Electronic Supplementary Material For:

Diminished arginine bioavailability during hyperglycaemia distinguishes individuals with $A^{-}\beta^{+}$ ketosis-prone diabetes from those with type 2 diabetes: implications for the pathogenesis of ketoacidosis in $A^{-}\beta^{+}$ ketosis-prone diabetes

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ESM Methods

Kinetic Calculations were made using tracer dilution and precursor- product equations at isotopic steady state. **ESM Fig. 2** shows the stable isotope enrichments of all tracers when samples were collected for analysis, confirming that plateau isotopic enrichments were achieved during this time period.

Protocol 1:

Total arginine, ornithine, citrulline, phenylalanine fluxes (Q_{Arg}, Q_{Orn} Q_{Cit}, Q_{Phe}) were calculated by:

Total Flux (μmol kg-LBM^{-1} h^{-1}) = (IE_{Inf} / IE_{Pl}) x i

where IE_{Inf} and IE_{Pl} are the isotopic enrichments of the tracer in the infusate and in plasma at isotopic steady state (M+6 isotopomer for arginine, M+2 isotopomer for ornithine, M+2 isotopomer for citrulline, M+5 isotopomer for phenylalanine) and i is the rate of infusion of $^{13}C_6$-arginine, $^{15}N_2$-ornithine, $^{2}H_2$-citrulline, or $^{2}H_5$-phenylalanine in μmol·kg LBM^{-1}·h^{-1}.

ESM Fig. 3 shows the major flux pathways for arginine metabolism that were measured in this study.

Endogenous flux was calculated by subtracting the tracer infusion rate from total flux. The major flux pathways were: for arginine (influx 1+6, efflux 2+3+7), ornithine (influx 3, efflux 4+5), and citrulline (influx 5+7, efflux 6).

Arginine Oxidation was calculated from its rate of conversion to expired CO_2:

Arg Oxidation (μmol kg-LBM^{-1} h^{-1}) = ($VCO_2 \times IE_{CO2} \times 44.6 \times 60$) / (LBM $\times$ 0.78 $\times$ IE_{Arg} $\times$ 6),

where *V*CO2 is the rate of CO_2 excretion in breath (mL/min), 0.78 is the adjustment factor for CO_2 recovered in the expired air(12; 13), IE_{CO2} is the plateau IE of breath CO_2 and IE_{Arg} is the plasma IE of arginine. The terms 44.6 and 60 convert mL/min to μmol/h and the term 6 compensates for the formation of 6 $^{13}CO_2$ molecules from the oxidation of one $^{13}C_6$-arginine molecule.

Arginine Bioavailability, i.e., arginine available for the synthesis of body proteins and other metabolites such as ornithine, citrulline, nitric oxide (NO), creatine and agmatine was calculated by subtracting its irreversible loss by oxidation to CO_2 from its flux:

Arg Bioavailability (μmol kg-LBM^{-1} h^{-1}) = Arg flux (μmol kg-LBM^{-1} h^{-1}) – Arg Oxidation (μmol kg-LBM^{-1} h^{-1}).

Arginine Hydrolysis, ornithine synthesis from both the arginases and L-arginine:glycine amidinotransferase (AGAT) catalyzed pathways, was calculated from its rate of conversion to ornithine:

Arg Hydrolysis (μmol kg-LBM^{-1} h^{-1}) = $Q_{Arg \rightarrow Orn} = Q_{Orn} \times (IE_{Orn} / IE_{Arg})$,

where Q_{Orn} is the flux of ornithine, IE_{Orn} is the plasma enrichment of the M+5 isotopomer of ornithine (that is, $^{13}C_5$-ornithine derived from $^{13}C_6$-arginine), and IE_{Arg} is the isotopic enrichment of the M+6 isotopomer of arginine.

De novo Arginine Synthesis from citrulline was calculated as:

Arg Synthesis (μmol kg-LBM^{-1} h^{-1}) = $Q_{Cit \rightarrow Arg} = Q_{Arg} \times (IE_{Arg} / IE_{Cit})$,

where Q_{Arg} is the total flux of arginine, IE_{Arg} is the plasma enrichment of the M+2 isotopomer of arginine (derived from $^2H_2$-citrulline), and IE_{Cit} is the plasma enrichment of the M+2 isotopomer of citrulline.

Because arginine flux in the fasting state consists solely of arginine released from proteolysis and *de novo* synthesis, *arginine derived from protein breakdown* was obtained as follows:

Arg from Protein Breakdown = Arg Flux – Arg Synthesis

Nitric Oxide (NO) Synthesis Rate was calculated from the rate of conversion of arginine to citrulline by the NO synthase reaction(10; 11):

NO Synthesis (μmol kg-LBM^{-1} h^{-1}) = $Q_{Arg \rightarrow Cit} = Q_{Cit} \times (IE_{Cit} / IE_{Arg})$,

where Q_{Cit} is the flux of citrulline, IE_{Cit} is the plasma enrichment of the M+6 isotopomer of citrulline (that is, $^{13}C_6$-citrulline derived from $^{13}C_6$-arginine), and IE_{Arg} is the isotopic enrichment of the M+6 isotopomer of arginine.

Protocol 2:

Total arginine and citrulline fluxes ($Q_{Arg,}$ Q_{Cit}) were calculated by:

Flux (μmol kg-LBM^{-1} h^{-1}) = $(IE_{Inf} / IE_{Pl}) \times i$

where IE_{Inf} and IE_{Pl} are the isotopic enrichments of the tracer in the infusate and in plasma at isotopic steady state (M+2 isotopomer for arginine, M+2 isotopomer for citrulline) and *i* is the rate of infusion of $^{15}N_2$-arginine or $^{2}H_2$-citrulline in μmol·kg $LBM^{-1}·h^{-1}$.

De novo arginine synthesis from citrulline was calculated from:

Arg synthesis (μmol kg-LBM^{-1} h^{-1}) = $Q_{Cit→Arg} = Q_{Arg}$ x (IE_{Arg} / IE_{Cit})

where Q_{Arg} is the total flux of arginine, IE_{Arg} is the plasma enrichment of the M+2 isotopomer of arginine (that is derived from $^{2}H_2$-citrulline), IE_{Cit} is the plasma enrichment of the M+2 isotopomer of citrulline.

NO synthesis rate was calculated from the rate of conversion of arginine to citrulline by the NO synthase reaction: NO synthesis (μmol kg-LBM^{-1} h^{-1}) = $Q_{Arg→Cit} = Q_{Cit}$ x (IE_{Cit} / IE_{Arg})

where Q_{Cit} is the flux of citrulline, IE_{Cit} is the plasma enrichment of the M+1 isotopomer of citrulline (ureido-^{15}N-citrulline derived from $^{15}N_2$-arginine), IE_{Arg} is the plasma enrichment of the M+2 isotopomer of arginine.

Underlying principles and assumptions of stable isotope tracer methodology used in the present study.

1. Stable isotope tracers: Because stable isotopes of elements such as carbon, nitrogen, hydrogen and oxygen occur naturally in several biomolecules of plants and animals they are metabolically and physiologically indistinguishable from the unlabeled biomolecules. Because of this unique property biomolecules containing an element, such as carbon (Atomic weight 12) highly labeled with its stable isotope (Atomic weight 13) can be used as a tracer to quantify the metabolic kinetics of the biomolecule (tracee) *in vivo*. This is particularly useful in human studies because unlike radioactive tracers it does not pose the adverse health risks of radiation exposure.

