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Developmental changes in the utilization of citrulline by neonatal pigs

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Abstract

Developmental changes in the renal expression and activity of argininosuccinate synthase (ASS1) and argininosuccinate lyase (ASL), enzymes that use citrulline for the production of arginine, have been reported. Thus, the ability of neonates, and especially premature neonates, to produce arginine may be compromised. To determine the utilization of citrulline in vivo, we measured renal expression of *ASS1* and *ASL* and conducted citrulline compartmental and noncompartmental kinetics using [^{15}N]citrulline in pigs of five different ages (from 10 days preterm to 5 wk of age). The tracer was given in substrate amounts to also test the ability of neonatal pigs to use exogenous citrulline. Preterm and term pigs at birth had lower *ASS1* and *ASL* expression than older animals, which was reflected in the longer half-life of citrulline in the neonatal groups. The production and utilization of citrulline by 1-wk-old pigs was greater than in pigs of other ages, including 5-wk-old animals. Plasma citrulline concentration was not able to capture these differences in citrulline production and utilization. In conclusion, the developmental changes in renal *ASS1* and *ASL* gene expression are reflected in the ability of the pigs to use citrulline. However, it seems that there is an excess capacity to use citrulline at all ages, including during prematurity, since the bolus dose of tracer did not result in an increase in endogenous citrulline. Our results support the idea that citrulline supplementation in neonatal, including premature, pigs is a viable option to increase arginine availability.

Keywords: arginine, citrulline, kinetics, neonatal, premature

INTRODUCTION

The endogenous synthesis of the conditionally essential amino acid arginine relies in the production of citrulline by the gut (23) and further conversion into arginine by other tissues and organs (20). Among these, the kidney is the main site for the net production of arginine (4, 7) due to the high expression and abundance of argininosuccinate synthase (ASS1) and argininosuccinate lyase (ASL), enzymes responsible for the conversion of citrulline into arginine. This intestinal-renal axis is responsible for most of "de novo" arginine synthesis, although additional extrarenal sources of arginine production have been identified (9, 15). The contribution of this de novo pathway to circulating arginine during the postprandial period accounts for ~10–15% of the total arginine flux (1, 5, 16), with the remainder originating from protein turnover.

During the neonatal period, ASS1 and ASL undergo developmental changes. Earlier in life, the renal abundance and activity of these two enzymes are low, but they increase toward the third week of life in rodents (10, 11) and pigs (14, 24). This, together with the presence of ASS1 and ASL in enterocytes during the neonatal period (normally absent in adults), led some researchers to postulate that the intestinal-renal axis was not functional in neonates (3, 11, 19). Recently, we have shown that the axis is present and functional in neonatal pigs and that the neonatal gut exports citrulline (14). Furthermore, we showed that the kidney uses this plasma citrulline to produce arginine. However, it is still unclear if neonatal pigs, especially those born prematurely, are able to use citrulline at the same rate as older animals. This is important because if citrulline utilization is impaired in neonatal pigs, this would prevent the use of citrulline supplementation to increase arginine availability. To test the hypothesis that the stage of development affects the rate of utilization of citrulline and ability to use exogenous citrulline, we determined citrulline kinetics in pigs of different ages.

MATERIALS AND METHODS

General

Domestic conventionally-reared crossbred pigs were obtained from a local commercial swine farm. All animal procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Animals and Instrumentation

Pigs in five different age groups were studied. Ten-day preterm (10PT) pigs (n = 8, 4 male and 4 female pigs) and five-day preterm (5PT) pigs (n = 8, 5 male and 3 female pigs) were obtained after cesarean section at *postconception day 104* (n = 4 sows) and *postconception day 109* (n = 4 sows), respectively (gestation length: 114 days). Piglets were resuscitated, and the umbilical artery was catheterized under isoflurane anesthesia. The catheter was advanced high into the thoracic aorta, and catheter placement (tip between the diaphragm and aortic arch) was verified during necropsy. Term (T) pigs (n = 6 from 2 litters, 3 male and 3 female pigs) were delivered naturally and catheterized immediately as indicated above. Preterm and T pigs were studied within 4–6 h of

birth and had no access to any source of feed. One-week-old (1W) pigs (n = 8 from 2 litters, 5 male and 3 female pigs) were littermates of T pigs and stayed with the sow until the day of the study. After a 4- to 6-h removal period from the sow (and feed deprivation), the pigs were implanted with a carotid catheter under isoflurane anesthesia. The carotid catheter was advanced, and the tip of the catheter was placed in the aortic arch. Five-week-old (5W) pigs (n = 8, 4 male and 4 female pigs) were weaned at the farm at 21 days of age and brought to the research facility at 28 days of age. 5W pigs were fed the same soybean meal-corn mix from weaning until the day of the study. After a 7-day acclimation period, feed was removed for 8 h, and pigs were implanted with a carotid catheter as indicated above.

