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ORIGINAL ARTICLE

Ascorbic acid supplementation improves postprandial glycaemic control and blood pressure in individuals with type 2 diabetes: Findings of a randomized cross-over trial

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Aim: The primary aim of this study was to investigate whether ascorbic acid (AA) supplementation improves postprandial glucose responses under free-living conditions in individuals with type 2 diabetes. A secondary aim was to investigate the effect of AA supplementation on blood pressure.

Materials and methods: A total of 31 individuals with type 2 diabetes (26 males and 5 females; aged 61.8 ± 6.8 years; duration of diabetes, 5.6 ± 4.6 years; HbA1c, $7.6\% \pm 0.7\%$ [mean $\pm$ SD]) were enrolled in a randomized cross-over study involving 4 months of supplementation with oral AA (2×500 mg/d) or placebo. Participants wore continuous glucose monitors for 48 hours and consumed standardized meals pre- and post-supplementation. Measurements included postprandial glucose incremental areas under the curve (iAUC), duration of day in hyper- and hypo-glycaemia status, average 24-hour and daily postprandial glucose concentrations, HbA1c, insulin, blood pressure (BP) and oxidative stress (F_2-isoprostanes).

Results: Following AA supplementation, significant decreases were observed in daily postprandial glucose iAUC (−36%), in duration of day with hyperglycaemia (−2.8 h/d) and postprandial hyperglycaemia (−1.7 h/d), in average 24-hour glucose (−0.8 mmol/L) and daily postprandial glucose (−1.1 mmol/L) concentrations, in systolic (−7 mm Hg) and diastolic (−5 mm Hg) blood pressures and in a specific fraction of free plasma F_2-isoprostanes (−47 pg/mL) as compared to placebo.

Conclusions: Individuals with type 2 diabetes experienced improved postprandial and 24-hour glycaemia and decreased BP after 4 months of AA supplementation as compared to placebo. These findings offer evidence for the proposed use of AA as an adjunct therapy to improve glycaemic and BP control in individuals with type 2 diabetes.

KEYWORDS
antidiabetic drug, clinical trial, continuous glucose monitoring (CGM), glycaemic control, randomised trial, type 2 diabetes

1 | INTRODUCTION

Ascorbic acid (AA) is a major water-soluble antioxidant that decreases cellular and tissue oxidative stress.[1–3] Oxidative stress has been proposed as an important underlying causative agent in the pathogenesis of insulin resistance[4] and hyperglycaemia-induced diabetes complications.[5] Furthermore, some studies have shown that supplementation with AA can improve whole body insulin action in individuals with type 2 diabetes (T2D).[6,7] We recently found that a 4-month period of of AA supplementation increases insulin-mediated peripheral glucose disposal and decreases skeletal muscle oxidative stress during hyperinsulinaemia in individuals with T2D.[8] Improvements in insulin sensitivity with AA supplementation may subsequently promote improvements in glycaemic control. Indeed, findings of some

studies,[7,9–12] but not others,[13] support the efficacy of AA supplementation in improving glycaemic outcomes, including lowering of HbA1c and fasting glucose concentrations in individuals with T2D.

Reliance on general measures such as HbA1c to characterize glycaemic control may fail to account for daily glycaemic excursions that can lead to acute hypoglycaemic events or transient postprandial hyperglycaemia.[14] Use of continuous glucose monitors (CGMs) can overcome such limitations by allowing assessment of daily glucose excursions into hyperglycaemia and hypoglycaemia under free-living conditions. Assessment of postprandial glycaemia is of particular importance clinically, given that evidence from epidemiological and interventional studies implicate postprandial hyperglycaemia as an independent risk factor for cardiovascular disease and cardiovascular events in individuals with T2D.[15–17] Assessment of hypoglycaemia is also clinically important, as severe hypoglycaemia may result in symptoms such as stupor, unconsciousness or even death, and is considered a potential risk with therapies that improve mean glycaemic control.[18] Currently, there is a paucity of data investigating the effects of AA supplementation on postprandial glycaemia and daily excursions into hyper- and hypo-glycaemia during free-living conditions in individuals with T2D.

Control of cardio-metabolic disease risk factors, including high blood pressure, is important in the management of T2D.[19] Systematic reviews of randomized controlled trials have reported potential beneficial effects of AA supplementation on blood pressure (BP) in individuals with T2D.[20,21] However, the number of studies is limited and, among these studies, further limitations include a lack of specificity for AA only and short durations of supplementation.[20,22,23] Thus, there is a need to further explore the efficacy of AA supplementation in individuals with T2D.