2. The single whole body pool model: The constant infusion tracer method utilized in this study assumes that the free *arginine, ornithine, citrulline and phenylalanine* present in all

organs, tissue beds and the circulation systems flow freely in and out of each other and as such can be represented by a single whole body pool. This pool receives inflow of these 4 amino acids from the diet, from *de novo* synthesis (except phenylalanine) and (in the case of phenylalanine and arginine) from cellular protein breakdown. Outflow of the four amino acids from the pool can undergo catabolism or be used to synthesize other metabolites or proteins in the case of arginine and phenylalanine.

Arginine is a unique exception to this assumption because its free body pool excludes the liver which is a net consumer of arginine as it takes up arginine (net inflow) but does not release it (zero outflow). This happens because free arginine in the liver is rapidly metabolized to urea via arginase within the urea cycle located in periportal hepatocytes. For this reason in the current study we refer to the arginine pool as the "*extrahepatic* whole body pool"

Further, in the current study we chose to make all kinetic measurements in the fasting state to avoid inflow of amino acids from the diet, can be highly variable in different individuals, into the whole body pool.

3. Measurement of flux (inflow and outflow from the pool): Flux through the pool, an index of production and utilization of the tracee can be quantified by administering its tracer by constant infusion. Because the stable isotope labeled tracer is indistinguishable from the tracee, its rate of inflow and outflow from the pool will be identical. Soon after the tracer infusion starts, the amount of tracer relative to tracee in the pool will be lower and the tracer will leave at a slower rate. However as the concentration of tracer increases there comes a time when both tracer and tracee will be entering and leaving the pool at the same rate, hence the isotopic enrichment (tracer/tracee ratio) of the tracer reaches a constant value (plateau). At this time, the rate of inflow and outflow of the tracer and tracee are the same and the constant isotopic enrichment of the tracer is a direct result of its dilution by the constant flow through the pool of the tracee. Hence, a simple steady state equation can be used to calculate flux of the tracee,

that is inflow (or rate of production) and outflow (or rate of utilization) from the pool by dividing the rate of infusion of the tracer (dose rate) by its measured plateau isotopic enrichment and subtracting the tracer dose to give the tracee flux.

In this study the pools of all four amino acids and their products of interest (in the case of arginine and citrulline) were primed with the appropriate tracer at the start of each infusion in order to shorten the time taken to achieve a plateau enrichment in both the precursor and product pools.

4**. Measurement of arginine conversion to ornithine (hydrolysis), citrulline and nitric oxide (NO), and its oxidation to carbon dioxide; and the conversion of citrulline to arginine (*de novo* arginine synthesis):** When the isotopic enrichments of both a tracer (precursor) and its product have achieved a plateau in the pool, the rate of transfer of isotope from the precursor to the product is constant. At this time, i.e., at isotopic steady state, its rate of conversion can be calculated using a precursor-product equation based on the measured isotopic enrichment of the product derived from the precursor tracer multiplied by the flux (rate of production) of the product. For example, in this study, $^{13}C_6$-arginine is hydrolyzed to form $^{13}C_5$-ornithine. By simultaneously measuring the flux of ornithine with a different tracer $^{15}N_2$-ornithine, the rate of conversion of arginine to ornithine was readily calculated. The conversions of arginine to NO plus citrulline and of citrulline to arginine were calculated similarly.

With respect to arginine oxidation rate, the oxidation of $^{13}C_6$-arginine produces 6 molecules of $^{13}CO_2$. When the isotopic enrichments of both precursor and product are at plateau, the oxidation rate can be obtained by dividing the rate of excretion of the product $^{13}CO_2$ (VCO_2 x isotopic enrichment) in expired air by the isotopic enrichment of the precursor $^{13}C_6$-arginine.

5. Bioavailability of arginine: Because arginine is irreversibly lost when it is converted to CO_2, its bioavailability for anabolic reactions was obtained by subtracting its oxidation rate from its flux, an index of its rate of production in the extrahepatic body pool.

Sample Size Calculation for Protocol 2

This was based on data from our previous study of citrulline supplementation and arginine flux measurements in 10 adult patients with MELAS syndrome (Reference 6 of the main manuscript). When supplemented with citrulline for 3 days, arginine production (flux) in the participants changed from 50.0±9.8 μmol kg-BW^{-1} h^{-1} (Mean ± SD) to 130±28.8 μmol kg-BW^{-1} h^{-1} (Mean ± SD), that is by 80 μmol kg-BW^{-1} h^{-1}. We conservatively assumed that the response in arginine flux in the present KPD subjects would be 50% less, that is 40.0 μmol kg-BW^{-1} h^{-1} (a clinically meaningful difference), and that there would be no change from baseline in Arg flux in response to placebo treatment. Using a standard deviation of 28.8 (based on the previous study cited above), a sample size of 7 subjects would be required to detect a difference in Arg flux (production) between supplementations (Citrulline vs. Alanine placebo) with 80% power with a two-sided significance level of 0.05. Given the complexity of the protocol (4 visits per participant, with two rounds of supplementation), we recruited 10 KPD participants to allow for dropouts. In the event, there were no dropouts and all 10 KPD participants completed the crossover study.

ESM Table 1 Plasma glucose and insulin concentrations in control, ketosis prone diabetes (KPD) and type 2 diabetes groups immediately before bolus arginine or glucose in the euglycaemic study, and plasma glucose concentrations in the hyperglycaemic study at the same experimental times (Experimental Protocol 1). Insulin concentrations during isotopic steady state during the two studies in Experimental Protocol 1 are also shown.

Concentration	**Controls**	**KPD**	**T2D**	One-way ANOVA *p*-value
		Before bolus arginine		
	n = 10	n = 10	n = 7	
Euglycaemic study				
Glucose, mmol/l	5.03 ± 0.20 *	5.29 ± 0.14 *†	5.74 ± 0.15 †	0.032
Insulin, pmol/l	51.0 ± 9.38 *	131 ± 27.4 †	104 ± 30.9 *†	0.048
Hyperglycaemic study				
Glucose, mmol/l	6.00 ± 0.16 *	5.66 ± 0.17 †	5.63 ± 0.36 †	0.001
		Before bolus glucose		
	n = 10	n = 10	n = 7	
Euglycaemic study				
Glucose, mmol/l	4.97 ± 0.10 *	5.35 ± 0.17 *†	5.90 ± 0.24 †	0.004
Insulin, pmol/l	43.8 ± 9.58 *	121 ± 22.7 †	92.0 ± 26.3 *†	0.025
Hyperglycaemic study				
Glucose, mmol/l	6.85 ± 0.49	4.77 ± 0.35	4.67 ± 0.28	0.409
		At plateau enrichment of stable isotope infusion (isotopic steady state) §		
	n = 11	n = 10	n = 7	
Euglycaemic study				
Insulin, pmol/l	59.0 ± 8.88 *	141 ± 28.5 †	124 ± 35.7 *†	0.044
Hyperglycaemic study				
Glucose, mmol/l	11.9 ± 1.02 *	15.7 ± 0.62 †	16.2 ± 1.93 †	0.016
Insulin, pmol/l	1699 ± 340	1291 ± 314	874 ± 249	0.236

Data expressed as means ± SEM. One-factor repeated-measures ANOVA was used to test the statistical differences between the groups. Between group comparisons made by one-way ANOVA; Means in a row without a common symbol are different, $p < 0.05$ (post hoc Tukey's multiple comparison test). T2D, type 2 diabetes

§ Values are means of insulin concentrations in plasma samples obtained at the 4th and 5th hours of the stable isotope infusions in both studies and means of glucose concentrations in plasma samples obtained at the same time points of the stable isotope infusions in the hyperglycaemic study. (For the euglycaemic study (performed in the fasted state), glucose concentrations were not measured at the 4th and 5th hours of the isotope infusions – they would be similar to those measured before the arginine bolus in the euglycaemic study.) Plasma samples for calculation of flux measurements were also obtained between 4h and 5h (4h, 4:20, 4:40 and 5h), at isotopic steady state.