Tracer Administration and Sampling

Pigs were allowed to recover for 1–2 h after surgery. After two background blood samples had been collected 15 min apart, a bolus dose of (ureido) [^{15}N]citrulline (50 μmol/kg) was given using the arterial (umbilical or carotid) catheter and flushed with 10 mL normal saline (9 g NaCl/L) to ensure that all the dose was delivered into the animal and that the infusate was thoroughly flushed from the catheter. The syringe used to deliver the bolus dose of tracer was weighed using an analytical balance before and after tracer administration. Additional blood samples were taken 2.5, 5, 7.5, 10, 15, 20, 30, 40, 50, 60, 70, and 80 min thereafter. Blood samples were centrifuged immediately, and the plasma was stored at −80°C. After the last sample had been taken, pigs were euthanized with a commercial phenytoin-pentobarbital solution; the kidneys were collected, weighed, and snap frozen in liquid nitrogen.

Sample Analysis

Citrulline and arginine enrichments were determined by liquid chromotography-tandem mass spectroscopy (LC-MS/MS) after their derivatization with dansyl chloride as previously described (13). The following mass-to-charge ratio (m/z) transitions were monitored to determine enrichments: citrulline 409 → 392, 410 → 392; arginine 408 → 391, 409 → 391. Internal standards ([$^{2}H_{7}$]citrulline and [$^{15}N_{4}{}^{13}C_{6}$]arginine) were used to calculate plasma concentrations in the same run.

Kidney samples were homogenized in guanidinium thiocyanate-phenol-chloroform extraction solution (TRIzol, Life Technologies, Gaithersburg, MD), and RNA was isolated using Qiagen RNeasy Mini elute columns (Qiagen, Chatsworth, CA). After cDNA synthesis, PCR amplification and gene differentiation expression for *ASS1* (forward: 5′-CCCTCTACAACGAGGAGCTG-3′ and reverse: 5′-TCTGGAGGCGATGATATTCC-3′) and *ASL* (forward: 5′-CAAGGTAGCTGAGGAGTG-3′ and reverse: 5′-GGCCGTATGGATGTCTTC-3′) were performed with SYBR Green 1on a multicolor Real-Time PCR detection system (CFX96, Bio-Rad Laboratories, Hercules CA). The geometric mean of GAPDH (forward: 5′-ACCTCCACTACATGGTCTACA-3′ and reverse: 5′-ATGACAAGCTTCCCGTTCTC-3′) and β-actin (forward: 5′-GGACCTGACCGACTACCTCA-3′ and reverse: 5′-GCGACGTAGCAGAGCTTCTC-3′) expression was used to normalize the expression of the genes of interest. Results are expressed as fold changes compared with the T group.

Calculations

Model fitting was performed by fitting different exponential equations to the tracer data. Based on visual inspection of the residuals and adjusted (pseudo)R^2, the following biexponential model best fitted the citrulline enrichment data:

$$Yt=M1\times e-g1\times t+M2\times e-g2\times t \quad (1)$$

where Y_t is the ureido [^{15}N]citrulline enrichment (tracer/tracee) at *time t* (in min), M_1 and M_2 are intercept parameters (tracer/tracee), and g_1 and g_2 are exponential parameters of the model (in min^{-1}) calculated for each individual pig using the NLMIXED procedure of SAS (version 9.4, SAS Institute, Cary, NC).

The citrulline rate of appearance (RaCit; in $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) was calculated by noncompartment analysis as follows:

$$RaCit=dose/AUCCit0-\infty\times 60 \quad (2)$$

and

$$AUCCit0-\infty=M1/g1+M2/g2 \quad (3)$$

where the dose was (nominally) 50 μmol/kg. $AUC_{Cit0\text{-}\infty}$ is the citrulline enrichment area under the curve (AUC; tracer/tracee × min) from *time 0* to infinity, (60) is a factor to convert minutes to hours, and M_1, g_1, M_2, and g_2 are the parameters of the biexponential function.