The main aims of this study were to investigate the effects of AA supplementation on postprandial glucose responses, and on the duration of the time spent in periods of hyper- and hypo-glycaemia using ambulant CGMs. We hypothesized that AA supplementation would attenuate the daily postprandial increase in blood glucose concentrations, thereby lowering the duration of the time spent in hyperglycaemia in individuals with T2D. An additional aim of this study was to investigate the effects of AA supplementation on blood pressure.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

A double-blind, placebo-controlled cross-over study (#ACTRN12616000276459) was undertaken in individuals with T2D. Recruitment was undertaken via local newspaper advertisements during the period 2016-2017. Of the individuals screened by phone or email, 43 underwent clinical screening to confirm study eligibility. Among these individuals, 31 were enrolled (Figure S1 and Table 1). Participants ingested a capsule containing AA (500 mg L-ascorbic acid, microcrystalline cellulose, vegetable cellulose and vegetable magnesium stearate) (Solgar, Inc., Leonia, New Jersey) or placebo (560 mg gelatine, 8 mg calcium carbonate, vegetable magnesium stearate and vegetable cellulose) (Solgar, Inc.) twice daily for 4 months (123 ± 8

TABLE 1 Baseline participant characteristics

Characteristic/measure	Mean ± SD
n (M, F)	31 (26, 5)
Age (y)	61.8 ± 6.8
Height (m)	1.74 ± 0.09
Weight (kg)	88.7 ± 12.1
BMI (kg/m^2)	29.1 ± 3.1
SBP (mm Hg)	139 ± 10
DBP (mm Hg)	85 ± 9
Duration of diabetes (y)	5.6 ± 4.6
Diabetes management (n)	
• Metformin	19
• Janumet (sitagliptin + metformin)	5
• Sulfonylurea	6
• SGLT2 inhibitor	2
• Lifestyle only	5
Anti-hypertensive (n)	
• ACE inhibitor	6
• AT receptor antagonist	5
• Beta blocker	1
• Calcium Channel blocker	1
• Diuretic	1
Other medications (n)	
• Anti-hypercholesterolaemic	18
• Cardiovascular-related	5
• GERD/gout/anti-depressive/anti-	10
• Inflammatory/other	
HbA$_{1c}$ (%) [mmol/mol]	7.6 ± 0.7 [59.6 ± 5.3]
Fasting glucose (mmol/L)	9.1 ± 1.8
Total cholesterol (mmol/L)	4.7 ± 1.3
LDL-C (mmol/L)	2.5 ± 1.1
HDL-C (mmol/L)	1.3 ± 0.3
Triglycerides (mmol/L)	2.1 ± 1.0
Plasma AA (μmol/L)	41.2 ± 18.4
eGFR (mL/min/1.73 m^{-2})	79.1 ± 12.3
Urea (mmol/L)	6.3 ± 1.9
ALP (U/L)	75.7 ± 19.0
GGT (U/L)	48.1 ± 39.9
AST (U/L)	24.1 ± 9.2
ALT (U/L)	34.5 ± 16.3

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotrasnferase; eGFR, estimated glomerular filtration rate; GGT, gamma glutamyltransferase.

[mean ± SD] days). AA and placebo capsules had an identical appearance and were provided to participants in identical opaque sealed bottles. A minimum one-month washout period (46 ± 1 6 days) separated the treatments.[7,8] Concealment and randomization of treatment order was undertaken by a third party with no direct involvement in the study.

Participation criteria included: diagnosis of T2D; managment with diet or oral anti-hyperglycaemic medications; age between 35 and 75 years; stable HbA1c ≥ 6.5% and <10.0%; total cholesterol ≤6.5 mmol/L; HDL cholesterol ≥0.9 mmol/L; triglycerides ≤4.0 mmol/

L; BMI < 35 kg/m^2; systolic BP (SBP) < 160 mm Hg; diastolic BP (DBP) < 90 mm Hg; non-smoking status; absence of heart murmur, bleeding disorder or haemochromatosis; absence of comorbid cardiovascular, renal or liver diseases; not taking vitamin supplements; not pregnant or planning a pregnancy.

The study was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2008. All procedures involving human subjects were approved by the Deakin University Human Research Ethics Committee. Written informed consent was obtained from all participants prior to participation.

2.2 | Study protocol

Participants attended the Burwood clinical laboratory over two consecutive days at pre- and post-supplementation time points. On Day 1, body composition was measured using dual-energy X-ray absorptiometry (Lunar Prodigy, GE Medical Systems, Madison, Wisconsin). Participants were fitted with a glucose sensor (Enlite Medtronic Minimed, Northridge, California) and recorder (iPro2, Medtronic Minimed). Participants received training in capillary blood sampling (Accu-chek Performa; Roche Diagnostics GmbH, Mannheim, Germany). CGM values were calibrated to capillary blood glucose concentrations, which were measured before main meals and before sleep at night. Continuous glucose data (48 hours) were extracted from recorders using online software (Medtronic CareLink), commencing at 12:00 AM on Day 2, and the average of each 24-hour period was used in subsequent analyses. BP was taken using an automated sphygmomanometer (HEM-907, Omron Healthcare, Hoofddorp, The Netherlands), calibrated to less than 5 mm Hg annually using multi-subject auscultation. The average of three consecutive measurements with a 2-minute interval between measurements was determined. Participants were given an accelerometer (GTX3+, Actigraph LLC, Pensacola, Florida) to wear around their waist throughout the 48-hour period of CGM recording. Data were extracted from the accelerometer using Actilife software (v6.13.3, Actigraph LLC) and average 24-hour physical activity energy expenditure was calculated.[24] A minimum daily recording of 8 hours was deemed valid for representing usual activity. On Day 2, participants underwent fasting blood sampling for measurement of HbA1c, glucose, lipids, plasma insulin, plasma AA, plasma F_2-isoprostanes and renal/liver function. Participants were instructed not to take trial supplements prior to blood sampling.

