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Increased Calcium Absorption From Synthetic Stable Amorphous Calcium Carbonate: Double-Blind Randomized Crossover Clinical Trial in Postmenopausal Women

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ABSTRACT

Calcium supplementation is a widely recognized strategy for achieving adequate calcium intake. We designed this blinded, randomized, crossover interventional trial to compare the bioavailability of a new stable synthetic amorphous calcium carbonate (ACC) with that of crystalline calcium carbonate (CCC) using the dual stable isotope technique. The study was conducted in the Unit of Clinical Nutrition, Tel Aviv Sourasky Medical Center, Israel. The study population included 15 early postmenopausal women aged 54.9 ± 2.8 (mean $\pm$ SD) years with no history of major medical illness or metabolic bone disorder, excess calcium intake, or vitamin D deficiency. Standardized breakfast was followed by randomly provided CCC or ACC capsules containing 192 mg elemental calcium labeled with ^{44}Ca at intervals of at least 3 weeks. After swallowing the capsules, intravenous $CaCl_2$ labeled with ^{42}Ca on was administered on each occasion. Fractional calcium absorption (FCA) of ACC and CCC was calculated from the 24-hour urine collection following calcium administration. The results indicated that FCA of ACC was doubled ($\pm$ 0.96 SD) on average compared to that of CCC ($p < 0.02$). The higher absorption of the synthetic stable ACC may serve as a more efficacious way of calcium supplementation.

KEY WORDS: AMORPHOUS CALCIUM CARBONATE; ABSORPTION; STABLE ISOTOPES; CALCIUM BIOAVAILABILITY; POSTMENOPAUSE

Introduction

Inadequate dietary calcium intake is prevalent worldwide, especially in elderly people and in women who have difficulty in maintaining calcium intake above 1000 mg per day.[1–3] Calcium supplementation with and without vitamin D has become a widely recognized strategy for achieving adequate calcium intake.[4] Calcium dietary supplements are derived from several origins, including natural sources, such as oyster shells, coral calcium, and dolomite minerals, and from synthetic precipitates, composed of both organic and inorganic calcium salts.[4] Calcium carbonate and calcium citrate salts are by far the most common forms of calcium supplements in use today.[4] The limited absorbability of calcium has resulted in an ongoing scientific debate on the level of bioavailability of different calcium supplements. Calcium citrate was reported to be absorbed better than calcium carbonate, and to cause a greater rise in serum calcium and a greater fall in serum parathyroid hormone (PTH).[5–7] However, to date, advanced sensitive isotopic tracer methods revealed comparable bioavailability of calcium salts, such as carbonate and citrate, in healthy adult men and women.[8]

Calcium carbonate, one of the most abundant minerals in nature, has six known polymorphs, of which the most thermodynamically stable form is calcite. The least stable polymorph is the amorphous form, amorphous calcium carbonate (ACC).[9] The amorphous polymorph is characterized by distinctive 40-nm to120-nm spherules, in contrast to the 1-μm to 10-μm crystals typical of the other polymorphs.[9] Solubility studies have suggested that there are dramatic differences between the calcium carbonate polymorphs. Whereas crystalline phases are considered poorly soluble, the amorphous polymorph is approximately 120 times more soluble than calcite.[10]

Stabilization of synthetic ACC can be a challenging task. Reported attempts at stabilizing synthetic ACC[9,11–13] used either toxic materials or various organic polymers to enable

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the stabilization of ACC.[9,11] Macromolecules that inhibit the crystallization of calcium carbonate and thereby stabilize the amorphous phase have been identified in several organisms.[14–16] Freshwater crayfish construct specialized transient mineral storage sites (gastroliths) that are composed of stabilized ACC embedded into an organic matrix comprising dense chitin fibers and proteins.[17] When needed, the thermodynamic instability of ACC is exploited to offer a highly bioavailable calcium source that enables rapid and effective transport of the mineral across the intestinal epithelium and into the hardening exoskeleton. Inspired by the molecular mechanism used by crayfish to stabilize ACC, a novel method for synthetic production of stabilized ACC using either phosphoamino acids or phosphorylated amino acids was developed.[18]