ESM Table 2 Summary of the main kinetic findings in Protocol 1.

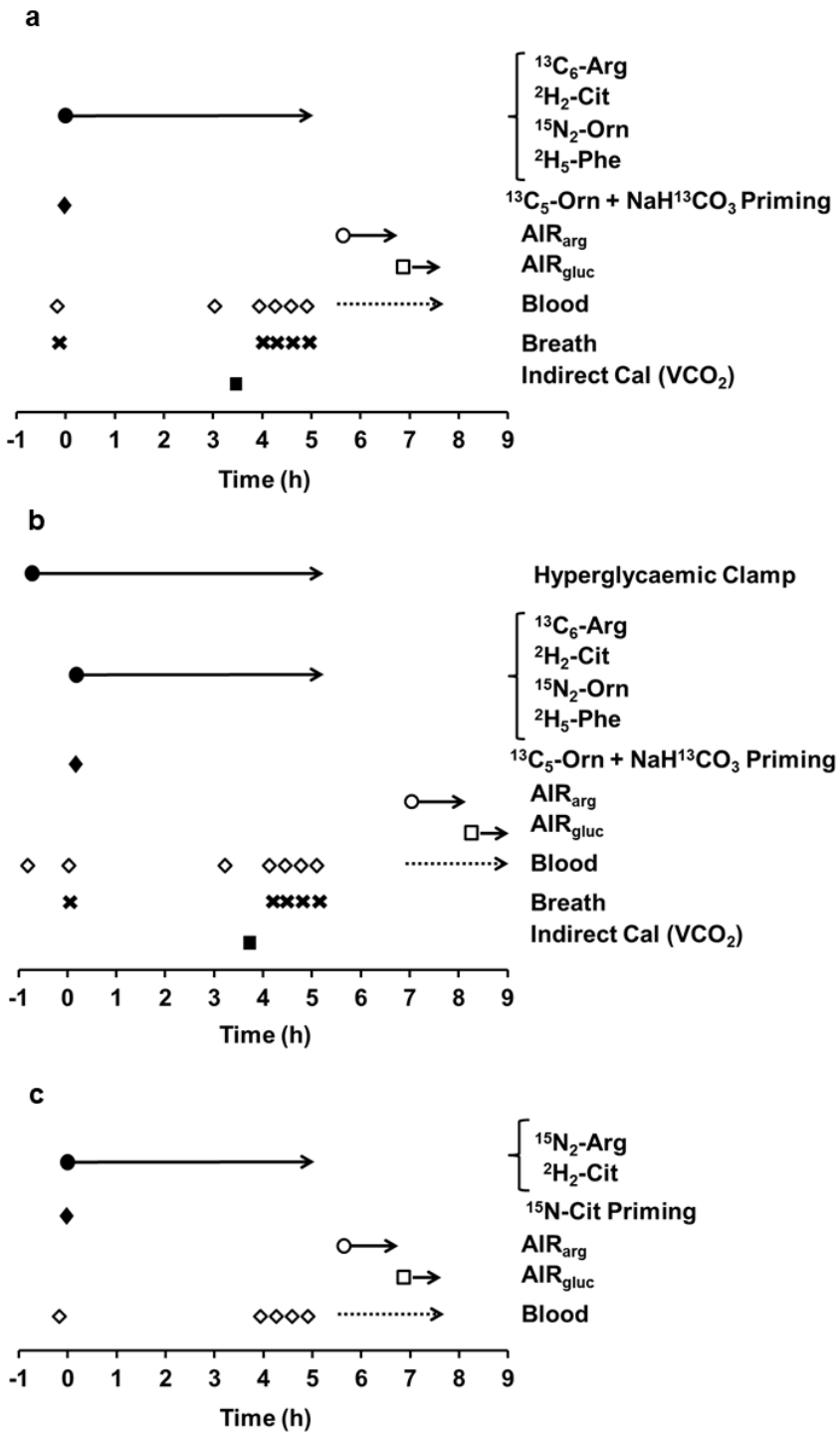
Kinetics	KPD compared to T2D and Controls
Euglycaemia	
Arginine flux	No difference between groups
Arginine synthesis	KPD and Controls are similar; KPD trend slower compared to T2D
Arginine from protein breakdown	No difference between groups
Arginine oxidation	No difference between groups
Arginine bioavailability	No difference between groups
Hyperglycaemia	
Arginine flux	KPD and Controls are similar; KPD trend slower compared to T2D
Arginine synthesis	No difference between groups
Arginine from protein breakdown	KPD and Controls are similar; KPD trend slower compared to T2D
Arginine oxidation	No difference between groups
Arginine bioavailability	KPD and Controls are similar; KPD significantly lower compared to T2D
Difference in moving from euglycaemic to hyperglycaemic state	
Arginine flux	KPD and Controls are similar; KPD trend slower compared to T2D
Arginine synthesis	KPD and T2D are similar; Controls trend slower compared to KPD
Arginine from protein breakdown	KPD and Controls are similar; KPD trend slower compared to T2D
Arginine oxidation	No difference between groups
Arginine bioavailability	KPD and Controls are similar; KPD significantly lower compared to T2D

T2D, type 2 diabetes

ESM Table 3 Summary of the main kinetic findings in Protocol 2.

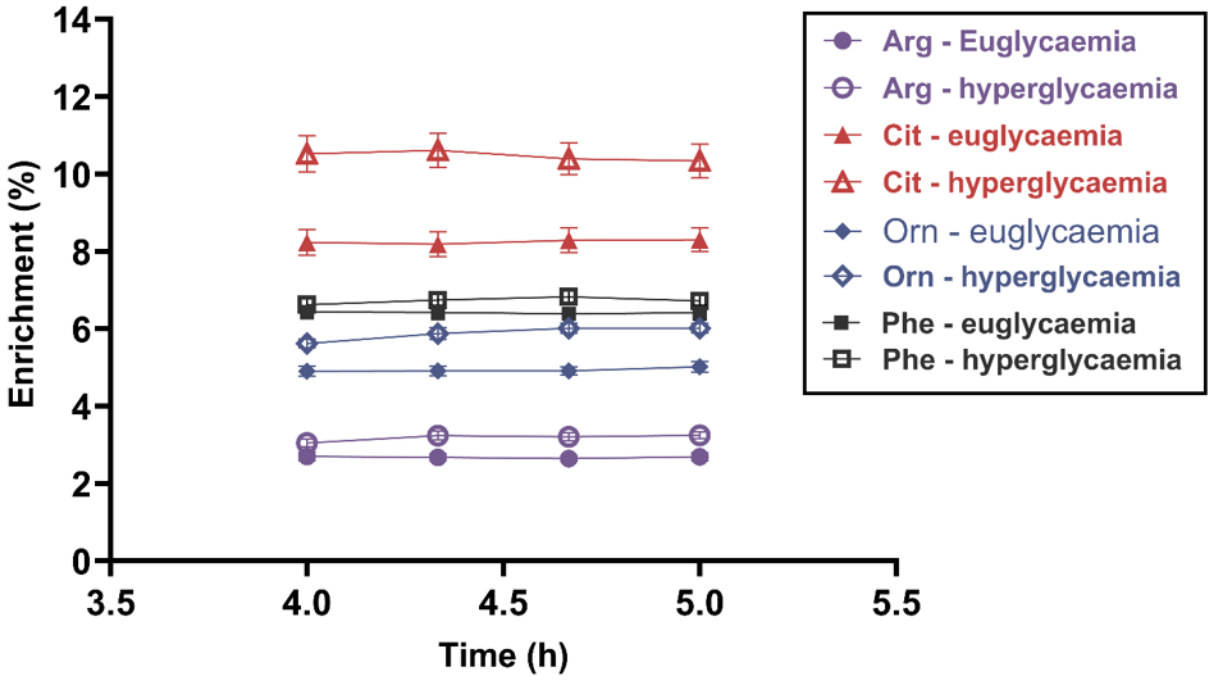
Kinetic Measurement	Effect of Supplementation (Change from pre-treatment to post-treatment)	
	Alanine	Citrulline
Arginine flux	↔	↑
Arginine synthesis	↔	↑
Arginine availability (arginine concentration)	↔	↑
NO Synthesis	↔	↑