2.3 | Standardized diet

A standardized diet (Table S1) was provided to participants, to consume over the 48-hour period of CGM recording. Energy content of the diet was based on individual estimated requirements using the Schofield equation,[25] with an activity factor of 1.4 applied. The macronutrient profile of the daily diet, main meals (n = 3) and snacks (n = 2) was approximately 55% carbohydrate, 25% fat and 20% protein.[26,27] The nutrient composition of the diet was established using Foodworks software (v.9.0 Xyris, Australia). The same diet was maintained for participants during each 48-hour testing period.

2.4 | Outcome measures, sample size determination and compliance

The primary outcome of the study was postprandial glucose incremental area under the curve (iAUC) above basal glucose, assessed as a cumulative total (10.5 hours) of the 3.5-hour post-prandial periods after each main meal.[26] A sample size of n = 22 was determined to provide 95% power (α = 0.05, two-tailed t-test) to detect a 50% reduction in post-prandial glucose iAUC after 4 months of AA supplementation.[26] Secondary outcomes were duration of the day (h/d) spent with hyperglycaemia (>10.0 mmol/L glucose) and hypoglycaemia (<4.0 mmol/L glucose), average 24-hour glucose concentration, mean 10.5-hour post-prandial glucose concentration, HbA1c, insulin, blood pressure, total 10.5-hour postprandial glucose AUC (iAUC above basal glucose plus basal glucose AUC) and duration of the day spent with post-prandial hyperglycaemia. All measures were assessed pre- and post-supplementation.

2.5 | Analytical methods

Total plasma AA concentration was measured using an Ultimate 3000 Thermofisher HPLC system with UV detection as described previously.[28] Plasma insulin was measured using a human insulin ELISA (Alpco, Salem, New Hampshire) according to the manufacturer's instructions. Free F_2-isoprostanes were measured in plasma samples using negative chemical ionization gas-chromatography-mass spectrometry (GC-MS) with an Agilent 7983 GC and Agilent 5975 MS according to prior methods,[29] with some modifications. F_2-isoprostanes (8,12-iso-iPF2α-VI, 5-iPF2α-VI, 8-iso PGF2α) were measured and quantified using added deuterated internal standards (0.5 ng each of 8,12-iso-iPF2α-VI-d11, 5-iPF2α-VI-d11 and 8-iso PGF2α-d4). F_2-isoprostanes were observed as two main chromatographic peaks, as shown previously.[29,30] Peak 1 contained 5-iPF2α-VI and 8-iso-PGF2α, while peak 2 contained 8,12-iso-iPF2α-VI. Peak 1 concentration was quantified using 569 m/z, corrected for the combined responses of 8-iso-PGF2α-d4 (573 m/z) and 5-iPF2α-VI-d11 (580 m/z), while peak 2 concentration was quantified using 569 m/z corrected for 8,12-iso-iPF2α-VI-d11 (580 m/z). The two peak concentrations were added together for total free F_2-isoprostanes concentration.

2.6 | Compliance and adverse effects

Compliance was determined by capsule counts, using the equation: *number of capsules consumed/number of capsules expected to be consumed for full compliance ×100%*. Participants completed a four-day diet record and a seven-day physical activity recall prior to commencement of each treatment[8] to monitor consistency of diet and physical activity. Participants were contacted monthly to follow-up on regular capsule ingestion, to inform them of any treatment changes, and to note any adverse effects to health that may have arisen subsequent to the supplementation.

2.7 | Statistics

AA and placebo trials were compared using ANCOVA (SPSS v.23; IBM, Armonk, New York) with the within-subject difference in post-

supplementation responses as the dependent variable and the corresponding difference in pre-supplementation responses as a covariate.[31] Adjusted mean differences between treatments and effect sizes (Cohen's d) were determined from ANCOVA data. Two-way repeated measures ANOVA with Fisher's LSD tests were used to assess within-treatment pre-post changes (Prism v.6.0; Graphpad Software Inc., La Jolla, California). Paired t-tests or Wilcoxon rank tests were used to compare survey measures of energy intake, vitamin C intake and physical activity energy expenditure[32] among treatments.

Both complete-case (CC) and per-protocol (PP) analyses were performed. Less than 5% of data were missing across randomized participants; therefore, we opted for CC rather than intention-to-treat main analyses as little bias is expected.[33] PP analyses included data concerning all participants for whom data was complete, who remained consistent in treatment, and who were at least 80% capsule compliant. To model the impact of missing data, sensitivity analyses of key outcomes were undertaken using "last-observation-carried-forward" (LOCF) imputations and multiple imputations (MI, n = 5 iterations) using linear regression. Data found to violate normality using a D'Agnostino and Pearson test were log-transformed prior to parametric testing and are presented within the results as geometric mean (SD factor). Potential order or carry-over effects were investigated using two-way ANOVA with treatment order as the between-subject factor. Significance was set at $P < 0.05$ for all analyses.