We recently confirmed the superior bioavailability of stable ACC over crystalline calcium carbonate (CCC) using a labeled radioactive model in rats, where increased bioavailability of up to 40% and 30% was measured in the serum and bone, respectively, as was a 26.5% increase in retention levels.[19] We also showed a beneficial effect of ACC-containing compounds (both natural gastrolith and synthetic stable ACC) over CCC and calcium citrate in preventing bone loss, inducing bone formation and maintaining bone mechanical strength in an ovariectomy-induced bone loss model in rats.[20] The present study sought to compare calcium gastrointestinal bioavailability of synthetic stable ACC to that of CCC in human subjects.

Subjects and Methods

Study design

The study was a single-center study conducted in a double-blind, randomized, crossover manner, using the precise and accurate dual-stable isotope technique[21] that compares the true fractional calcium absorption (FCA) from each calcium source. The study took place between June 2011 and January 2012 at the Unit of Clinical Nutrition, Tel-Aviv Sourasky Medical Center, Tel-Aviv, Israel and its protocol was approved by the internal ethical committee of the medical center. The study was sponsored by Amorphical Ltd and was executed by the research center personnel. Allocation numbers for randomization were computer generated and participants, investigators, and their staff remained blinded to treatment assignments throughout the study. The treatments were prepared by the sponsor and labels were affixed to the vials prior to shipping. The research staff was instructed to dispense the products according to the cohort assignment lists. The procedure was explained verbally and in writing to suitable candidates by the investigators and written informed consent was obtained from each participant.

Participants

Fifteen postmenopausal women (age 54.9 ± 2.8 years, mean $\pm$ SD) who were no more than 5 years from menopause and who met the eligibility criteria were recruited via advertisement in a local newspaper. This study population was chosen to ensure a reasonably homogeneous group and one most likely to be needing calcium supplementation to maintain bone health. All participants were apparently healthy and did not suffer from any major medical illness or metabolic bone disorder. The patients' characteristics are given in Table 1. Inclusion criteria were: absence of menstrual period for 12 months but not more than 5 years, or absence of menstrual period for 6 to 12 months and follicle stimulating factor (FSH) greater than 40 IU/L, and subjects with body mass index (BMI) 18 to 29 (inclusive). Exclusion criteria included women who, on the basis of 3-day food diary records, had an estimated daily calcium intake >1100 mg through combined diet and supplements (Based on the Food and Nutrition Services and Public Health Services, Israel Ministry of Health [2008], Israeli Nutrient Database [BINAT]. Derived from Tzameret software on consumption of food and nutrients). Women who had vitamin D deficiency <20 ng/mL, hypercalcemia, nephrolithiasis, inflammatory bowel disease, malabsorption, or chronic diarrhea, those who had taken antibiotics within the past month, and those suffering from digestive, hepatic, renal, or inflammatory diseases were also excluded. Other exclusion

Table 1. Baseline Characteristics of the Study Participants

Participant #	Age (year)	Height (cm)	Weight (kg)	BMI (kg/cm^2)	PTH (pg/mL)	FSH (mIU/mL)	25-(OH)D (ng/mL)
1	56	162	90.7	34.3[a]	17.6	57.0	23
2	54	159	63.1	24.9	62.1	119.0	33
3	59	160	72.8	28.5	9.0	39.8	45
4	56	163	62.5	23.3	28.3	117.0	25
5	53	156	60.2	24.7	24.8	27.7	31
6	57	161	71.2	27.4	30.8	50.8	27
7	55	166	67.9	24.7	28.8	51.8	23
8	56	159	68.5	26.9	33.0	68.5	26
9	47	156	60.5	25.0	39.3	64.0	37
10	51	161	85.4	32.0[a]	23.4	60.6	21
11	55	152	65.4	28.0	18.7	71.2	22
12	56	159	53.9	21.3	40.0	80.2	33
13	58	161	75.8	29.0	28.2	42.6	30
14	55	160	54	21.1	16.9	161.0	69
15	56	151	68.2	29.9	17.9	44.9	29
Mean	55	159	68.0	26.7	27.9	70.4	32
SD	3	4	10.0	3.6	12.3	34.7	12

