_1$ versus $H_a: p_2 \neq p_1$

Parameters: α = .05, N = 144000, N_1 = 48000, N_2 = 96000, N_2/N_1 = 2, p_1 = .12

2) *Models were adjusted: 1) for age/gender and 2) multiple major CV risk factors, including lifestyle factors, and medication quite comprehensively (n=7). (alcohol consumption, …). The details are not shown. Overfitting may impact results. Please explain how overfitting was tested and ruled out? Please consult a statistican if uncertain.

<u>**Response**</u>: Thank you for your comment. We used multivariable adjusted models to adjust for potential confounders that might influence the association between ketonuria categories and CACS. In our study, we defined confounding variables using the following criteria: 1) causally associated with the outcome (CACS) or 2) non-causally or causally associated with the exposure (ketonuria) and 3) is not an intermediate variable in the causal pathway between the exposure (ketonuria) and outcome (CACS). We have described how we selected the covariates for adjustment in the Methods section (page 8, 1st paragraph) of the revised manuscript as follows:

> "Confounding variables were selected according to the following criteria: 1) causally associated with the outcome (CACS) or 2) non-causally or causally associated with the exposure (ketonuria) and 3) is not an intermediate variable in the pathway between the exposure (ketonuria) and outcome (CACS)."

As suggested, overfitting was tested using the "overfit" command in Stata (StataCorp LP), which calculates shrinkage statistics to measure the amount of overfitting generated by an estimated model as defined by Bilger and Manning (2015). The results were as follows:

Shrinkage statistics (expressed as percentages)

	Out-of-sample predictive bias	In-sample predictive bias	Overfitting
Estimate	2.77	2.61	0.16
Standard error	0.02	0.01	0.02

As shown in the above results, the amount of overfitting generated by the final model was only 0.16%.

References

Bilger M, Manning WG, Measuring overfitting in nonlinear models: a new method and an application to health expenditures, Health Economics, 2015,24(1):75-85.

Szklo M, Nieto J, Epidemiology: Beyond the Basics, Jones & Bartlett Learning, 2007.

3) Medication of statins was higher in those without ketosis- which is an inverse relationship. Because statins reduce progression of atherosclerosis - this rather enhances the study results.

<u>Response</u>: Thank you for your comment. As noted, the use of lipid-lowering agents, which we assumed consisted of mostly statins, appeared to be the highest in the no ketonuria category (3.6%) when compared with categories 1 and ≥ 2 (2.6% and 2.8%, respectively). However, these differences were marginal and partly attributed to age and sex differences. After adjustment for age, sex, and BMI, the lipid-lowering agent use did not differ among the ketonuria categories (p for trend = 0.333, Supplementary Table 1). We have added this point to the Results section (page 9, 1st paragraph) of the revised manuscript.

"Although lipid-lowering agent use appeared to be higher in the no ketonuria category

than categories 1 and ≥2, there was no difference after adjustment for age, sex, and BMI

(*p* for trend = 0.333, **Supplementary Table 1**)"

Strengths

4) *very large cohort

***novel topic with potential impact on clinical management and primary prevention of**

CHD guidelines, that needs more research.

***uniform ethic population- Koreans.**

Further comments:

introduction:

OK- well written

methods:

*** CACS CT scanning: correct.**

<u>Response</u>: Thank you for your appreciation of our study. We appreciate the supportive

comments.

5) *FFQ questionäre:

5-1) specific food products/plants have a higher potential of inducing ketosis than others

due to their composition (eg. MCT). Does the FFQ questionnaire used allow to stratify

the food into "pro-ketogenic" and "non-ketogenic?" or not?

Please give a bit more details on typical "korean" dietary patterns with regards to their

pro- ketogenic potential. Does the typical Korean diet include many MCT dense

ingredients? (or 1,3 butanediol)? would be interesting.

<u>Response</u>: Thank you for your comment. We agree that dietary information is a critical

component in evaluating the role of ketosis. Unfortunately, specifics about the recent dietary

patterns or changes of the study participants were not available in our study. However, we did have information on the participants' habitual diets collected via a 103-item self-administered food frequency questionnaire (FFQ) reflective of food intake over the past year, which was designed for use in South Korea. These data are presented in the revised Table 1.