3 | RESULTS

3.1 | Participants

Among the 31 participants who were initially enrolled, 27 completed the study, and their data were used in the main analyses. Two participants withdrew from the study prior to treatment order randomization, while another participant withdrew after completing the first treatment arm only. Family obligations and/or work commitments were cited as reasons for withdrawal. Another participant was withdrawn during the first treatment because of development of a serious illness that was unrelated to study participation. Capsule compliance was high and similar between the AA group (geometric mean [SD factor]: 92.5% [1.1]) and the placebo group (94.3% [1.1]) ($P = 0.94$). Exclusions from PP analyses included two participants with relatively poor (<80%) capsule compliance and five participants who altered their anti-hyperglycaemic treatment during the study. No changes in compliance with any other treatments were reported during the study. No significant differences were observed between treatments for self-reported measures of energy intake (AA mean ± SD, 8732 ± 2467 kJ/d vs placebo, 8943 ± 1957 kJ/d; $P = 0.69$), vitamin C intake (AA geometric mean [SD factor], 70.6 mg/d [2.3] vs placebo, 61.7 mg/d [3.3]; $P = 0.46$) or physical activity energy expenditure (AA mean ± SD, 624 ± 441 kcal/d vs placebo, 825 ± 711 kcal/d; $P = 0.13$). The only self-reported adverse
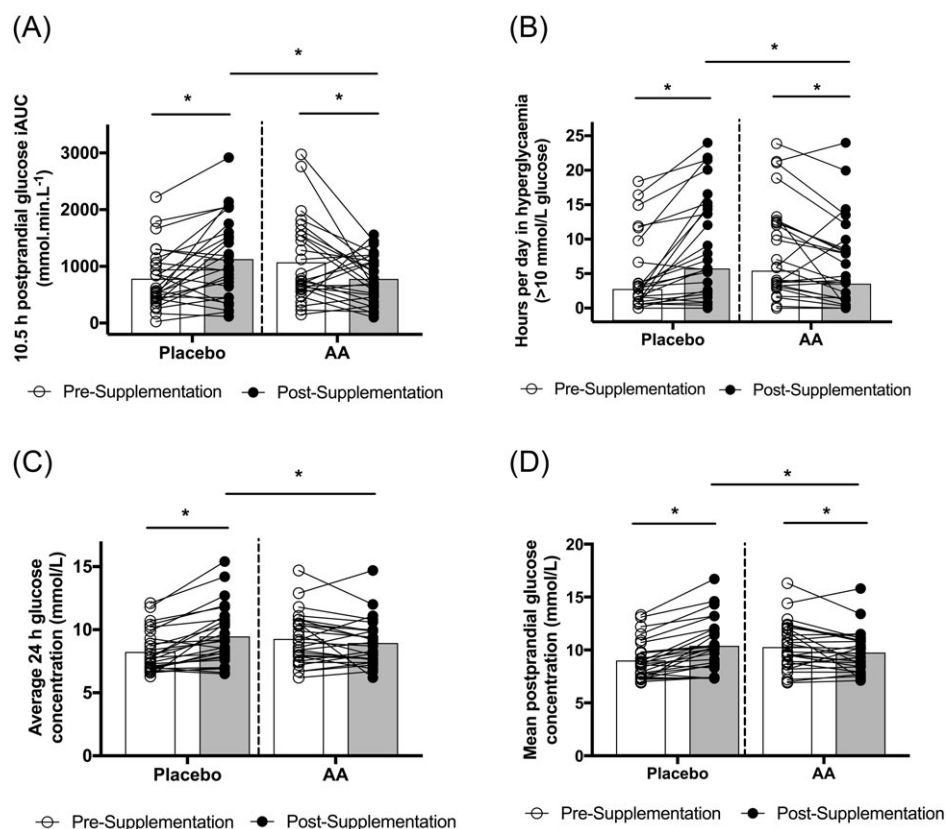

FIGURE 1 Effect of AA supplementation on key glycaemic outcomes in main analyses ($n = 27$). A, 10.5-Hour post-prandial glucose iAUC. B, Duration of day spent with hyperglycaemia. C, Average 24-hour glucose concentration. D, mean daily postprandial glucose concentration. *Denotes significant difference ($P < 0.05$) between points. Statistical analysis for duration of the day spent with hyperglycaemia was made using log-transformed normalized data. Bars represent the mean (or geometric mean for duration of day with hyperglycaemia). Open circles represent individual pre-supplementation data, while closed circles represent post-supplementation data

effect was a feeling of depression in one participant during placebo administration.

3.2 | Main analyses (n = 27)

3.2.1 | Postprandial glucose

Daily 10.5-hour postprandial glucose iAUC decreased significantly ($P < 0.01$) during AA supplementation relative to a significantly increased ($P < 0.01$) response during placebo administration ($P < 0.01$ between the two) (Figure 1). Mean daily 10.5-hour post-prandial glucose concentration also decreased significantly ($P = 0.04$) during AA supplementation relative to an increased ($P < 0.01$) response during placebo administration ($P < 0.01$ between the two) (Figure 1). Adjusted mean changes in 10.5-hour post-prandial glucose iAUC and mean post-prandial glucose with AA supplementation compared to placebo administration were -410 mmol.min.L^{-1} (Cohen's $d = 1.32$) and -1.1 mmol/L (Cohen's $d = 1.27$), respectively. Total 10.5-hour post-prandial glucose AUC (iAUC above basal glucose plus basal glucose AUC) decreased during AA supplementation relative to a significantly increased ($P < 0.01$) response during placebo administration (Table 2). Raw post-prandial glucose data are provided in Figure S1.