We were unable to fit a unique function to the arginine data; for this reason, the [^{15}N]arginine [originating from the (ureido) [^{15}N]citrulline infused] AUC was calculated using the trapezoidal rule. The fraction of plasma arginine from citrulline (Arg from Cit, in %) was calculated for the 80-min observation period using the following precursor-product equation:

$$Arg\ from\ Cit=AUC0-80\times 100/AUCCit0-80 \quad (4)$$

where $AUC_{Arg0-80}$ is the area under the tracer/tracee guanidino [^{15}N]arginine curve (tracer/tracee × min) from *time 0* to 80 min, 100 is a factor to express the fractional contribution as a percentage, and $AUC_{Cit0-80}$ is the area under the tracer/tracee ureido [^{15}N]citrulline curve (tracer/tracee × min) from *time 0* to 80 min calculated using the parameters of the biexponential function (*Eq. 1*).

The plasma citrulline clearance rate (i.e., the virtual volume of plasma cleared of citrulline per unit of time, in $\text{L}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) was calculated as follows:

$$CitClearance=RaCit/Cit \quad (5)$$

where [Cit] is the plasma concentration of citrulline (in μmol/L).

The half-life of citrulline (λ_{Cit}, in min; i.e., the time to dispose 50% of the citrulline molecules from the body) and the mean retention time (MRT, in min; the average time a molecule stays in the body) were determined as follows:

$$\lambda Cit = \ln 2 / g2 \quad (6)$$

$$MRT = AUMC / AUC \quad (7)$$

$$AUMC = M1/g12 + M2/g22 \quad (8)$$

where AUMC is the area under the momentum curve (tracer/tracee × min^2) and the other variables are as defined for *Eqs. 1 and 3*.

To gain a better understanding of citrulline metabolism, a compartmental analysis was performed using the standard formulas and notation scheme of Shipley and Clark (22) (see APPENDIX 1), where Q_a and Q_b are the sizes of *compartments A* and *B* (in mmol/kg body wt), respectively (Fig. 1), k_{yx} is the rate of transfer (in min^{-1}) of citrulline to *compartment Y* from *compartment X*, and F_{yx} is the flow rate (in $\mu mol \cdot kg^{-1} \cdot h^{-1}$) of citrulline to *compartment Y* from *compartment X*. In contrast to noncompartmental analysis, which is assumption free, compartmental analysis presupposes some previous knowledge. Based on the fitted model and the known metabolism of citrulline, we postulated the following structure for the two-compartment model (Fig. 1). The production of citrulline takes part (mostly) in the small intestine, and it is represented by the entry of citrulline (F_{ao}; Fig. 1) into the compartment (*compartment A*), where the tracer is given and samples are collected. Citrulline is then distributed and equilibrates (F_{ba} and F_{ba}; Fig. 1) with a second compartment (*compartment B*) from where it is disposed of (F_{ob}; Fig. 1). Note that at steady state $F_{ao} = F_{ob}$ and represents the total production and disposal of citrulline, respectively. Although the assumption of steady state over the period studied is not absolutely necessary (6), it was tested by monitoring changes in the concentration of endogenous (tracee) citrulline over time.

The volume of distribution of *compartment A* (V_a; in L/kg) can be determined as follows:

$$Va = Qa / Cit \quad (9)$$

Similarly, the volume of distribution of *compartment B* (V_b; in L/kg) can be calculated (assuming that plasma concentration reflects the concentration in this compartment) as follows:

$$Vb = Qb / Cit \quad (10)$$

Statistics

Data were analyzed using the proc mixed statement of SAS with age and sex (and their interaction) as fixed effects and litter as the random effect of the model. In addition, linear and quadratic preplanned orthogonal contrasts were used to assess the shape of the response to age. Because of the uneven spacing of "age," contrast coefficients were obtained using the ORPOL function of proc IML in SAS. Post hoc pairwise comparisons were conducted using Tukey's procedure. Plasma amino acid steady state was tested by fitting linear and quadratic orthogonal contrasts using the proc mixed statement of SAS with time as the fixed effect and pig (within age) as the random effect of the model. Pearson correlations between ASS1 and ASL and body weight and kidney weight were calculated using SAS's proc correlation procedure. Values are presented as means ± SE. Type I error was set at 0.05.