In our additional analysis of the baseline macronutrient composition of the participants' diets, the overall proportions of carbohydrate, fat, and protein were 68.1%, 18.2%, and 13.6%, respectively, indicating a relatively high proportion of carbohydrate intake. These findings are in line with those of other studies in South Korea and are within the recommended baseline range at the time of the study (Kwon, YJ, et al., 2020; Paik, HY, 2008). In 2015, the Korean Nutrition Society lowered the maximum acceptable macronutrient distribution range (AMDR) for carbohydrates from 70% in 2010 to 65%, increased the maximum AMDR for fat from 25% to 30%, and maintained the AMDR for protein at 7–20% in adults aged > 19 years (Kwon, YJ, et al., 2020; Paik, HY, 2008). Asian countries are known for the cultural tendency toward carbohydrate-rich foods, such as rice and noodles, which constitute the basis of traditional diets making ketogenic diets difficult to maintain (Kossoff, EH and McGrogan, JR, 2005). In contrast, the dietary recommendations for Americans are carbohydrate, 45–65%; fat, 25–35%; and protein 10–30% of the total daily calories.

We observed that carbohydrate intake was relatively lower and that fat intake was slightly higher in the ketonuria group than in the non-ketonuria group; however, these differences were marginal. Low carbohydrate intake, defined as < 50 g/day, was also marginally higher in the ketonuria group ($\geq$ 2) than in the non-ketonuria group (7.2% and 5.4%, respectively). The FFQ evaluates average dietary habits within the past year (Ahn, Y, et al., 2007); therefore, it may not reflect the most recent dietary characteristics as nutrition-induced ketosis can temporarily occur on a ketogenic diet. The South Korean diet also typically includes pre-seasoned dishes with various kinds of seasonings, including oils;

however, seasonings and oils are not included in the FFQ. Therefore, nutrient intake estimates according to the FFQ, especially for fat and cholesterol, were reported as lower than those of the dietary records, which is the reference standard (Ahn, Y, et al., 2007). This may have influenced the accuracy of our dietary data. In addition, data on other eating patterns such as intermittent fasting that can affect ketosis were not available, which is a major limitation of our study. We have incorporated and further elaborated on this point in the Results (page 9, 1st paragraph) section, Table 1, and the Discussion section (page 14, last paragraph – page 15, 1st paragraph) of the revised manuscript as follows:

"Participants in the ketonuria category ≥2 showed lower total energy intake, and slightly lower carbohydrate and slightly higher fat intake than participants in the no ketonuria category."

"Third, information on fasting time, recent dietary habits, and intermittent fasting, which may affect ketonuria levels, was not available. Dietary information was collected through self-administered FFQs, which reflect usual food intake throughout the previous year, and may not reflect recent diet compositions.[45] The FFQ is also limited in assessing macronutrient composition and may underestimate fat and cholesterol intake compared with dietary records, because it does not include seasonings and oils, which are used in pre-seasoned dishes typical in South Korean diets."

References

Kossoff EH, McGrogan JR. Worldwide use of the ketogenic diet. Epilepsia. 2005;46(2):280-289.

Kwon, YJ, Lee, HS, Park, JY, et al., Associating Intake Proportion of Carbohydrate, Fat, and

Protein with All-Cause Mortality in Korean Adults, Nutrients, 2020;12.

Paik, HY, Dietary reference intakes for Koreans (KDRIs), Asia Pacific journal of clinical nutrition, 2008;17.

Ahn, Y, Kwon, E, Shim, JE, et al., Validation and reproducibility of food frequency questionnaire for Korean genome epidemiologic study, Eur J Clin Nutr, 2007;61:1435-1441.

5-2) The FFQ collected dietary patterns from "the previous year". Were the patients asked if they recently changed their diet markedly?

<u>Response</u>: Thank you for your comment. Before proceeding with the FFQ, the participants were asked whether their diet had changed markedly during the past year compared with their previously maintained diet. If they answered "No," they proceeded with the FFQ. If they answered "Yes," they were instructed to answer according to their usual diet before proceeding with the FFQ. The reasons for any recent changes in diet characteristics were not further assessed, limiting our ability to evaluate them in our analysis. We have added this information to the Methods section (page 5, last paragraph – page 6, 1st paragraph) of the revised manuscript as follows:

> "Information on dietary patterns was collected through a 103-item food frequency questionnaire (FFQ). Participants were first asked whether their diet had changed markedly during the past year compared with a previously maintained diet; if they answered "No," they proceeded with the FFQ and if they answered "Yes," they were instructed to answer according to their usual diet."

6) *p6, line 2/3: Blood and urine samples for ketone bodies were collected after 10h of fasting.:

1) this was done only once (1x) baseline, and not serial during the entire F/U period of 4years, annually? Please add as study limitation.