3.2.2 | Duration of day spent with hyper- and hypoglycaemia

Duration of the day spent with hyperglycaemia significantly decreased ($P = 0.03$) during AA supplementation relative to an increased ($P < 0.01$) response during placebo administration ($P < 0.01$ between the two; adjusted mean change vs placebo, -2.8 h/d; Cohen's $d = 1.15$) (Figure 1). Duration of time within the 10.5-hour post-prandial period with hyperglycaemia significantly decreased during AA supplementation ($P = 0.04$) relative to a significantly increased ($P < 0.01$) response during placebo administration (Table 2). There was no significant effect of AA supplementation on duration of the day spent with hypoglycaemia (Table 2).

3.2.3 | Average 24-hour glucose and general glycaemic measures

Average 24-hour glucose decreased during AA supplementation relative to a significantly increased ($P < 0.01$) response during placebo administration ($P < 0.05$ between the two; adjusted mean change vs placebo, -0.8 mmol/L; Cohen's $d = 0.83$) (Figure 1). There were no

TABLE 2 Effect of supplementation on body composition, glycaemia, general biochemistry and physical activity (main analysis, $n = 27$)

Measure	Placebo		AA		P value[a]	Adjusted mean difference (AA – placebo)[b]	Cohen's d[c]
	Pre	Post	Pre	Post			
Weight (kg)	86.5 ± 12.3	86.9 ± 12.1	86.8 ± 12.0	87.1 ± 12.2	0.62	+0.2	0.20
BMI (kg/m^2)	29.2 ± 3.3	29.3 ± 3.2	29.2 ± 3.0	29.3 ± 3.0	0.75	+0.0	0.13
% Body fat	34.3 ± 5.5	34.6 ± 5.5	35.0 ± 5.2	34.8 ± 5.4	0.76	+0.1	0.13
Fat-free mass (kg)	57.8 ± 8.5	57.4 ± 8.3	57.5 ± 8.6	57.5 ± 8.4	0.68	+0.1	0.17
Hours per day with hypoglycaemia (<4 mmol/L glucose)[d]	0.0 (1.1)	0.0 (1.1)	0.0 (1.0)	0.0 (1.1)	0.25	+0.1	0.47
10.5-hour post-prandial glucose (total) AUC (mmol/min/L)	5690 ± 1167[e]	6538 ± 1558	6442 ± 1422	6169 ± 1267[e]	0.02	−640	1.02
Hours per day with post-prandial hyperglycaemia (>10 mmol/L glucose)[d]	2.0 (2.1)[e]	3.7 (2.3)	3.5 (2.3)	2.5 (2.5)[e,f]	0.01	−1.7	1.10
HbA1c (%) [mmol/mol]	7.4 ± 0.7 [57.4 ± 3.7]	7.6 ± 0.9 [59.6 ± 5.1]	7.7 ± 0.9 [60.7 ± 5.1]	7.6 ± 0.9 [59.6 ± 5.1]	0.36	−0.2%	0.37
Fasting glucose (mmol/L)	8.5 ± 1.9	9.0 ± 2.1	9.0 ± 1.8	8.7 ± 1.9	0.15	−0.5	0.59
Fasting insulin (μIU/mL)[d]	9.4 (1.5)	9.4 (1.8)	9.4 (1.5)	9.2 (1.6)	0.73	−0.3	0.14
Total cholesterol (mmol/L)	4.6 ± 1.0	4.7 ± 1.1	4.7 ± 1.0	4.9 ± 1.3	0.50	+0.1	0.28
LDL-cholesterol (mmol/L)	2.4 ± 0.8	2.5 ± 0.9	2.5 ± 0.8	2.6 ± 1.0	0.78	+0.0	0.11
HDL-cholesterol (mmol/L)	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	0.40	−0.0	0.35
Triglycerides (mmol/L)[d]	1.8 (1.6)	1.8 (1.6)	1.9 (1.5)	2.1 (1.7)	0.06	+0.3	0.79
eGFR (mL/min/1.73 m^{-2})[d]	75.9 (1.2)	73.9 (1.2)	76.3 (1.2)	75.3 (1.2)	0.39	+1.0	0.00
ALP (U/L)	70.0 ± 16.7	72.1 ± 20.5	72.9 ± 18.9	74.1 ± 23.2	0.37	+1.9	0.37
GGT (U/L)[d]	38.6 (1.9)	38.7 (1.8)	34.1 (1.8)	35.7 (1.8)	0.11	+4.1	0.67
AST (U/L)[d]	22.9 (1.4)	22.7 (1.4)	24.2 (1.4)	23.8 (1.5)	0.35	+1.1	0.39
ALT (U/L)	31.5 ± 15.9	34.1 ± 14.8	34.9 ± 16.3	36.7 ± 18.2	0.40	+1.8	0.35
24-hour accelerometer physical activity energy expenditure (kcal)	396 ± 206	432 ± 277	415 ± 247	419 ± 241	0.68	−20.3	0.13

Data are given as mean ± SD.
[a] P value for ANCOVA test between treatments.
[b] Mean difference between treatments after adjusting for pre-supplementation differences using ANCOVA test.
[c] Effect size of ANCOVA test.
[d] Data was analysed using log-transformed normalised data and is expressed as geometric mean (SD factor).
[e] Denotes significantly different to Placebo Post.
[f] Denotes significantly different to AA Pre, $P < 0.05$.

significant differences between or within treatments for HbA1c, fasting glucose or insulin (Table 2).