ESM Fig. 1



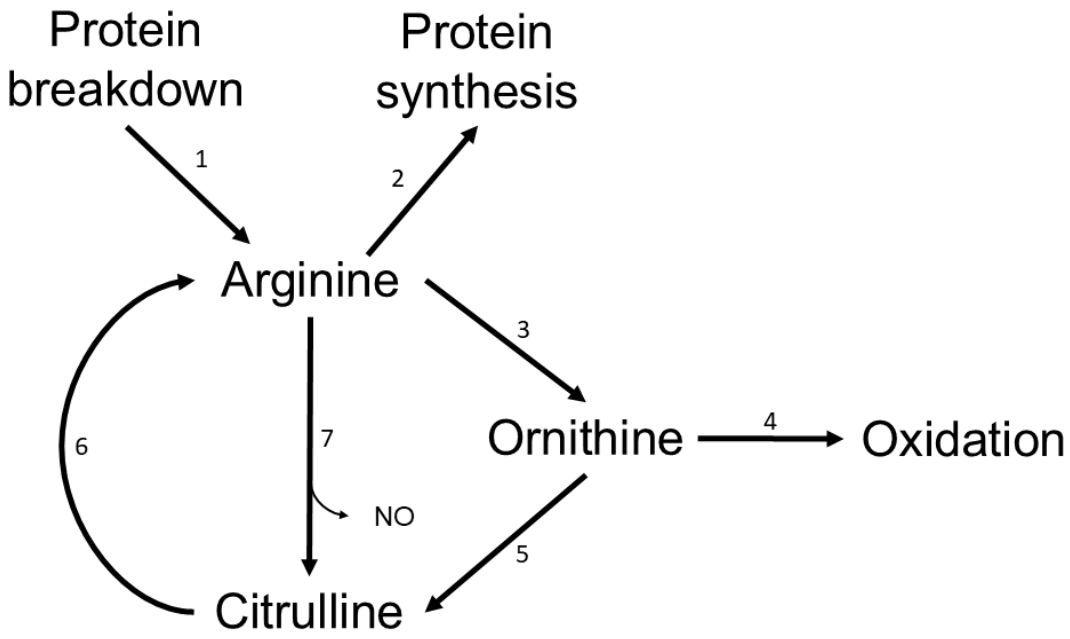
(**a, b**) Schema of Protocol 1 under euglycaemic (**a**) and hyperglycaemic (**b**) conditions. (**c**) Schema of Protocol 2 (pre-supplement and post-supplement).

ESM Fig. 2



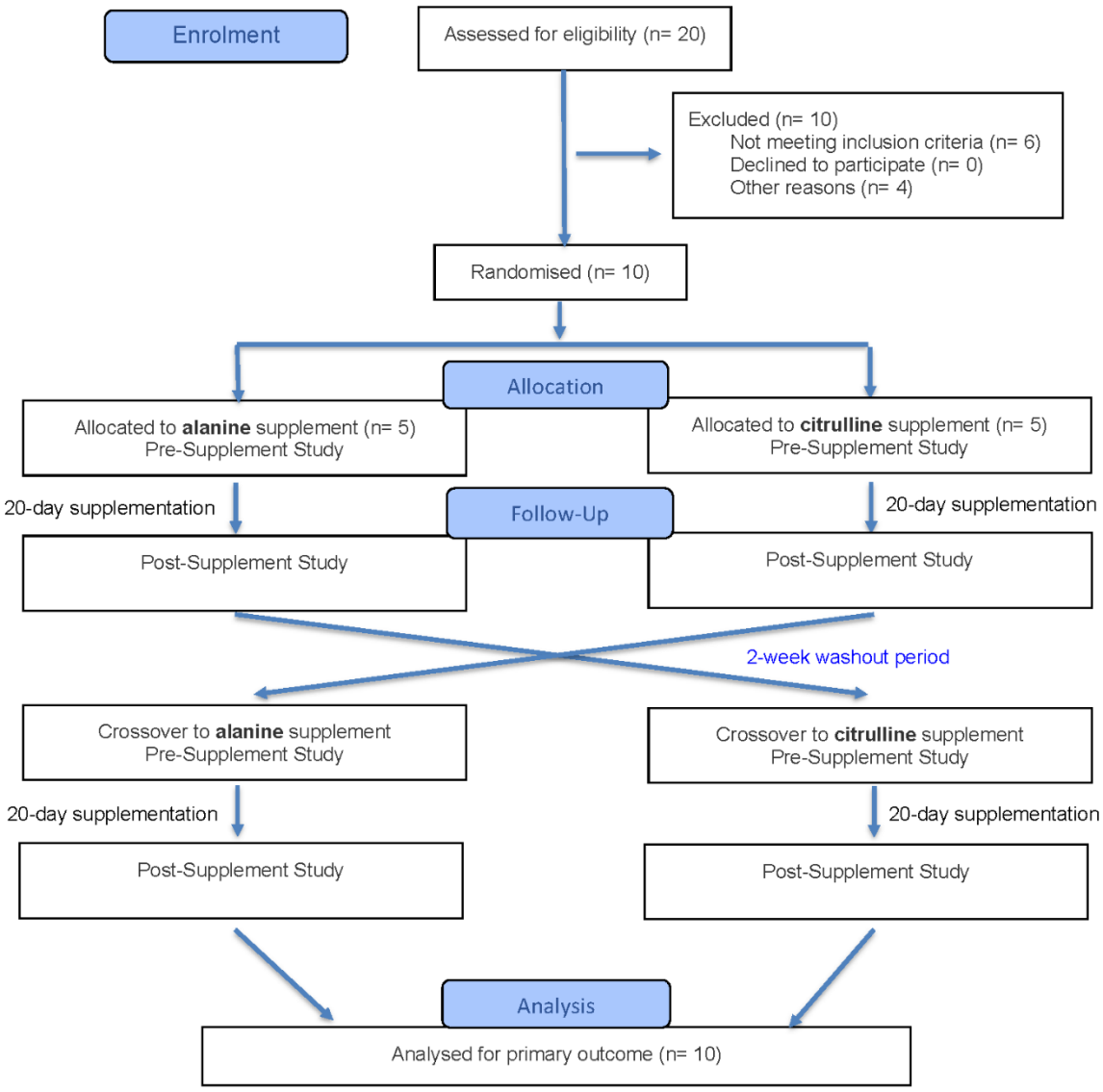
Plateau enrichment of $^{13}C_6$-Arginine, $^{2}H_2$-Citrulline, $^{15}N_2$-Ornithine and $^{2}H_5$-Phenylalanine during infusion in Protocol 1.