<u>**Response**</u>: Thank you for your comment. As suggested, we have mentioned this point in the limitations section of the Discussion (page 15, 1st paragraph) section in the revised manuscript as follows:

> "Fourth, information on ketonuria was collected once at baseline; thus, the changing status of ketonuria was not incorporated into the analysis."

7) *10h of fasting is appropriate for inducing ketosis, but a rather short time window. Most regimen recommend a 16h fasting /8h eating pattern (16:8) - or even longer periods of fasting- which may be more beneficial. Please discuss briefly - in the discussion.

<u>**Response**</u>: Thank you for your comment. As suggested, we added that 10 h of fasting is a rather short time window for inducing ketosis to the Discussion (page 12, last paragraph – page 13 , 1st paragraph) section of the revised manuscript as follows:

> "In addition, the 10 hours of fasting in our study participants may have been a relatively short time window for inducing ketosis, since most intermittent fasting regimens recommend longer periods of fasting, such as 16 hours of fasting between 8 hours of eating.[41, 42] Studies with longer periods of fasting may provide more insight into the association between fasting ketonuria and CAC."

8) Discussion:

*well discussed.

*Please add to section 4.1. clinical impact a statement (prior to the last sentence) like:

"Our results indicate that ketosis may have an underestimated impact on primary prevention of coronary heart disease and the progression of coronary atherosclerosis".

Further studies are required…

<u>Response</u>: Thank you for your comment. As suggested, we added that ketosis may have an underestimated impact on the primary prevention of coronary heart disease and coronary atherosclerosis progression to the Discussion section (page 14, 1st paragraph) of the revised manuscript as follows:

> "our results indicate that ketosis may have an underestimated impact on the primary prevention of coronary heart disease and progression of coronary atherosclerosis."

9) I agree, further data are needed. However in my opinion ketosis - and intermittent fasting as intervention- could have positive and currently underestimated impact on primary CHD prevention. This topic certainly needs further investigations.

*Data whether or not the subjects were actively practicing "intermittent fasting" or during the 4 years period, or prior to the baseline scan, were not collected. This would be interesting. However this is acknowledged appropriately.

<u>Response</u>: Thank you for your comment. As noted, we acknowledged that the lack of data on whether participants were practicing intermittent fasting was a limitation of our study in the Discussion section.

10) *lipid-lowering medication intake was higher in the 0= non-ketosis group, which should slow down progression of CHD- however it did not. Please discuss the inverse

relationship- which further points at how powerful ketosis might be.

Response: Thank you for your comment. As noted, the use of lipid-lowering agents appeared to be the highest in the no ketonuria category (3.6%) when compared with categories 1 and ≥ 2 (2.6% and 2.8%, respectively). However, we performed additional analysis by adjusting for age, sex, and BMI, and found that lipid-lowering agent use did not differ among the ketonuria categories (p for trend = 0.333, Supplementary Table 1). We have added this point to the Results section (page 9, 1st paragraph) of the revised manuscript.

> "Although lipid-lowering agent use appeared to be higher in the no ketonuria category than categories 1 and ≥2, there was no difference after adjustment for age, sex, and BMI (*p* for trend = 0.333, **Supplementary Table 1**)."

11) *p.12 last para/p13, 1st sentence. I agree high fat diet pose a risk for higher total cholesterol- but also LDL.

There is data suggesting that ketosis (especially, if induced by high-fat diet) may increase LDL. However, LDL was lower in those with higher levels of ketosis. Why? Low-fat diet? Lower TG levels in ket 2 group indeed appear suggestive. Please discuss…. Composition of the diet may play a role, or a genetic predisposition.

Response: Thank you for your comment. Indeed, in our study, ketonuria category ≥ 2 appeared to show slightly lower low-density lipoprotein cholesterol (LDL-C) and triglyceride levels than ketonuria categories 0 and 1. Lower triglyceride levels may be due to lower carbohydrate intake, and although this may decrease small dense LDL concentrations, it is unlikely to affect total LDL-C concentrations. Previous studies have shown that ketogenic diets are associated with lower triglyceride and small dense LDL particles; however, LDL-C levels generally increase with ketogenic diets (Bhanpuri, NH, et al., 2018; Yancy, WS, et al.,