BMI = body mass index; PTH = parathyroid hormone; FSH = follicle-stimulating hormone.
[a]Inclusion criteria deviation.

criteria were use of oral steroids, anticonvulsants, bisphosphonates, estrogen compounds, calcitonin, or teriparatide within the past 6 months. An additional exclusion criterion was any acute medical situation (eg, acute infection) within 48 hours of study start, which is considered of significance by the principal investigator.

Production of products

ACC (Amorphical Ltd., Beer-Sheva, Israel; Table 1) was produced by dissolving appropriate amounts of precipitated calcium carbonate (Zifroni Chemicals Suppliers Ltd., Rishon-Lezion, Israel) in 32% HCl together with $^{44}CaCO_3$ (enriched to 96.1%; CMR, Moscow, Russia), after which it was re-precipitated to an ACC powder containing 192 mg elemental calcium (600 mg powder) labeled with 15 mg ^{44}Ca per treatment dose. This treatment dose was divided into two gelatin capsules. Overall, three batches of ^{44}ACC were prepared and sent to magnetic sector thermal ionization spectrometry (MAT 261; Finnigan, Bremen, Germany) for 44:42 molar ratio analysis, calcium content measurement by atomic absorption (Analytical Research Services, Ben-Gurion University, Beer-Sheva, Israel), X-ray diffraction (XRD; The Nanotechnology Institution, Ben-Gurion University), and loss on drying (LOD) tests, which were used to evaluate the amorphous content of each ^{44}ACC batch. ^{44}ACC batches that contained $<5\%$ crystalline $CaCO_3$ were deemed suitable for administration. The capsules were sealed in a container filled with silica gel to avoid humidity and kept at room temperature. The composition of the ACC capsule is given in Table 2.

CCC (Zifroni Chemicals Suppliers Ltd., Rishon-Lezion, Israel) was labeled by homogenizing appropriate amounts of it with $^{44}CaCO_3$ (enriched to 96.1%; CMR) to reach CCC powder containing 192 mg elemental calcium (480 mg powder) labeled with a 15-mg ^{44}Ca per treatment dose. Due to the weight difference between CCC and ACC, 60 mg sucrose (Fagron Gmbh & Co. KG, Rotterdam, The Netherlands) was added to each CCC capsule. One batch of ^{44}CCC was prepared and sent to magnetic sector thermal ionization spectrometry (MAT 261; Finnigan) for 44:42 molar ratio analysis.

Intravenous (iv) isotopic $^{42}CaCl_2$ solution was produced by Concept for Pharmacy Ltd. Kfar-Saba, Israel under a laminar flow hood to ensure sterility. The appropriate amount of $^{42}CaCO_3$ (enriched to 96.3%, CMR) was dissolved in 37% HCl and mixed with 0.45% NaCl. The pH was adjusted to 5.5 with 10 N NaOH. The solution was forced through a 0.22-μm sterilization filter into a sterile container. Individual doses (1.5 mg ^{42}Ca/dose) were transferred into sterile vials for later use. Aliquots were sent for sterility and pyrogenicity testing (Aminolab, Nes-Ziona, Israel), and for calcium content measurement by atomic absorption (Analytical Research Services, Ben-Gurion University, Beer-Sheva, Israel) before use. All of the iv solutions used in this study were sterile and free of pyrogens.