RESULTS

As expected, a strong linear effect ($P < 0.001$) of age on body and kidney weight was observed (Table 1); however, no effect of sex on body ($P = 0.70$) or kidney ($P = 0.37$) weight was detected. Kidney weight showed a high correlation with body weight ($r = 0.955$, $P < 0.001$), but there was no effect ($P > 0.15$) of either age or sex on the weight of kidney as a percentage of body weight (~7.5 g kidney/kg body wt).

Renal ASS1 and ASL Expression

There was an effect of age ($P < 0.001$) on the kidney expression of *ASS1* and *ASL* (Fig. 2). This effect was linear ($P < 0.013$) and quadratic ($P < 0.004$) for *ASS1*, reaching a maximal expression in 1W pigs (Fig. 2*A*). The expression of *ASL* increased ($P < 0.03$) linearly with age (Fig. 2*B*). The expression of these two enzymes was highly correlated ($r = 0.83$).

Plasma Citrulline and Arginine Concentrations

There was no effect of sex on any of the variables shown below, and, for this reason, sex was removed from the model. The plasma concentration of endogenous citrulline (i.e., not labeled) decreased ($P < 0.001$) linearly in T, 1W, and 5W pigs, increased ($P < 0.001$) in 10PT pigs, and remained stable ($P = 0.54$) in 5PT pigs during the 80-min sampling period (Fig. 3*A*). Although there were no linear or quadratic effects ($P > 0.33$) of age on plasma citrulline concentration, citrulline concentration was greater ($P < 0.005$) in 1W pigs compared with T pigs (Fig. 3*B*). Plasma endogenous arginine concentration increased linearly ($P < 0.001$) in 10PT and 5PT pigs, decreased linearly ($P < 0.001$) in 1W pigs, and remained stable ($P > 0.10$) in T and 5W pigs during the 80-min sampling period (Fig. 4*A*). The plasma arginine concentration was greater ($P < 0.001$) in 1W pigs than in pigs from the other age groups (Fig. 4*B*).

Fit of the Biexponential Model

A biexponential model provided a good fit for the citrulline enrichment data (pseudo $R^2 > 0.994$, and no evident pattern for the residuals; Fig. 5 and Table 2). The intercept of the function (sum of M_1 and M_2; i.e., tracer/tracee enrichment at *time 0*) showed an age effect ($P < 0.002$), with a differ-

ence between 1W versus T and 5W pigs. Whereas there was no effect of age for parameter g_1 of the biexponential equation ($P = 0.59$), parameter g_2 was affected by age ($P < 0.001$).

Citrulline Kinetics

Noncompartmental analysis. The actual tracer dose administered to the pigs is shown in Table 3. Despite a similar dose for all pig ages, there was an effect of age ($P < 0.001$) for the areas under the enrichment curves ($AUC_{Cit0-\infty}$, $AUC_{Cit0-80}$, and $AUC_{Arg0-80}$) with the lowest values for the 1W age group (Table 3). The fraction of the AUC for [^{15}N]citrulline during the 80-min experimental period ranged from 73.7% for the 10PT group to 90% of the total AUC in the 1W age group ($P < 0.001$). The contribution of [^{15}N]citrulline (and thus, by inference, endogenous citrulline) to circulating arginine was maximal for 5PT and T pigs, reaching almost 14% of circulating arginine (Table 3). The rate of appearance of citrulline was greater ($P < 0.001$) for the 1W age group, but no differences were observed among the other age groups (Table 3).

Age had similar linear and quadratic effects ($P < 0.001$) on citrulline half-life (Fig. 6) and MRT with 1W pigs showing the lowest values (Table 3). A quadratic effect of age was detected for plasma clearance of citrulline ($P < 0.001$) with the 1W pig group displaying the greater values for this variable (Table 3).

Compartmental analysis. There was an effect of age on Q_a ($P < 0.002$) and Q_b ($P < 0.01$; Table 4); whereas Q_a was larger ($P < 0.008$) in 1W pigs than in T and 5W pigs, Q_b was greater ($P = 0.03$) in 5PT pigs compared with 5W pigs. The volume of distribution of *compartments A* and *B* was affected ($P < 0.004$; Table 4) by age; pigs in the 1W group had a larger distribution of *compartment A* ($P < 0.05$) than 10PT, 5PT, and 5W pigs (Table 4). In contrast, the volume of distribution of *compartment B* was larger for 5PT pigs than pigs in the 1W and 5W age groups.