3.2.4 | Blood pressure

Both SBP ($P < 0.05$) and DBP decreased during AA supplementation compared to placebo ($P < 0.01$ between the two) (Figure 2). Adjusted mean differences in SBP and DBP with AA supplementation compared to placebo were − 7 mm Hg (Cohen's $d = 1.50$) and − 5 mm Hg (Cohen's $d = 1.39$), respectively. Prevalence of hypertension (ie, SBP ≥ 140 mm Hg and/or DBP ≥ 90 mm Hg) decreased during AA supplementation (pre, 12/27 vs post, 7/27) and increased during placebo administration (pre, 9/27 vs post, 14/27).

3.2.5 | Body composition, lipids, energy expenditure and renal and liver function

There were no significant differences between or within treatments for measures of body composition, serum lipids and accelerometer-measured physical activity energy expenditure (Table 2). AA supplementation had no effect on serum renal or liver function measures, including creatinine, urea, uric acid, bilirubin, albumin (data not shown), estimated glomerular filtration rate, alkaline phosphatase, gamma glutamyltransferase, aspartate aminotransferase and alanine aminotransferase (Table 2).

3.2.6 | Plasma AA concentration

AA concentration significantly increased ($P < 0.01$) during AA supplementation compared to placebo ($P < 0.01$ between treatments; adjusted mean change vs placebo, +50.0 μmol/L; Cohen's $d = 4.23$) (Figure 3).

3.2.7 | F_2-Isoprostanes

Total free plasma F_2-isoprostanes concentration (peak 1 + peak 2) decreased significantly ($P = 0.03$) during AA supplementation, but was not significantly different as compared to placebo ($P = 0.11$ between the two) (Figure 3). Peak 1 concentration (containing both 5-iPF2α-VI and 8-iso PGF2α) did not change significantly ($P = 0.17$ between treatments) (Figure 3). Peak 2 (containing 8,12-iso-iPF2α-VI) significantly decreased ($P < 0.01$) with AA supplementation compared to placebo ($P = 0.01$ between treatments) (Figure 3). Adjusted mean changes in F_2-isoprostanes concentrations with AA supplementation compared to placebo were − 37.9 (Cohen's $d = 0.65$), +11.3 (Cohen's $d = 0.57$) and − 46.7 pg/mL (Cohen's $d = 1.05$) for total (peak 1 + 2), peak 1 and peak 2, respectively.

3.2.8 | PP analyses (n = 20)

Overwhelmingly, findings of the PP analyses were quantitatively and statistically similar to those of the main analyses (Tables S2 and S3). An exception concerned fat free mass, which was found to decrease with placebo relative to AA supplementation ($P < 0.05$ for comparison between the two). However, the relatively small magnitude of difference between AA supplementation and placebo concerning fat-free mass (+0.6 kg) is unlikely to be of clinical significance.

3.3 | Sensitivity analyses

Sensitivity analyses that evaluated missing data using LOCF and MI confirmed the statistical significance of key outcome measures reported in the main analyses (Table S4). Two-way ANOVA using pre-supplementation values and within-supplementation changes with treatment order as the between-subject factor revealed no effect of treatment order on any key outcome measure. This suggests an absence of carry-over effects of treatments.

4 | DISCUSSION

After 4 months of supplementation with AA, participants with T2D had a 36% lower daily postprandial glucose iAUC and a − 1.1 mmol/L lower mean post-prandial glucose concentration as compared to placebo. Improvements in post-prandial glycaemia with AA supplementation likely contributed to the significant decreases also observed in duration of the day spent with hyperglycaemia (−2.8 h/d), time spent
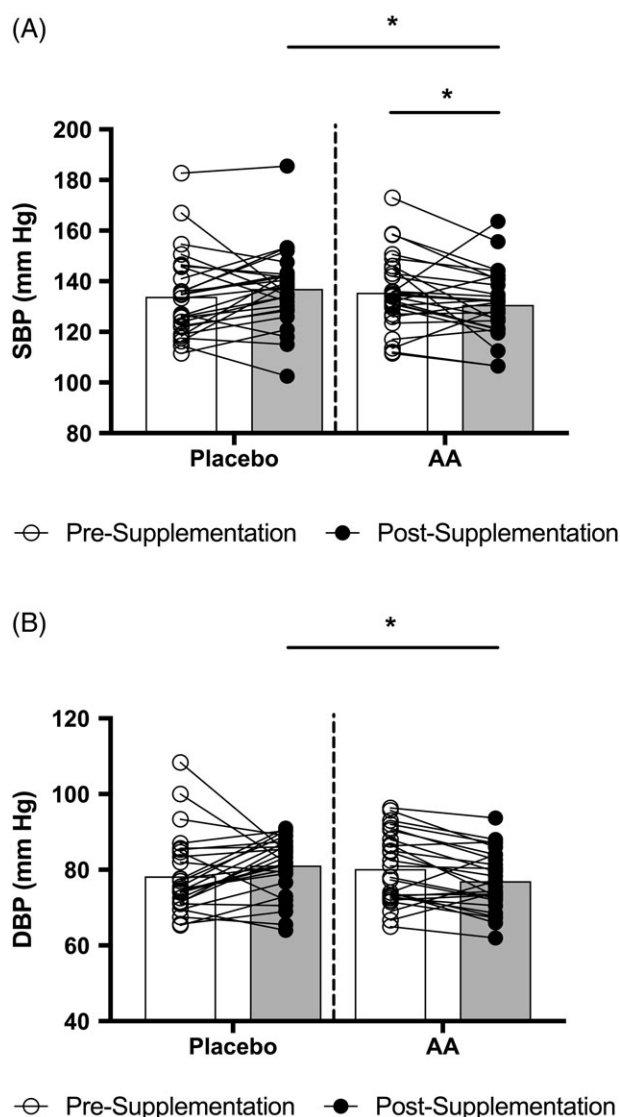