Table 2. Stable Synthetic Amorphous Calcium Carbonate Product Content

Material	Weight (%)
Ca	32
CO_3	47.25
Phosphoserine	0.64
H_2O	20.11

Study protocol

The participants visited the Unit of Clinical Nutrition of the medical center on three occasions. At the first visit, they signed an informed consent form and their general health was evaluated by medical history and physical examinations. After a 12-hour fast, suitable participants provided blood specimens at 7:30 am for FSH, 25-OH vitamin D, and general kidney and liver function evaluation, including: calcium, phosphorus, albumin, alkaline phosphatase, creatinine, glutamic-oxaloacetic transaminase (GOT), glutamic/glutamate pyruvic transaminase (GPT), lactate dehydrogenase (LDH), and PTH levels. All blood tests analyses were performed in the general laboratory of the medical center. The participants completed a 3-day food diary and reported calcium supplementation. Based on these data, daily calcium intake was estimated in order to ensure a total calcium intake of approximately 1100 to 1300 mg/d. Three days prior to the administration of the designated test capsules, the participants were asked to maintain a diet similar to the one they had previously reported. On the day of the study visits, the participants arrived to the Unit after an overnight fast and all were given the planned breakfast with the exception of participant #9 who failed to eat breakfast. They then randomly received two gelatin capsules, overall containing 192 mg elemental calcium of either CCC or ACC, intrinsically labeled with 15 mg of ^{44}Ca. Five minutes after swallowing the capsules, they were intravenously infused over 10 minutes with 1.5 mg $CaCl_2$ labeled with ^{42}Ca. After returning home, they continued eating meals as instructed by the dietitian (based on their food records), and collected all the urine excreted over the 24 hours following the infusion. After at least a 21-day washout period (27.7 ± 5.1 days, mean $\pm$ SD; median $= 27$ days), spot urine samples were collected from all participants and tested to confirm full washout. The identical procedure was repeated with the alternative formulation.

Sample collection and analysis

A 24-hour urine sample was collected to determine the amounts of calcium, sodium, potassium, urea, and creatinine excretion and for stable isotope analysis. Subjects were asked to void and collect their first urine before the iv administration of $CaCl_2$ and to come the next day just before the end of the 24 hours to void and collect their last urine at the unit of clinical nutrition. Creatinine measurements were used to assess complete 24-hour urine collection. Calcium absorption analyses were performed at Baylor College of Medicine (Houston, TX, USA) as described.[22] Ammonium oxalate was used to precipitate Ca isotopes in the urine samples.[23] The amount of extracted calcium was then used for calcium isotope ratio measurements.

Calculations

FCA (α_{24h}) was calculated as described in Yergey and colleagues.[22] In brief, ^{42}Ca, ^{44}Ca, ^{43}Ca, and other measurable stable Ca isotopes were measured in the 24-hour urine collection by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500 cs; Agilent Technologies, Inc., Tokyo, Japan). The ratio of each stable Ca isotope to ^{43}Ca was calculated using ^{43}Ca as an internal standard of the stable Ca isotopes. The FCA of the ^{44}Ca-enriched compound was obtained by dividing the ratio of the actual amount of the enrichment of ^{44}Ca by the corresponding amount of the enrichment of ^{42}Ca.

Statistical analysis

The sample size was determined so that the study would have a power of 80% to detect a clinically relevant difference in calcium absorption of at least 30% between the two treatments when testing at an alpha level of 0.05. In a previous study,[24] the standard deviation for FCA between postmenopausal women was 8% and within women was less than 1%. Assuming an SD of 7%, 13 participants were needed for the study. Given an anticipated dropout rate of 5%, we enrolled 15 participants into the study. The statistical analyses were eventually performed on the data of 13 participants after 2 participants dropped out.

The trial was designed as a crossover study that is analyzed using a matched pairs test that compares differences between two dependent samples. The primary endpoint with respect to efficacy was mean change in fractional calcium absorption from ACC versus CCC in each woman. Due to the small sample size, the difference between FCA from the two treatments was tested using the Wilcoxon matched pairs test, and Spearman's correlation coefficient was calculated for determining the relationships between calcium absorption and other parameters. Wilcoxon matched pairs test and Spearman's correlation test were performed using Prism 5 software (San Diego, CA, USA). Differences were considered significant at $p < 0.05$.