There was no effect ($P > 0.11$) of age on the rate constants for citrulline transfer between *compartments A* and *B* (k_{ba} and k_{ab}; Table 4). The rate constant for the irreversible loss of citrulline (k_{ob}), however, was affected ($P < 0.001$) by age, with 1W pigs having the highest rate followed by T and 5W animals (Table 4).

The transfer of citrulline (flux) between the two compartments was affected ($P < 0.007$) by age. The transfer of citrulline from *compartment A* to *compartment B* (F_{ba}) was greater in 1W pigs than in T and 5W pigs, and the reverse transfer (F_{ab}) was greater in 5PT pigs compared with 5W pigs ($P < 0.05$; Table 4). The irreversible loss of citrulline (F_{ob}) was greater ($P < 0.005$) for 1W pigs than for pigs from the other age groups (Table 4).

DISCUSSION

The developmental changes in the presence and abundance of the enzymes needed for the synthesis of citrulline and de novo production of arginine led researchers to believe that the intestinal-renal axis was not present in neonates (3, 11, 19). We have shown that the presence of intestinal ASS1 and ASL during the neonatal period does not colocalize with the enzymes that produce citrulline, and, for this reason, citrulline is exported from the gut into portal blood (14). Part of the

argument supporting the hypothesis of a lack of the intestinal-renal axis for arginine synthesis was that the renal expression, abundance, and activity of ASS1 and ASL are lower during the neonatal period than later in life (10–12). We have previously shown that the renal abundance of these two enzymes is greater in young animals than in neonatal animals (14). In the present study, the gene expression of *ASS1* and *ASL* further supports the concept of a developmental increase in the renal ability to use citrulline for arginine synthesis. However, the capacity to use citrulline and produce arginine by the kidney has been shown to greatly exceed the endogenous citrulline production. For example, young healthy human volunteers were able to clear a large single citrulline dose (equivalent to ~100 times their endogenous hourly production and resulting in a two order of magnitude increase in plasma citrulline concentration) within ~6–8 h with minimal spillage into the urine (18). Therefore, despite a reduced renal ASS and ASL enzymatic activity during the neonatal period, the ability to use citrulline may not only be sufficient to use all the endogenous citrulline produced but also to metabolize supplemental exogenous citrulline.

To determine the capacity of pigs of different ages to use endogenous and exogenous citrulline, we used tracer methodology. We provided the tracer at substrate amounts (roughly the equivalent to the hourly RaCit), which resulted in tracer-to-tracee ratios between 2 and 4. Despite this sudden increase in plasma total citrulline, the endogenous citrulline (i.e., unlabeled) concentration remained relatively constant over the period studied. Although there were significant linear effects in plasma endogenous citrulline concentrations over the 80-min study window, these effects were small (<5 μmol/L) and not consistent across the different ages. Steady-state conditions are not absolutely necessary to estimate citrulline kinetics (15, 17), provided that the production of the tracee is not altered (6). We have shown in mice that when renal utilization of citrulline is impaired, there is an immediate increase in plasma citrulline (15). For these reasons, a quasi-steady state for citrulline kinetics was assumed.

The estimation of citrulline production either by the noncompartmental (RaCit) or compartmental approach (F_{ao}) yielded identical values. The estimates of citrulline production in the present study are similar to our previous estimations made with a constant infusion approach (14, 16, 21), which have shown greater citrulline production in 1W pigs. Engelen et al. (8) reported greater citrulline production when a bolus dose of citrulline followed by compartmental analysis was used instead of a constant infusion of tracers; however, the estimation of production (flux) is model dependent. In their model, Engelen et al. (8) assumed that citrulline enters *compartment B* and that the disposal also occurs from this compartment. Given that the metabolism of citrulline is interorgan (production by the gut, utilization by the kidney), we believe that our model (Fig. 1) is a better approximation to the underlying physiology. Although rate constants are identical for both models, Q_b is larger for the model proposed by Engelen et al. and thus F_{ob} (and F_{ao}) is also larger (APPENDIX 2).