ESM Fig. 3



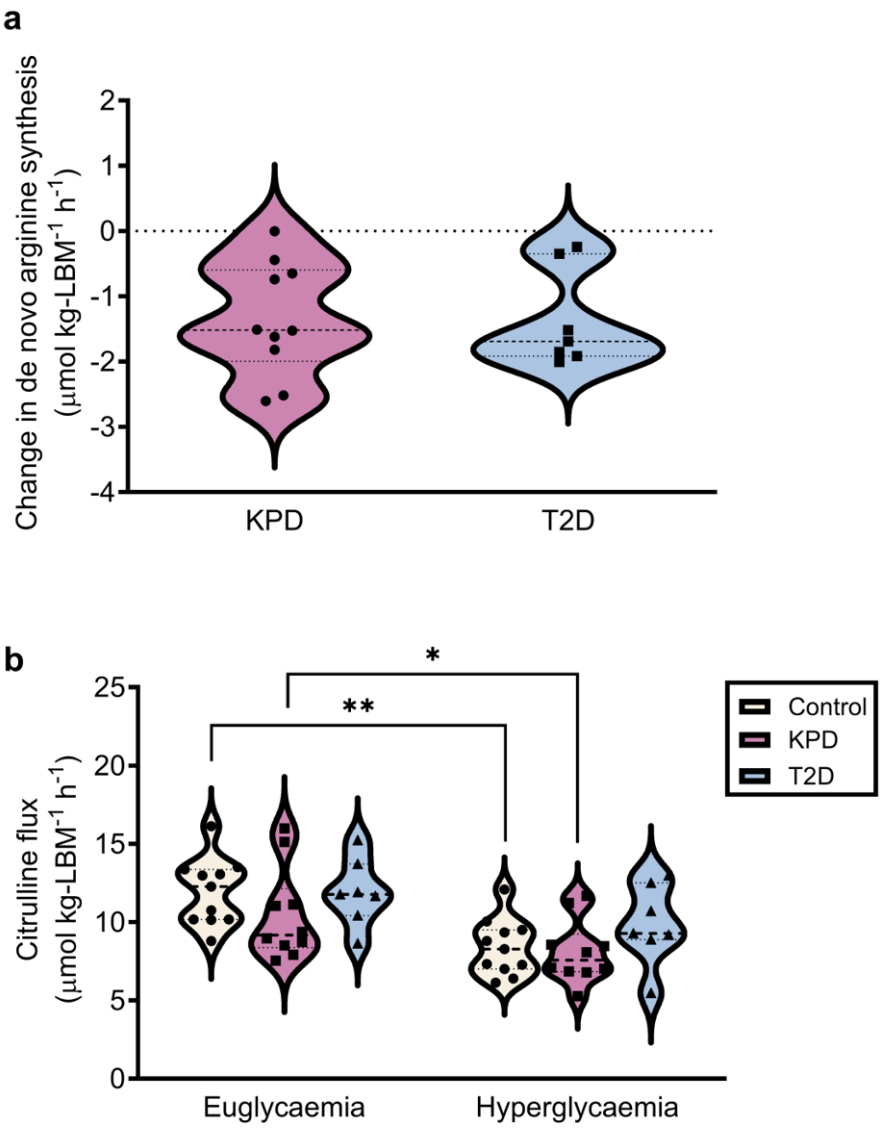
Schematic diagram of major pathways involved in arginine metabolism in the fasting state. Numbered arrows correspond to pathways measured in the study as described in Methods (Supplementary Material pages 3-5).