Results

The study was conducted between June 2011 and January 2012. A total of 15 participants were recruited (Fig. 1). One participant (#15) dropped out from the study between treatments due to a traumatic fracture, and another (#14) was excluded from the analysis due to administration of ^{44}ACC capsules that had been exposed to humidity, resulting in the fusion of two capsules. Ten of the 13 participants (77%; ± 22.8 confidence interval) had a higher FCA from ACC compared to that from CCC, whereas the other 3 had an insignificant elevation in FCA from CCC compared to that from ACC, which was under the detection limit of the apparatus (ie, <5% alteration in FCA value) (Fig. 2). Overall, FCA from ACC was doubled (± 0.96 SD) on average, compared to CCC, in each woman. These results were found to be statistically significant by the Wilcoxon matched pairs test ($p < 0.01$). Mean FCA value for each calcium supplement administration

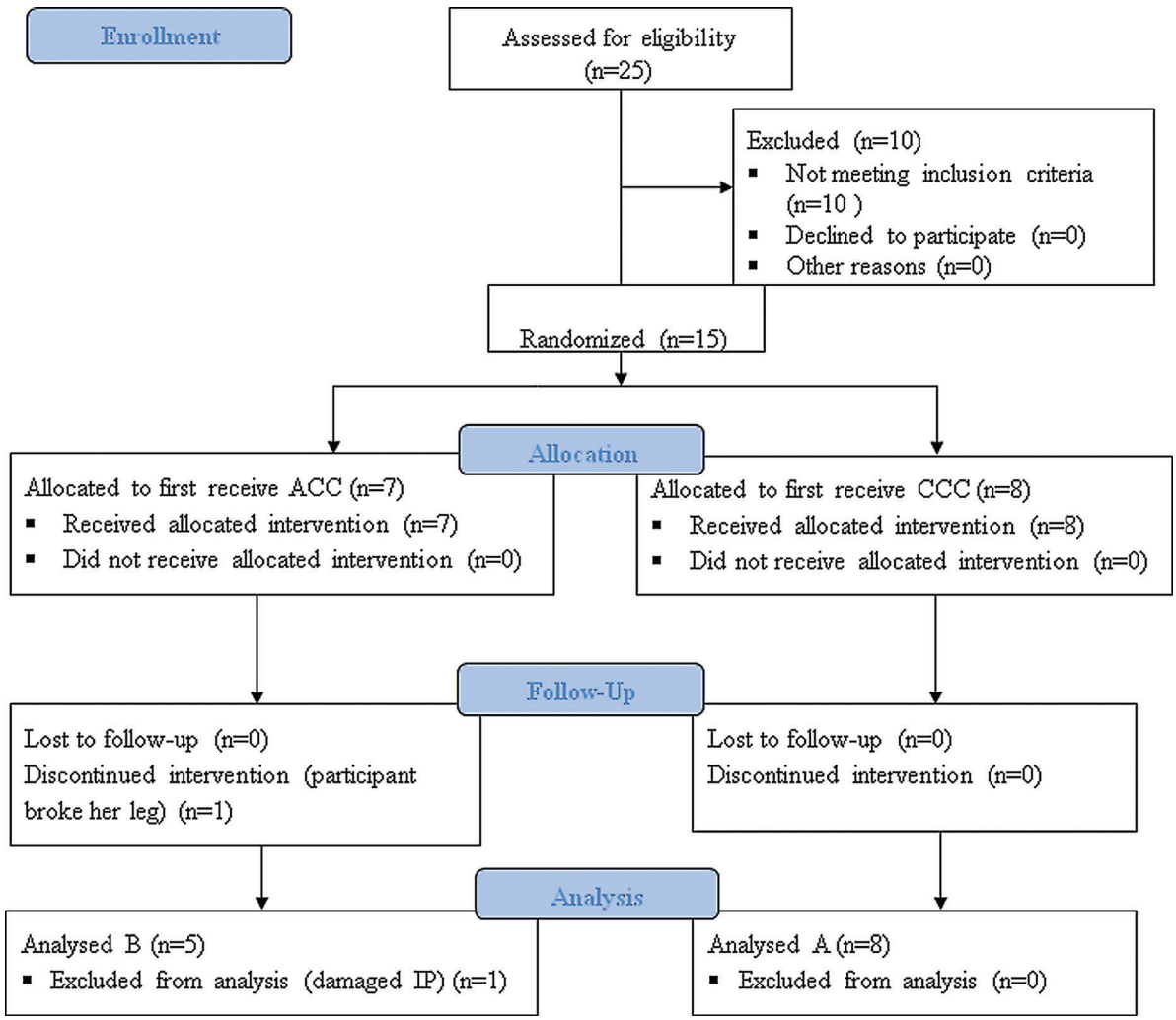

Fig. 1. Clinical study flow diagram. ACC = amorphous calcium carbonate; CCC = crystalline calcium carbonate.

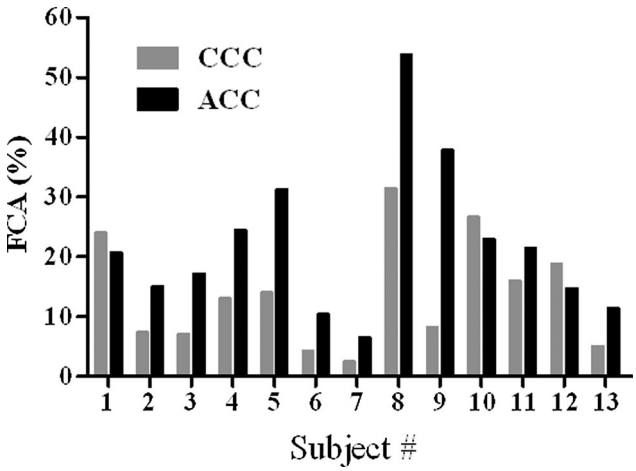

Fig. 2. FCA from ACC versus CCC for each woman following 24-hour urine collection. $n = 13$; Wilcoxon matched pairs test, two-tailed $p < 0.01$. FCA = fractional calcium absorption; ACC = amorphous calcium carbonate; CCC = crystalline calcium carbonate.

calculated as two independent groups was 13.7 ± 9.2 for CCC and 22.1 ± 12.8 for ACC.

The study's participants presented with a BMI of $26.7 \pm 3.6\ kg/m^2$ on average. All 10 participants who demonstrated an increase in their FCA following administration of ACC compared to CCC had a BMI ≤ 29. Out of the 3 participants who presented similar FCA from both formulations, 2 participants (#1 and #10) had the highest BMI