The advantage of compartmental modeling is that provides additional insights on the metabolism of citrulline. Here, we demonstrated an extensive movement of citrulline between the two compartments and that their volumes of distribution reflect the extracellular and intracellular spaces for *compartments A* and *B*, respectively. The measurements of citrulline disposal ($AUC_{Cit0-80}/AUC_{Cit0-\infty}$, half-life, MRT, and clearance) indicate that 1W pigs can use citrulline faster than pigs from other age groups. However, plasma citrulline concentrations in this age group were virtually

identical to the concentrations measured in premature (10PT and 5PT) pigs due to the fact that 1W pigs also had the greatest citrulline production. Because plasma concentration is the result of production and utilization, plasma citrulline concentrations need to be interpreted with caution mainly during the neonatal period when both processes may be affected. Regardless, the utilization of citrulline contributed ~9–14% to circulating arginine, a value similar to our previous determinations (14, 16).

In conclusion, there are developmental changes in renal *ASS1* and *ASL* gene expression that are reflected in the ability of the pigs to use citrulline. However, it seems that there is an excess capacity to use citrulline at all ages, including during prematurity, since the bolus dose of tracer did not result in an increase in endogenous citrulline. Although it is not possible to determine a safe upper citrulline supplementation level from the present data, the almost complete disposal of the tracer within an hour suggests that a supplementation level that doubles the endogenous production should be safe and efficacious. This supplementation level is equivalent to to 0.2–0.25 g citrulline/(kg·day) and is comparable to a high dose supplementation regime in adult humans (2). Our results support the idea that citrulline supplementation in neonatal, including premature, pigs is a viable option to increase arginine availability.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

D.G.B. and J.C.M. conceived and designed research; M.A.M., I.C.D., X.W., B.S., and J.C.M. performed experiments; M.A.M. and J.C.M. analyzed data; J.C.M. interpreted results of experiments; J.C.M. prepared figures; J.C.M. drafted manuscript. M.A.M., D.G.B., and J.C.M. edited and revised manuscript; M.A.M., I.C.D., X.W., B.S., D.G.B., and J.C.M. approved final version of manuscript.

APPENDIX 1

Calculation of Citrulline Kinetics From a Bolus Dose of Citrulline Tracer

The compartmental analysis was performed after normalizing the sum of the intercepts in *Eq. 1* to unity, as follows:

$$Yt=H1\times e-g1\times t+H2\times e-g2\times t \quad \text{(A1)}$$

where H_1 and H_2 (unitless) are calculated as follows:

$$H1=M1/M1+M2 \quad (A2)$$

$$H2=M2/M1+M2 \quad (A3)$$

Calculation of the rate constants. Each rate constant (in min^{-1}) is related mathematically to the entire group of parameters. All effluxes from *compartment A* (k_{aa}) and *compartment B* (k_{bb}) are calculated as follows:

$$kaa=H1\times g1+H2\times g2 \quad (A4)$$

$$kaa+kbb=g1+g2 \quad (A5)$$

$$kbb=g1+g2-kaa \quad (A5')$$

and the exchange rates between *compartments A* and *B* as follows:

$$kaa\times kbb-kab\times kba=g1\times g2 \quad (A6)$$

$$kab=kaa\times kbb-g1\times g2/kba \quad (A6')$$

Because in the model proposed (Fig. 1) k_{ba} is the sole exit from *compartment A*, then

$$kba=kaa \quad (A7)$$

From the model (Fig. 1 and *Eq. A8*), the rate of irreversible loss of citrulline (k_{ob}) can be calculated by difference as follows:

$$kbb=kob+kab \quad (A8)$$

$$kob=kbb-kab \quad (A8')$$

Calculation of the compartment sizes and flow rates. At *time 0* (i.e., before any exchange between the compartments takes place), the whole tracer dose is in *compartment A* and thus the size of the compartment (Q_a; in μmol/kg) can be estimated by the dilution of the dose as follows:

$$Qa=dose/M1+M2 \quad (A9)$$

For the model proposed in Fig. 1, the total flow rate (F_{aa}; in $\mu mol\cdot kg^{-1}\cdot min^{-}1$) as well as the inflow (F_{ao} + F_{ab}) and outflow (F_{ba}) rates for *compartment A* at steady state can be calculated as follows:

$$Faa=Fao+Fab=Fba \quad (A10)$$

$$Qa \times kaa = Fao + Qb \times kab = Qa \times kba \quad (A10')$$

The total flow (F_{bb}), inflow (F_{ba}), and outflow ($F_{ab} + F_{ob}$) rates for *compartment B* are then calculated as follows:

$$Fbb = Fba = Fab + Fob \quad (A11)$$

$$Qb \times kbb = Qa \times kba = Qb \times kab + Qb \times kob \quad (A11')$$

Rearranging this last equation, the size of *compartment B* can then be calculated as follows:

$$Qb = Qa \times kba / kbb \quad (A12)$$

Flow rates are then obtained by multiplying the corresponding rate constant (F_{xy}) by the size of the compartment from which originates (Q_y); multiplying by 60 min/h, the flow rates are expressed in micromoles per kilogram per hour.