2004). Aging is associated with increased LDL-C concentrations, and it is possible that the slightly lower LDL-C concentrations in the ketonuria group in our study were due to a slightly lower mean age in this group, rather than because of any dietary intake component. We performed additional analysis for lipid levels according to ketonuria categories with adjustment for age, sex, and BMI. We found that triglyceride levels continued to decrease with increasing ketonuria category, but LDL-C levels appeared to be lowest in the no ketonuria category compared to the ketonuria categories 1 and ≥2. (**Supplementary Table 1**) We have added these comments to the Discussion section (page 14, 1st paragraph in the revised version) as follows:

> "Although LDL-C appeared to decrease with increasing ketonuria category in our study participants, after adjustment for age, sex, and BMI, LDL-C was lowest in the no ketonuria category. Furthermore, it is unclear whether any predisposing factors for ketonuria played a role in our study population, as information on the reason for ketonuria, such as genetic predisposition, longer fasting duration, recent dietary characteristics, or adherence to intermittent fasting, was not available."

References

Bhanpuri, NH, Hallberg, SJ, Williams, PT, et al., Cardiovascular disease risk factor responses to a type 2 diabetes care model including nutritional ketosis induced by sustained carbohydrate restriction at 1 year: an open label, non-randomized, controlled study, Cardiovasc Diabetol, 2018;17:56.

Yancy, WS, Jr., Olsen, MK, Guyton, JR, et al., A low-carbohydrate, ketogenic diet versus a low-fat diet to treat obesity and hyperlipidemia: a randomized, controlled trial, Ann Intern Med, 2004;140:769-777.

Atherosclerosis style guide checklist

Atherosclerosis applies format guidelines to all accepted papers, with the aim of improving their readability.

Manuscripts that do not conform to the format guidelines of the *Atherosclerosis* Journal will be returned to the authors for reformatting.

Please find below a questionnaire to guide authors to comply with the formatting requirements for revised submissions. For more detailed information, visit [our website](#).

Please note that when you answer "No" to a question, editing of your manuscript is required before submission to *Atherosclerosis*.

Manuscript structure and style

Does your manuscript contain all the below essential elements, in this order?

   Yes

(please stick to the headers as indicated below)

- Title – **Yes**
- Authors, Affiliations, Contact Information – **Yes**
- Abstract <u>in the Atherosclerosis format</u> (*Background and aims*, *Methods*, *Results*, *Conclusions*) –**Yes** Introduction – **Yes**
- Materials and methods (or Patients and methods) – **Yes**
- Results – **Yes**
- Discussion – **Yes**
- Conflict of interest (mandatory) – **Yes**
- Financial support (if applicable) – **Yes**
- Author contributions (mandatory) – **Yes**

- Acknowledgements (if applicable) – **N/A**
- References – **Yes**
- Figures and Tables (with legends in the suitable style) – **Yes**

Abstract style

Is the Abstract structured in the below sections?

    Yes

- *Background and aims* – **Yes**
- *Methods* – **Yes**
- *Results* – **Yes**
- *Conclusions* – **Yes**

Figure and table legends

Are figure and table legends formatted as described below?

    Yes

Each figure and table legend should have a brief overarching title that describes the entire figure without citing specific panels, followed by a description of each panel, and all symbols used. – **Yes**

If a figure or table contains multiple panels, the letter describing each panel should be capitalized and surrounded by parenthesis: i.e. (A)(B)(C)(D). – **N/A**

Please make sure to apply the formatting requirements to figures and tables where necessary (e.g. style of p values, gene and protein nomenclature). – **Yes**

Footnotes to tables

Are footnotes to tables formatted as described below?

      Yes

Footnotes to tables should be listed with superscript lowercase letters, beginning with "ᵃ." – **Yes**

Footnotes must not be listed with numbers or symbols. – **Yes**

Abbreviations

Are abbreviations defined when first used in the text?

      Yes

Use of abbreviations should be kept at a minimum. – **Yes**

Units

Are units expressed following the international system of units (SI)?

      Yes

If other units are mentioned, please provide conversion factors into SI units. – **Yes**

DNA and protein sequences

Are gene names italicized?