FIGURE 2 Effect of AA supplementation on blood pressure in main analyses (*n* = 27). A, systolic blood pressure. B, diastolic blood pressure. *Denotes significant difference (*P* < 0.05) between points. Bars represent the mean. Open circles represent individual pre-supplementation data, while closed circles represent post-supplementation data
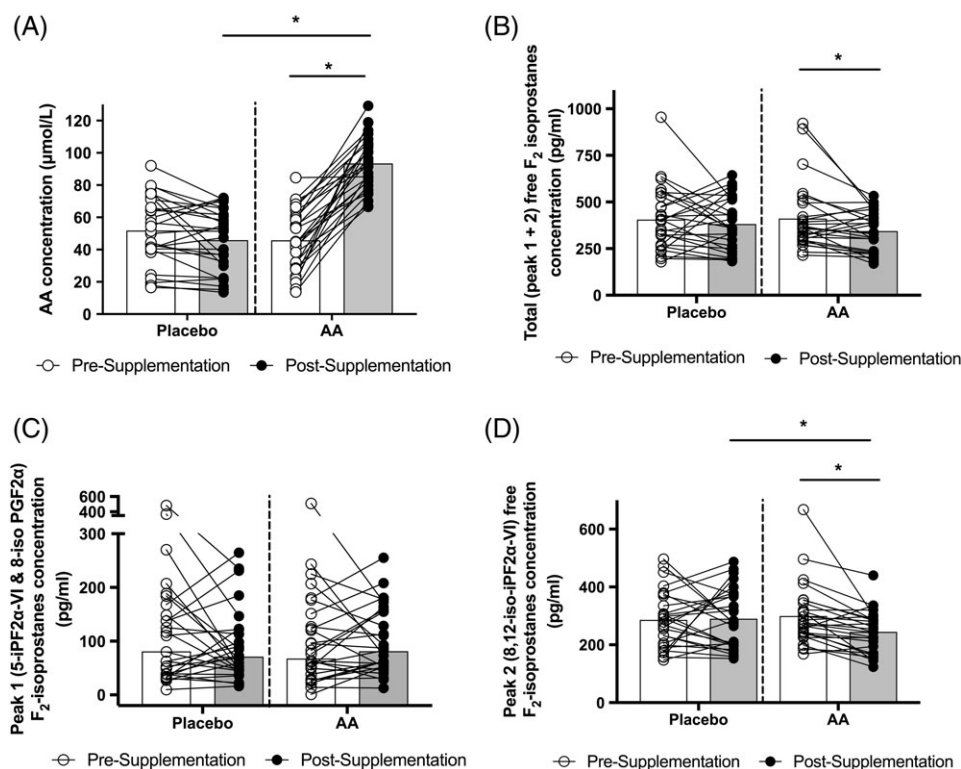
FIGURE 3 Effect of AA supplementation on plasma concentrations of ascorbic acid and F_2-isoprostanes in main analyses ($n = 27$). A, plasma ascorbic acid concentration. B, total free F_2-isoprostane concentration (peak 1 + peak 2). C, F_2-isoprostane peak 1 concentration (containing 5-iPF2α-VI and 8-iso PGF2α) 4D shows F_2-isoprostane peak 2 concentration (containing 8,12-iso-iPF2α-VI); *Denotes significant difference ($P < 0.05$) between points. Statistical analysis for peak 1 was made using log-transformed normalized data. Bars represent the mean (or geometric mean for peak 1). Open circles represent individual pre-supplementation data, while closed circles represent post supplementation data

with post-prandial hyperglycaemia (−1.7 h/d) and average 24-hour glucose concentration (−0.8 mmol/L) as compared to placebo. These findings are of potential clinical importance given that post-prandial hyperglycaemia has been considered an independent risk factor for cardiovascular disease and cardiovascular events in individuals with T2D.[15-17] The improvement in total daily time spent with hyperglycaemia is also of potential clinical importance as the risk of complications in T2D is strongly associated with previous hyperglycaemia.[34] Calculated effect sizes of these glycaemic improvements with AA supplementation indicate that they were all large improvements (Cohen's $d > 0.80$). Magnitude of changes and statistical findings were consistent across CC and PP analyses concerning these key measures and the vast majority of measures. Moreover, sensitivity modelling of data consistent with intention-to-treat approaches using imputations for missing data confirmed the robustness of the key findings.