values (≥ 32). These values are above the BMI inclusion criteria of 18 to $29\ kg/m^2$. One participant (#9), failed to eat breakfast before capsule ingestion and swallowed the calcium capsules from each source on an empty stomach. This participant demonstrated the highest elevation of FCA from CCC to ACC (ie, an increase of 360%). This high increase may suggest a possible role of food ingestion specifically over CCC absorption.

Spearman's correlation test revealed no correlation between FCA values of CCC, ACC or the relative ratio of ACC/CCC and the baseline parameters of age, BMI, PTH, FSH, and 25-(OH) vitamin D (Table 3). A significant inverse correlation was found between the baseline FCA from CCC and the relative ACC/CCC FCA ratio ($r = -0.81$ $p < 0.001$; Fig. 3), suggesting that the relative increase of FCA from ACC compared to CCC is affected by the baseline calcium gastrointestinal absorption from CCC. The outlier value of participant #9, who failed to eat breakfast, may indicate the possible role of food ingestion over CCC absorption.

No investigational product-related adverse events were recorded during the study.

Discussion

The results of the present study demonstrate a significantly higher bioavailability of ACC over CCC, with FCA values that doubled on average. Ten of the 13 study participants (77%) had a higher FCA from ACC compared to that from CCC, whereas the other 3 participants showed an insignificant higher FCA from CCC compared to ACC, which was within the detection limit of the apparatus. Although the sample size is relatively small, the participants recruited to this study were quite homogeneous and apparently free of any comorbidity that could potentially influence the test results, thereby allowing the establishment of statistical significance of the findings.