      N/A

Gene names should be italicized; protein products of the loci are not italicized. – **N/A**

For murine models, the gene and protein names are lowercase except for the first letter. – **N/A**

(e.g., gene: *Abcb4;* protein: Abcb4)

For humans, the whole gene name is capitalized. – **N/A**

(e.g., gene: *ABCB4;* protein ABCB4)

Mouse strains and cell lines

Are knock-out or transgenic mouse strains and cell lines italicized and the symbol superscripted? N/A

(e.g. *ob/ob* , $p53^{+/+}$, $p53^{-/-}$)

p values

Are *p* values consistently formatted according to the below style throughout the manuscript (including figures and tables)?
       Yes

p <X

p >X

p=X

Language

Is your manuscript written in good English?
                    Yes

Please make sure that you consistently use either American or British English, but not a mixture of them. – **Yes**

Please make sure that words are written consistently in the same way throughout the manuscript. – **Yes**

e.g. non-significant or nonsignificant

e.g. down-regulation or downregulation

Artwork

Have you submitted high-resolution versions of your original artwork?
            Yes

Please make sure to use uniform lettering and sizing in your original artwork, including letters to indicate panels, consistently throughout all figures. – **Yes**

Atherosclerosis policy on the use of proper terminology when referring to intima-media thickness (IMT) N/A

Atherosclerosis has recently embraced a new editorial policy to clarify the use of proper terminology when referring to intima-media thickness (IMT):

IMT should be referred to as "arterial injury" or "arteriopathy", not atherosclerosis.

For more details, please see the following letter to the editor and reply published in *Atherosclerosis*

"IMT is not atherosclerosis", Spence 2020 (https://doi.org/10.1016/j.atherosclerosis.2020.09.016)

"Carotid intima-media thickness should not be referred to as subclinical atherosclerosis: A recommended update to the editorial policy at *Atherosclerosis*", Raggi and Stein 2020 (https://doi.org/10.1016/j.atherosclerosis.2020.09.015)

Supplementary Material

Fasting ketonuria is inversely associated with coronary artery calcification in non-diabetic individuals

In Young Cho, Yoosoo Chang, Eunju Sung, Yejin Kim, Jae-Heon Kang, Hocheol Shin, Sarah H. Wild, Christopher D. Byrne, Seungho Ryu

Supplementary Table 1. Estimated[a] mean values (95% CI) and adjusted[a] proportion (95% CI) of baseline characteristics by fasting ketonuria category

Characteristics	Fasting ketonuria category			*p* for trend
	0	1	≥2	
Total cholesterol (mg/dL)	198.9 (198.8-199.1)	200.8 (199.7-201.8)	199.9 (198.8-201.1)	0.003
LDL-C (mg/dL)	130.0 (129.8-130.1)	131.8 (130.9-132.7)	131.6 (130.6-132.6)	<0.001
HDL-C (mg/dL)	63.9 (63.8-64.1)	66.2 (65.3–67.0)	66.1 (65.1–67.0)	<0.001
Triglycerides (mg/dL)	132.6 (132.2-133.1)	97.7 (95.3–100.1)	91.0 (88.4–93.7)	<0.001
Lipid lowering agent (%)	3.6 (3.5-3.7)	2.9 (2.4-3.4)	3.6 (2.9-4.4)	0.333

CI, confidence interval; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein cholesterol

[a]Adjusted for age, sex and BMI

Supplementary Table 2. Odds ratios[a] (95% CIs) for CACS ≥10 by fasting ketonuria category in 144,346 health checkup examinees at Kangbuk Samsung Hospital between 2011 and 2019

	Fasting ketonuria category			*p* for trend
	0	**1**	**≥2**	
Number	136,159	4,486	3,701	
Cases (N) of CACS ≥10	11,078	293	155	
Age- and sex-adjusted prevalence [b] (95% CIs)	8.0 (7.9–8.2)	7.3 (6.6-8.1)	6.0 (5.1–6.8)	
Adjusted odds ratios [a] (95% CIs)				
Age- and sex-adjusted	1.00 (reference)	0.89 (0.78–1.02)	0.68 (0.57–0.81)	<0.001
Multivariable-adjusted	1.00 (reference)	0.93 (0.81-1.06)	0.74 (0.62-0.88)	0.001

Abbreviations: CACS, coronary artery calcium score; CI, confidence interval.

[a] Estimated from binomial logistic regression models used with prevalent CACS ≥10 as the outcome

The multivariable model was adjusted for age, sex, center, year of screening exam, smoking status, alcohol intake, educational level, total energy intake, medication for hyperlipidaemia, history of hypertension, SBP, and LDL-C.

[b] per 100 persons.