ESM Fig. 4



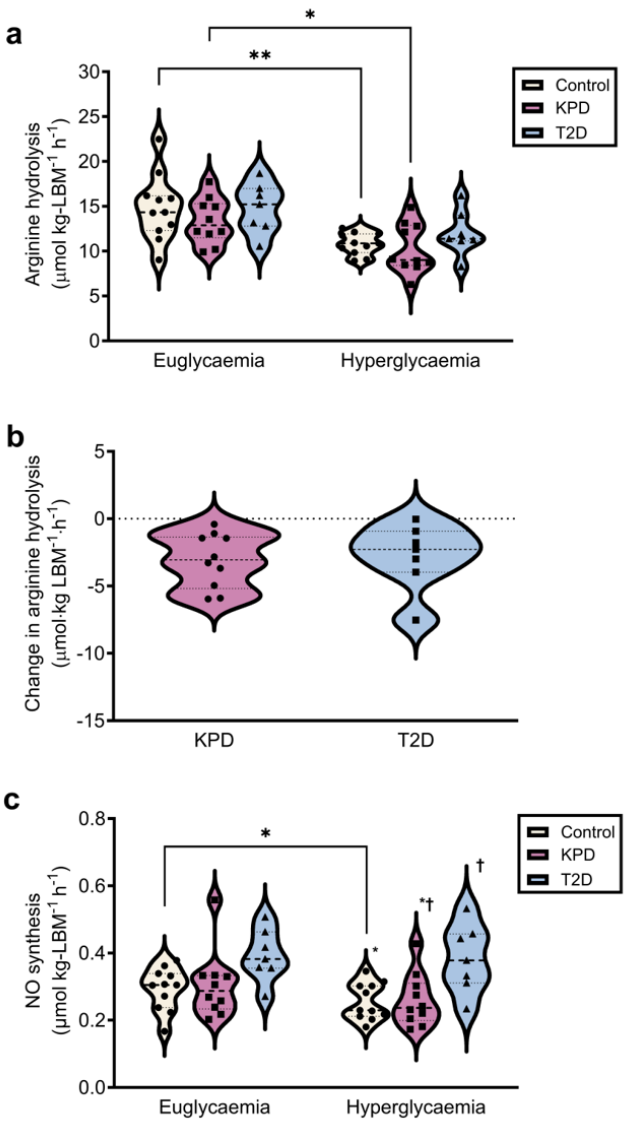
Protocol 2 CONSORT Flow Diagram

ESM Fig. 5



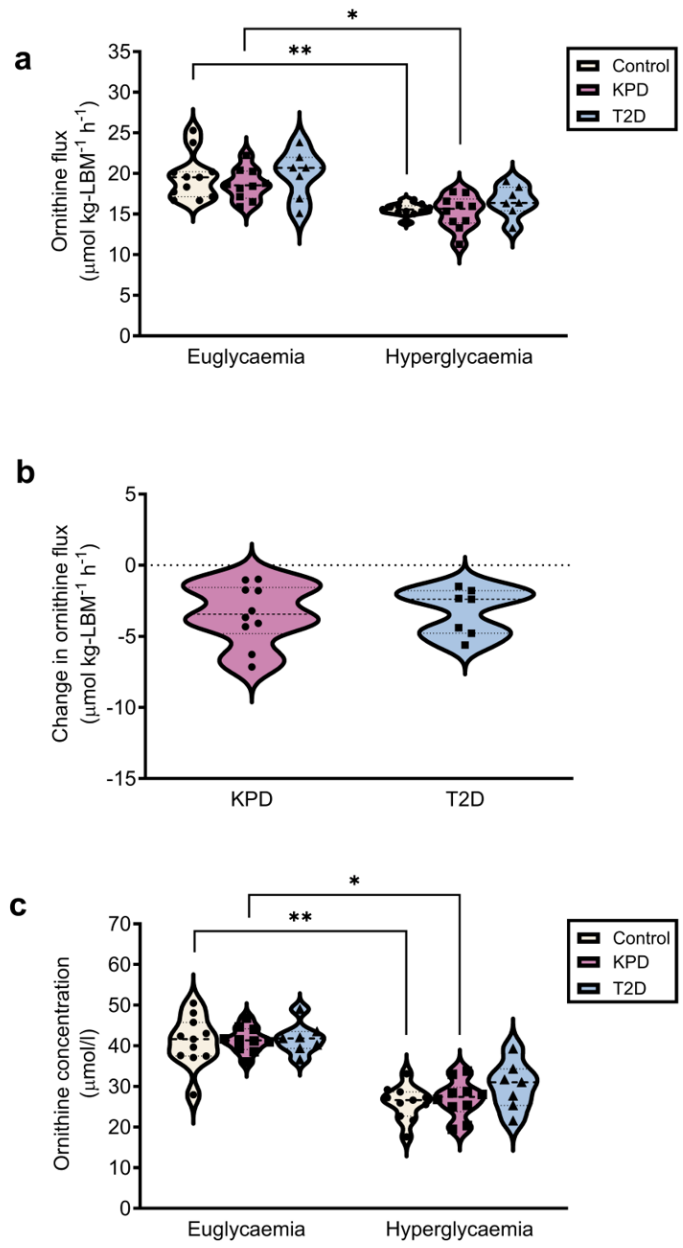
Change in de novo arginine (**a**) from the euglycaemic to the hyperglycaemic condition of participants in the two diabetes groups (KPD and type 2 diabetes). Significant difference in change between the two diabetes groups was determined by Wilcoxon rank-sum test. Citrulline flux (**b**) of participants in control, ketosis prone diabetes (KPD) and typical type 2 diabetes (T2D) groups. Wilcoxon signed-rank test was used to test for statistical difference between euglycaemic and hyperglycaemic conditions in each group and exact Wilcoxon rank-sum was used to test for significant differences between groups in each condition. A Bonferroni correction for 9 hypothesis tests was applied to obtain adjusted *p*-value (*$p<0.05$, **$p<0.01$). T2D, type 2 diabetes

ESM Fig. 6



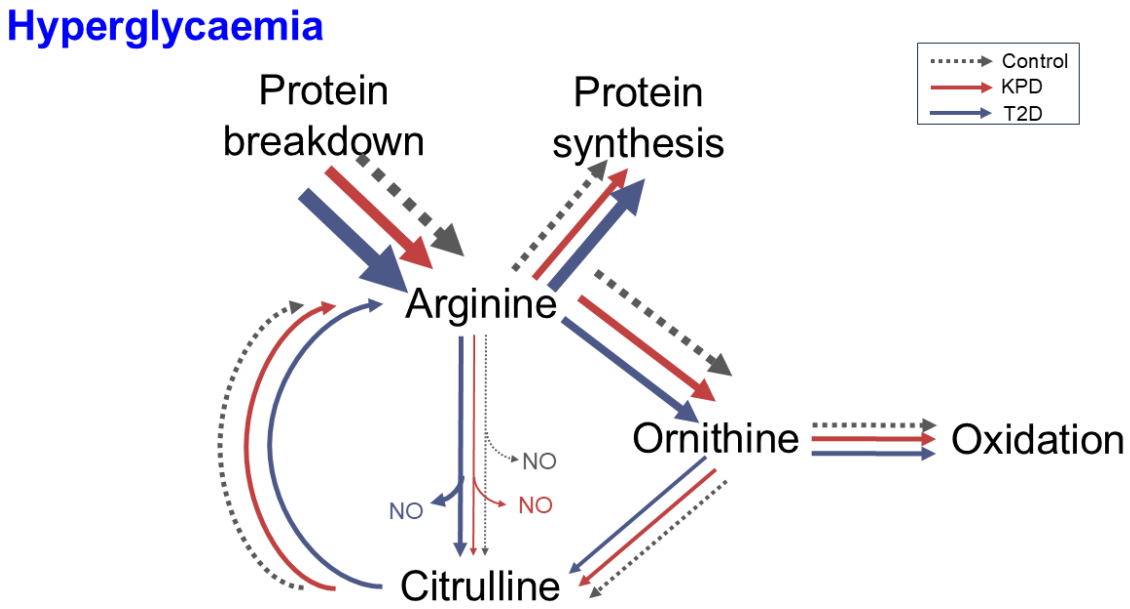
Arginine hydrolysis (**a**) and nitric oxide synthesis (**c**) rates of participants in control, KPD and type 2 diabetes groups. Wilcoxon signed-rank test was used to test for statistical difference between euglycaemic and hyperglycaemic conditions in each group and exact Wilcoxon rank-sum was used to test for significant differences between groups in each condition. A Bonferroni correction for 9 hypothesis tests was applied to obtain adjusted *p*-value (**p*<0.05, ***p*<0.01). Change in arginine hydrolysis (**b**) from the euglycaemic to the hyperglycaemic condition of participants in the two diabetes groups (KPD and T2D). Significant difference in change between the two diabetes groups was determined by Wilcoxon rank-sum test. In panels c, bars without a common symbol are different, *p*<0.05 within the same condition. T2D, type 2 diabetes

ESM Fig. 7



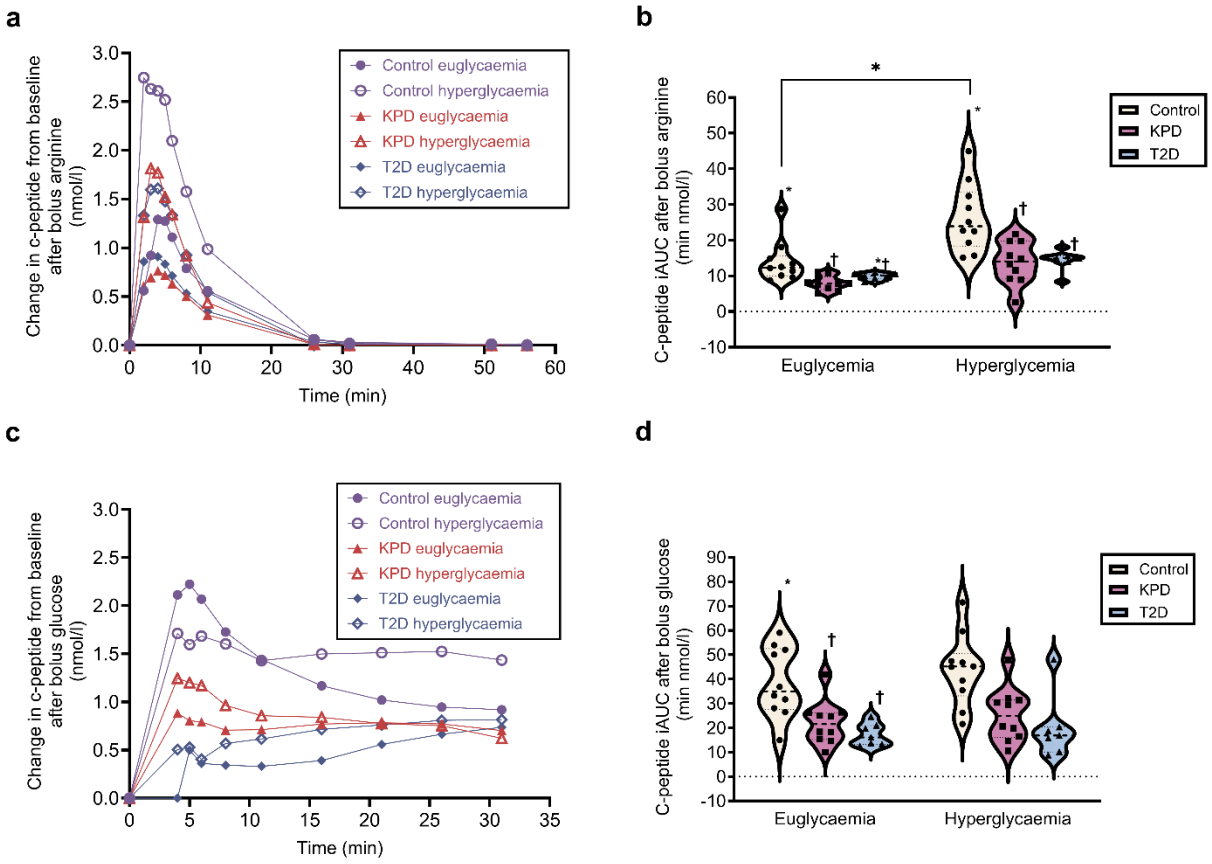
Ornithine flux (**a**) and plasma ornithine concentration (**c**) of participants in control, KPD and type 2 diabetes groups. Wilcoxon signed-rank test was used to test for statistical difference between euglycaemic and hyperglycaemic conditions in each group and exact Wilcoxon rank-sum was used to test for significant differences between groups in each condition. A Bonferroni correction for 9 hypothesis tests was applied to obtain adjusted p-value (*p<0.05, **p<0.01). Change in ornithine flux (**b**) from the euglycaemic to the hyperglycaemic condition of participants in the two diabetes groups (KPD and T2D). Significant difference in change between the two diabetes groups was determined by Wilcoxon rank-sum test. T2D, type 2 diabetes