Calcium carbonate and calcium citrate salts are by far the most common forms of calcium supplements in use today.[4] In our study, CCC, rather than calcium citrate, was selected as a control because both ACC and CCC are polymorphs of calcium carbonate.[9] Moreover, when taken with a meal, CCC is absorbed and tolerated as efficiently as calcium citrate in most individuals.[8]

The greater FCA of ACC compared to CCC supports our previous results of greater gastrointestinal and bone bioavailability of synthetic stable ACC than of CCC in a rat model.[19] There are several explanations for the higher bioavailability of ACC found in the current study. It is plausible that the differences in particle size, surface area, and solubility[9] affect intestinal absorption of calcium ion, a condition that would enhance passive diffusion of calcium.[25] It is generally assumed that the most soluble and therefore the most absorbable form of any element is its simple ionic state; ie, the Ca^{2+} ion in this case. Therefore, in order to release the maximum amount of ion from a salt, disintegration of the salt[26] and subsequent solubility and dissociation of the ion can be enhanced by reducing the particle size and increasing the amount of surface area, as in the case of ACC. Another possible explanation for the higher calcium absorption from ACC is the nanometric nature of its particles, which might enable them to pass the intestinal epithelia as intact calcium carbonate complexes. This hypothesis has been suggested previously, by the observation of a rat's ability to absorb small, intact complexes, such as calcium oxalate.[27] Nanotechnology has been reported to improve the absorption rate of drugs or nutrients due to an increase in surface area and a resulting higher solubility of the particles.[28–30] Compared with micrometer-sized pearl powder, nanometer-sized pearl powder showed a higher bioavailability of calcium in humans, as

Table 3. CCC, ACC, and ACC/CCC Correlation with Baseline Characteristics of the Study Participants

	CCC FCA		ACC FCA		ACC/CCC relative FCA	
	r	*p**	*r*	*p**	*r*	*p**
Age (years)	−0.12	0.70	−0.44	0.13	0.03	0.9
BMI (kg/cm^2)	0.17	0.57	0.01	0.97	−0.12	0.69
PTH (pg/mL)	−0.12	0.69	−0.07	0.82	0.14	0.64
FSH (mIU/mL)	0.36	0.23	0.19	0.53	−0.46	0.12
25-(OH)D (ng/mL)	−0.33	0.26	−0.01	0.96	0.42	0.15

CCC = crystalline calcium carbonate; ACC = amorphous calcium carbonate; FCA = fractional calcium absorption; BMI = body mass index; PTH = parathyroid hormone; FSH = follicle-stimulating hormone.

*Spearman's correlation test.

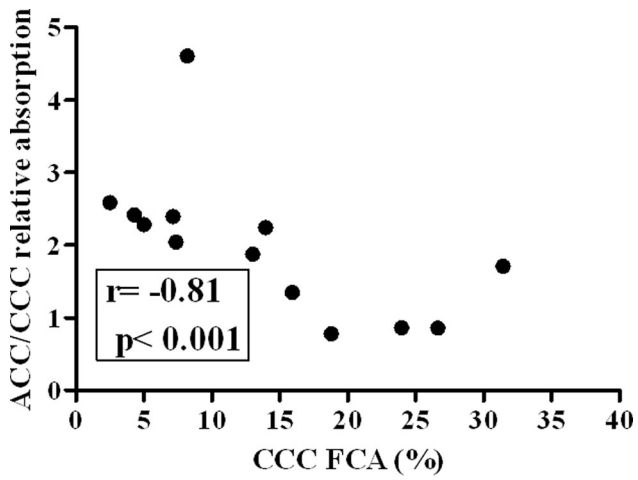

Fig. 3. Relative increase in FCA from CCC to ACC inversely correlating with CCC FCA. $n = 13$; Spearman's correlation test: $r = -0.81$, $p < 0.001$. FCA = fractional calcium absorption; ACC = amorphous calcium carbonate; CCC = crystalline calcium carbonate.