SBP and DBP were significantly decreased by 7 and 5 mm Hg, respectively, after AA supplementation compared to placebo. Calculated effect sizes of these improvements in BP with AA supplementation indicate that they were all large improvements (Cohen's $d > 0.80$). Moreover, the calculated prevalence of hypertension was 50% lower following AA supplementation (7/27) as compared with placebo (14/27). Biologically plausible mechanisms of AA in improving BP relate to its potential to enhance nitric oxide (NO) synthesis and bioavailability through its antioxidant actions. AA is thought to scavenge the oxidant superoxide[1] and, therefore, may decrease NO reactions with superoxide to limit formation of the potential vasculature-

damaging reactive species peroxynitrite.[35] AA has also been shown to preserve concentrations of the endothelial nitric oxide synthase (eNOS) co-factor tetrahydrobiopterin, in turn maintaining NO production via eNOS.[36] Similar to our findings, previous systematic reviews found AA supplementation to lower SBP and DBP by approximately 4-5 and 3-4 mm Hg, respectively, in individuals with T2D.[20,21] Consistent with prior studies,[22,23] many participants in our study population (~40%) were regularly using anti-hypertensive medications. Thus, our findings lend support to current evidence that AA supplementation can improve BP in individuals with T2D, even when managed with a primary anti-hypertensive agent(s).

While we cannot confirm the mechanism(s) of post-prandial glucose improvement based on our data, we speculate, based on prior findings, that the improved response occurred via an increase in insulin-mediated peripheral glucose disposal.[8] In that study, AA supplementation also increased skeletal muscle AA concentrations and decreased muscle oxidative stress during hyperinsulinaemia, which is suggestive of an ameliorative antioxidant effect on muscle insulin sensitivity.[8] Paolisso et al.[7] reported decreased plasma-free radicals (O_2) along with improved insulin-mediated whole-body glucose disposal in elderly individuals with T2D who were undergoing an AA dosage regimen similar to that of our study. We observed a significant decrease in the concentration of plasma-free F_2-isoprostanes during AA supplementation, which was largely the result of a decreased concentration of the plasma F_2-isoprostanes fraction co-eluting with 8,12-iso-iPF2α-VI. There has been limited specific investigation of 8,12-iso-iPF2α-VI

in individuals with T2D, although its concentration appears to be sensitive to oxidative stress[37,38] and it is an F_2-isoprostanes isomer that is relatively prevalent in human plasma.[39] Thus, 8,12-iso-iPF2α-VI might be a quantitatively important marker of oxidative stress in plasma. Although AA might decrease oxidative stress via antioxidant actions, the relationship between oxidative stress and post-prandial hyperglycaemia[40] also implicates potential indirect lowering of oxidative stress following improvements in post-prandial glycaemia. Our findings, in combination with those mentioned above, offer plausible evidence that AA supplementation may decrease oxidative stress and potentially improve glycaemic control in individuals with T2D.

Interestingly, we did not find a significant improvement in HbA1c despite improvement in post-prandial glucose responses. Previous studies that reported significant improvements in HbA1c after AA supplementation included more participants,[7,9–11] enrolled participants with a higher baseline HbA1c[7,9–11] and/or undertook AA supplementation for a longer period[11] than we did. Nonetheless, other findings we report suggest that HbA1c may be improved with a more prolonged supplementation period. The significantly decreased average 24-hour glucose and total post-prandial glucose AUC following AA supplementation as compared with placebo are predictive of an improvement in HbA1c, given that these measures have been found to correlate strongly with HbA1c variability.[41,42] On average, the wash-out period between treatments was 6.5 weeks, suggesting that there may not have been a complete wash-out of prior treatment effects with respect to HbA1c. However, of note, we did not observe any order or carry-over effect for HbA1c, or any other measures, in the study.

Another potential limitation of our study is that our cohort was predominantly male. Thus, the number of females was not adequate to undertake a reliable gender subgroup analysis. Despite this, it was apparent that all female participants followed the same general pattern of improvement across key outcome measures as did the cohort on whole.

Compliance in the present study was high and the number of reported adverse effects was low, suggesting that AA may be safely and consistently used in addition to a primary diabetes treatment. Given the potential side effects with common anti-diabetic medications, such as hypoglycaemia and weight gain, the idea that a relatively benign and inexpensive vitamin supplement might play a role in managing diabetes has particular appeal. Thus, findings of the current study implicate AA supplementation as a potentially useful adjunct therapy in individuals with T2D for management of both glycaemia and blood pressure.

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CONFLICTS OF INTEREST

No author has any conflicts of interest to disclose.

Author contributions

SAM recruited participants, collected data, performed analytical and statistical tests and wrote the manuscript. BR contributed to study design, provided clinical guidance and reviewed/edited the manuscript. LJCL contributed to conceptual ideas, the discussion and reviewed/edited the manuscript. JS contributed to conceptual ideas and reviewed/edited the manuscript. GDW conceptualized and devised the study, collected data and reviewed/edited the manuscript.

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SUPPORTING INFORMATION

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