ESM Fig. 8



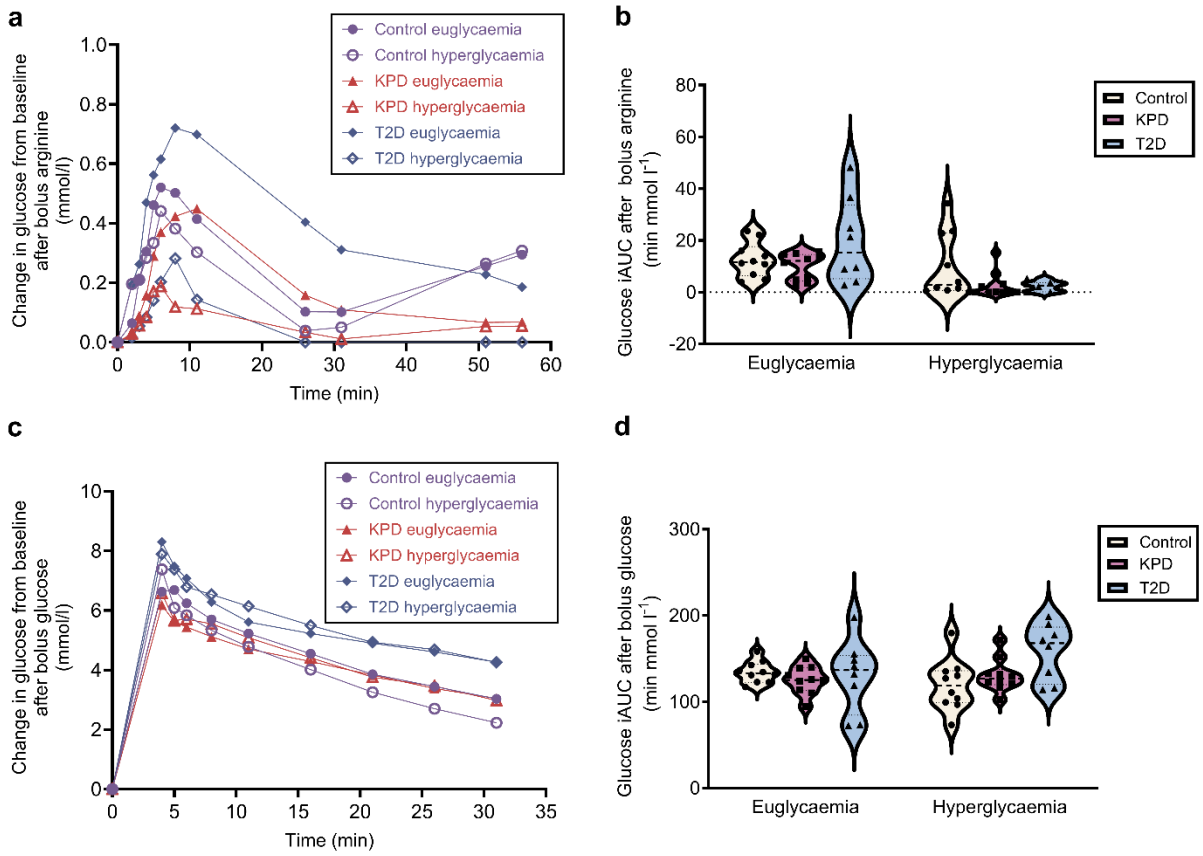
Schematic diagram showing relative differences between Control, KPD and type 2 diabetes patients in kinetic measurements of the pathways of arginine metabolism delineated in ESM Fig. 3, during sustained hyperglycaemia. Quantitative differences are reflected in the thickness of the arrows. See text for details. T2D, type 2 diabetes

ESM Fig. 9



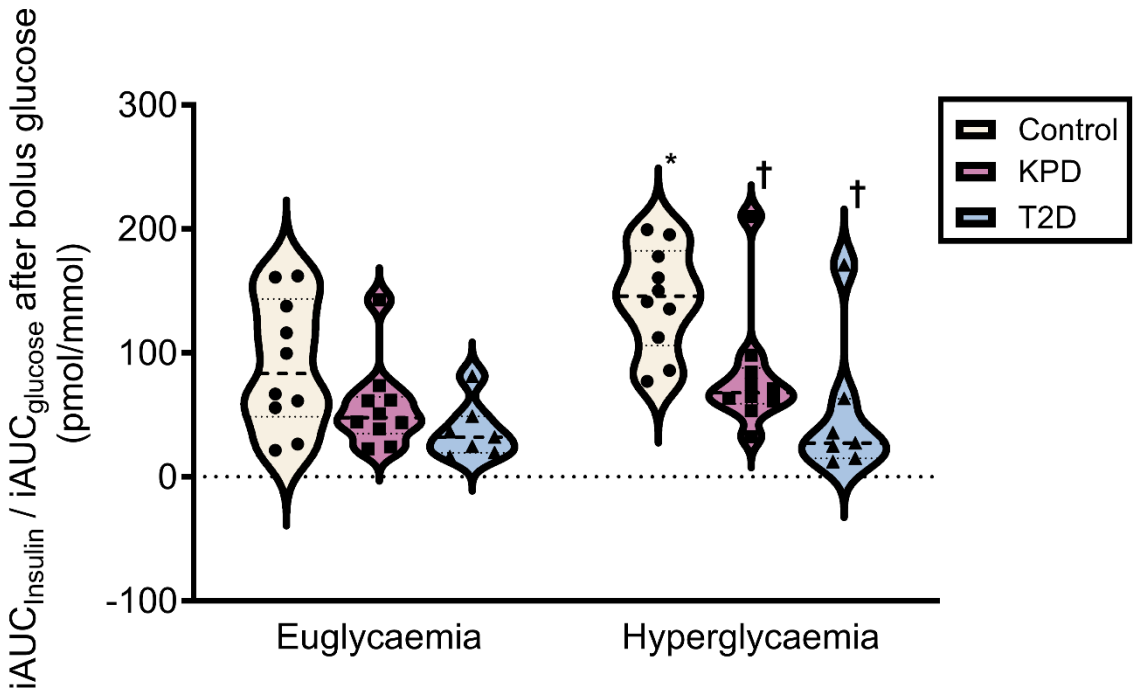
(**a**, **c**) Curves of plasma C-peptide concentrations (secretory response) during 56 minutes after an arginine bolus (**a**) and 31 minutes after a glucose bolus (**c**) in participants of control, KPD and type 2 diabetes groups under euglycaemic and hyperglycaemic conditions. Each point represents the mean value of the group at that time. (**b**, **d**) Incremental C-peptide area under the curve (iAUC) during 56 minutes after an arginine bolus (**b**) and during 31 minutes after a glucose bolus (**d**) in participants of control, KPD and T2D groups. Wilcoxon signed-rank test was used to test for statistical difference between euglycaemic and hyperglycaemic conditions in each group and exact Wilcoxon rank-sum was used to test for significant differences between groups in each condition. A Bonferroni correction for 9 hypothesis tests was applied to obtain adjusted p-value (*p<0.05). In panels b and d, bars without a common symbol are different, p<0.05 within the same condition. T2D, type 2 diabetes