Supplementary Table 3. Coronary artery calcium score (CACS) ratios[a] (95% CI) by fasting ketonuria category in clinically relevant subgroups

Subgroup	Fasting ketonuria category			*p* for	*p* for

	0	1	≥2		trend	interaction
Age						0.106
<50 years (N=125,028)	Reference	0.67 (0.49-0.90)	0.45	(0.31-0.63)	<0.001	
≥50 years (N=19,318)	Reference	1.01 (0.65-1.56)	0.76	(0.41-1.39)	0.481	
Sex						0.532
Women (N=34,896)	Reference	0.89 (0.51-1.55)	0.89	(0.48-1.65)	0.621	
Men (N=109,450)	Reference	0.91 (0.70-1.19)	0.60	(0.43-0.83)	0.003	
Current smoking						0.967
No (N=107,642)	Reference	0.92 (0.69-1.22)	0.63	(0.45-0.88)	0.008	
Yes (N=30,471)	Reference	0.93 (0.56-1.54)	0.69	(0.36-1.35)	0.294	
Alcohol intake						0.516
<20 g/day (N=102,123)	Reference	0.81 (0.60-1.09)	0.68	(0.48-0.96)	0.010	
≥20 g/day (N=34,727)	Reference	1.11 (0.70-1.76)	0.70	(0.39-1.27)	0.450	
HEPA						0.838
No (N=118,928)	Reference	0.87 (0.66-1.15)	0.66	(0.47-0.93)	0.011	

Yes (N=23,297)	Reference	1.03 (0.63-1.68)	0.65 (0.35-1.20)	0.255	
BMI					0.521
<18.5 kg/m² (N=3,337)	Reference	2.16 (0.44-10.74)	1.21 (0.21-6.94)	0.555	
18.5-22.9 kg/m² (N=47,399)	Reference	0.86 (0.57-1.30)	0.84 (0.52-1.35)	0.336	
23-24.9 kg/m² (N=37,656)	Reference	0.85 (0.54-1.32)	0.38 (0.20-0.69)	0.002	
25-29.9 kg/m² (N=48,581)	Reference	0.91 (0.60-1.39)	0.75 (0.45-1.24)	0.707	
≥30 kg/m² (N=7,373)	Reference	1.12 (0.39-3.18)	0.35 (0.09-1.37)	0.409	
Glucose					0.226
<100 mg/dL (N=103,735)	Reference	1.00 (0.77-1.30)	0.71 (0.52-0.98)	0.068	
≥100 mg/dL (N=40,610)	Reference	0.65 (0.38-1.11)	0.47 (0.23-0.97)	0.011	
HOMA-IR					0.554
<2.5 (N=118,849)	Reference	0.94 (0.73-1.21)	0.70 (0.51-0.94)	0.024	
≥2.5 (N=25,283)	Reference	0.79 (0.33-1.90)	0.39 (0.13-1.14)	0.095	
hsCRP					0.632

<1.0 mg/L (N=92,491)	Reference	1.05 (0.77-1.44)	0.78 (0.53-1.15)	0.370
≥1.0 mg/L (N=34,364)	Reference	0.83 (0.53-1.30)	0.67 (0.39-1.14)	0.090
Fatty liver				0.180
No (N=88,020)	Reference	0.88 (0.65-1.19)	0.78 (0.55-1.11)	0.115
Yes (N=56,033)	Reference	0.99 (0.67-1.44)	0.44 (0.26-0.74)	0.007

[a] Estimated from robust Tobit regression models performed with natural log(CACS + 1) as the outcome. The multivariable model was adjusted for age, sex, center, year of screening exam, smoking status, alcohol intake, educational level, total energy intake, medication for hyperlipidaemia, history of hypertension, SBP, and LDL-C.

Abbreviations: CI, confidence interval; HEPA, health-enhancing physical activity; BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein

Supplementary Table 4. Hazard ratios[a] (95% CIs) for coronary artery calcium score (CACS) ≥10 by fasting ketonuria category among participants with CACS = 0 (n=33,774)

| | Fasting ketonuria category | | | *p* for |
	0	1	≥2	trend
Person-year	130004.7	4441.0	3644.7	
Incident CACS ≥10	1411	46	25	
Incident density (/10^3)	10.9	10.4	6.9	
Multivariable adjusted hazard ratios [a] (95% CIs)	1.00 (reference)	1.11 (0.83-1.49)	0.80 (0.53-1.18)	0.259

Abbreviations: CI, confidence interval.

[a] Estimated from Cox proportional hazard models

The multivariable model was adjusted for age, sex, center, year of screening exam, smoking status, alcohol intake, educational level, total energy intake, medication for hyperlipidaemia, history of hypertension, SBP, and LDL-C.