assessed by serum and urinary calcium and serum PTH concentrations.[31] Recent studies have also suggested that amorphous materials can potentially be absorbed throughout larger parts of the intestine (ie, jejunum, ileum) allowing a total higher absorption.[32] Indeed, enhanced bioavailability was demonstrated specifically for nanosized amorphous substances.[33]

FCA has been reportedly higher in obese women compared to nonobese women[34] and indeed, 2 of our study women (participants #1 and #10) out of the 3 whose absorption did not improve following ACC supplementation were obese (BMI $\geq$29) and had a higher FCA from CCC compared to the rest of the group. We also found a significant negative correlation between the baseline FCA from CCC and the relative ratio of FCA, indicating that when absorption is relatively low, the beneficial effect on calcium absorption of a product with a better bioavailability will be greater. This finding is in agreement with a previous report that showed a greater treatment-related increase in FCA among subgroups of women with lowest baseline FCA after treatment with alendronate combined with vitamin D_3.[35] Thus, our results suggest that subjects with relatively low calcium absorption would benefit more from ACC treatment than those with intact calcium gastrointestinal absorption (ie, obese women).

The greatest improvement in calcium absorption after ACC ingestion (360% increase from baseline) was found in participant #9, who failed to eat breakfast before capsule administration on both occasions. This may indicate a possible role of food intake on CCC absorption. It has been previously shown that CCC absorption and solubility are proportional to gastric acid secretion.[36] Therefore, this result calls for further studies that control for fasting and nonfasting states.

We chose to perform the study on early postmenopausal women (up to 5 years from menopause) to ensure a reasonably homogeneous group, and one most likely to be needing an effective calcium supplementation to maintain bone health. Active 1,25-dihydroxyvitamin D has long been established as an important promoter of absorption of calcium in the gut, and studies involving larger cohorts have found significant positive correlations between levels of its inactive form, serum 25-(OH) vitamin D, and FCA.[37,38] However, considering the exclusion criteria of vitamin D deficiency (<20 ng/mL), the lack of correlation between FCA from either CCC or ACC to 25-(OH) vitamin D levels is not surprising.

There are a few limitations to this study that need to be acknowledged. The study was conducted as an outpatient trial, in which urine collections were done at home and participants were instructed to strictly keep to their diet during the study period. These may harbor potential risks to the validity of the results. However, the participants were carefully selected and paid for their enrollment assuming that they would fully cooperate. Other limitations have to do with two participants who had a higher BMI, outside the predefined values (18 to 29 kg/m^2) and one participant failed to eat breakfast. Also, a technical problem (instability of ACC capsules in batch #1) forced us to exclude 1 participant. Nevertheless, the highly statistically significant result of this trial cannot be disregarded.

In summary, this study demonstrates a higher bioavailability of calcium from synthetic stable ACC compared to CCC. These results are intriguing in light of our recent findings in an ovariectomized rat model, which showed a beneficial effect of ACC over common calcium supplements on bone loss prevention, induction of bone formation, and maintenance of bone mechanical strength.[20] Further clinical studies for testing the efficacy of synthetic stable ACC in the prevention of postmenopausal-related bone loss as well as a potential treatment for calcium malabsorption-related conditions are warranted.

Disclosures

GS, MD, OEM, and AS are employees of Amorphical Ltd.; AS owns stock in Amorphical Ltd. and received funding from the company; NV, SAA, EN, and YS state that they have no conflicts of interest.

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Authors' roles: NV, AS, SA, and AS designed the research (project conception, development of overall research plan, and study oversight). NV, GS, EN, and YS conducted research (hands-on conduct of the experiments and data collection). OM, AS, and AS provided essential reagents or provided essential materials (applies to authors who contributed by providing animals, constructs, databases, etc. necessary for the research). NV, GS, MD, SA, EN, and YS analyzed data or performed statistical analysis. NV, GS, and AS wrote the manuscript (only authors who made a major contribution). NV, GS, MD, and AS had primary responsibility for final content. All authors read and approved the final manuscript.

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