ESM Fig. 10



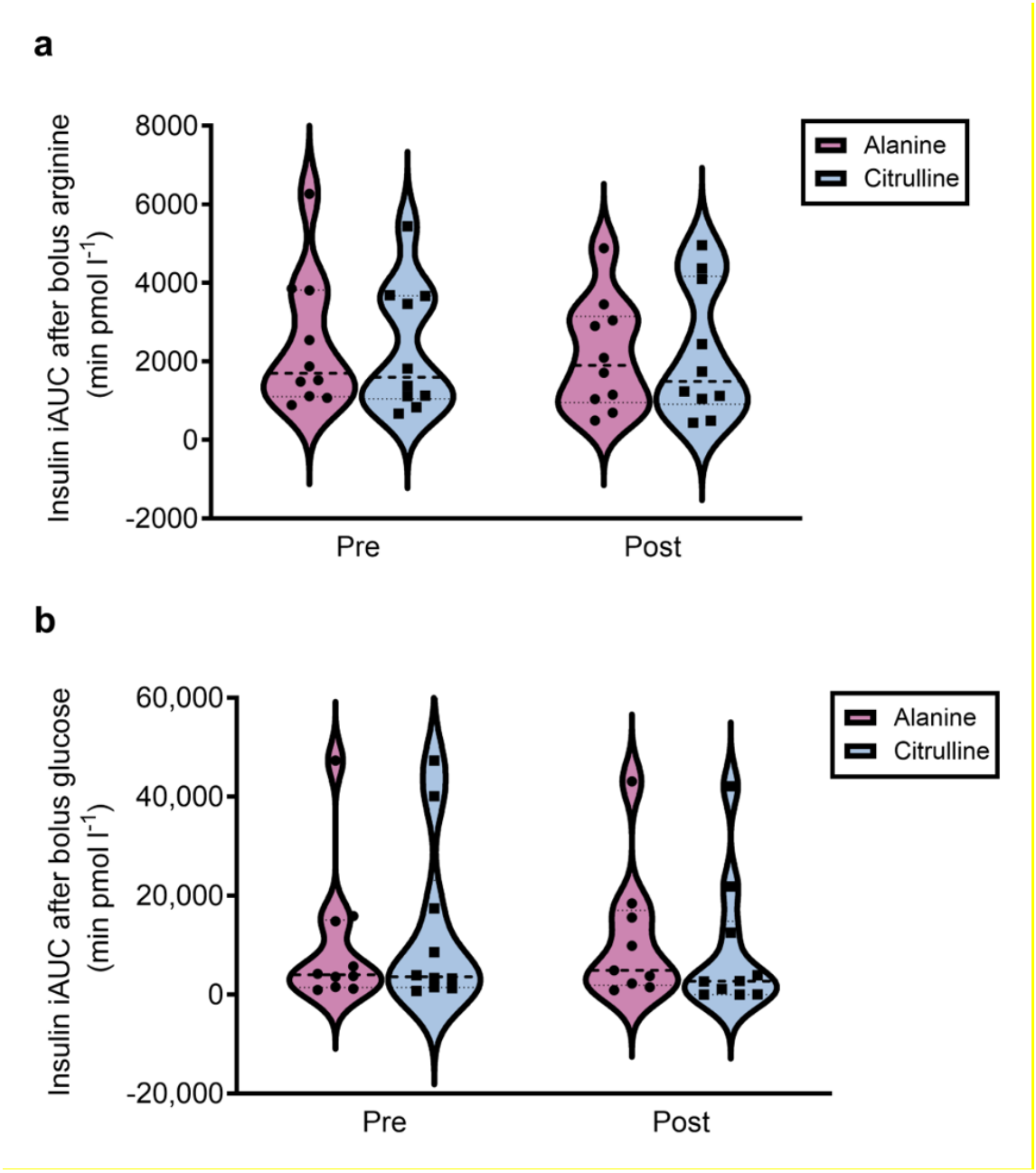
(**a**, **c**) Curves of incremental glucose concentrations during 51 minutes after an arginine bolus (**a**) and during 31 minutes after a glucose bolus (**c**) in participants of control, KPD and type 2 diabetes groups under euglycaemic and hyperglycaemic conditions. Each point represents the mean value of the group at that time. (**b**, **d**) Incremental glucose area under the curve (iAUC-glucose) during 56 minutes after an arginine bolus (**b**) and during 31 minutes after a glucose bolus (**d**) in participants of control, KPD and T2D groups. Wilcoxon signed-rank test was used to test for statistical difference between euglycaemic and hyperglycaemic conditions in each group and exact Wilcoxon rank-sum was used to test for significant differences between groups in each condition. A Bonferroni correction for 9 hypothesis tests was applied to obtain adjusted p-value. T2D, type 2 diabetes

ESM Fig. 11



Incremental insulin area under the curve (iAUC-insulin) / incremental glucose (iAUC-glucose) during 31 minutes after a glucose bolus in participants of control, KPD and T2D groups in euglycaemic and hyperglycaemic conditions. Wilcoxon signed-rank test was used to test for statistical difference between euglycaemic and hyperglycaemic conditions in each group and exact Wilcoxon rank-sum was used to test for significant differences between groups in each condition. A Bonferroni correction for 9 hypothesis tests was applied to obtain adjusted p-value. Without a common symbol are different, p<0.05 within the same condition. T2D, type 2 diabetes

ESM Fig. 12



(**a**, **b**) Incremental insulin area under the curve (iAUC) during 56 minutes after an arginine bolus (**a**) and during 31 minutes after a glucose bolus (**b**) in KPD participants after supplementation with alanine or citrulline in Experimental Protocol 2. Wilcoxon signed-rank test was used to test statistical difference between pre and post supplementation in each supplement. Wilcoxon matched-pairs signed-rank test was used to test statistical differences between two supplements in the same condition. A Bonferroni correction for 4 hypothesis tests was applied to obtain adjusted p-value.