APPENDIX 2

Note that for a different model structure, as proposed by Engelen et al. (8), the calculations are slightly different, resulting in a larger citrulline flux (F_{ob}).

Rate constants are identical, but the size of *compartment B* is larger (22). The total flow rate as well as the inflow (F_{ab}) and outflow rates (F_{ba}) for *compartment A* are now calculated as follows:

$$Faa = Fab = Fba \quad (A13)$$

$$Qa \times kaa = Qb \times kab = Qa \times kba \quad (A13')$$

and the total flow rate (F_{bb}) as well as the inflow ($F_{ba} + F_{bo}$) and outflow ($F_{ab} + F_{ob}$) rates for *compartment B* are as follows:

$$Fbb = Fba + Fbo = Fab + Fob \quad (A14)$$

$$Qb \times kbb = Qa \times kba + Fbo = Qb \times kab + Qb \times kob \quad (A14')$$

Note that the entry of citrulline into the system shifts from *compartment A* (F_{ao}) to *compartment B* (F_{bo}) between our model and the model used by Engelen et al. The calculation of the size of *compartment B* is then obtained by rearranging *Eq. A13′* as follows:

$$Qb = Qa \times kba / kab \quad (A15)$$

Because $k_{bb} > k_{ab}$, then it follows that the size of the *compartment B* is larger for the model proposed by Engelen et al. compared with the model proposed by us. As a consequence, their estimation of citrulline production is also greater.

REFERENCES

1. Agarwal U, Didelija IC, Yuan Y, Wang X, Marini JC. Supplemental citrulline is more efficient than arginine in increasing systemic arginine availability in mice. *J Nutr* 147: 596–602, 2017. doi: 10.3945/jn.116.240382. [PMCID: PMC5368575] [PubMed: 28179487] [CrossRef: 10.3945/jn.116.240382]

2. Allerton TD, Proctor DN, Stephens JM, Dugas TR, Spielmann G, Irving BA. l-Citrulline supplementation: impact on cardiometabolic health. *Nutrients* 10: E921, 2018. doi: 10.3390/nu10070921. [PMCID: PMC6073798] [PubMed: 30029482] [CrossRef: 10.3390/nu10070921]

3. Bertolo RF, Burrin DG. Comparative aspects of tissue glutamine and proline metabolism. *J Nutr* 138: 2032S–2039S, 2008. doi: 10.1093/jn/138.10.2032S. [PubMed: 18806120] [CrossRef: 10.1093/jn/138.10.2032S]

4. Borsook H, Dubnoff JW. The conversion of citrulline to arginine in the kidney. *J Biol Chem* 140: 717–738, 1941.

5. Castillo L, Ajami A, Branch S, Chapman TE, Yu YM, Burke JF, Young VR. Plasma arginine kinetics in adult man: response to an arginine-free diet. *Metabolism* 43: 114–122, 1994. doi: 10.1016/0026-0495(94)90166-X. [PubMed: 8289668] [CrossRef: 10.1016/0026-0495(94)90166-X]

6. Cobelli C, Toffolo G, Foster DM. Tracer-to-tracee ratio for analysis of stable isotope tracer data: link with radioactive kinetic formalism. *Am J Physiol Endocrinol Metab* 262: E968–E975, 1992. doi: 10.1152/ajpendo.1992.262.6.E968. [PubMed: 1616030] [CrossRef: 10.1152/ajpendo.1992.262.6.E968]

7. Dhanakoti SN, Brosnan JT, Herzberg GR, Brosnan ME. Renal arginine synthesis: studies in vitro and in vivo. *Am J Physiol Endocrinol Metab* 259: E437–E442, 1990. doi: 10.1152/ajpendo.1990.259.3.E437. [PubMed: 1975989] [CrossRef: 10.1152/ajpendo.1990.259.3.E437]

8. Engelen MPKJ, Com G, Anderson PJ, Deutz NEP. New stable isotope method to measure protein digestibility and response to pancreatic enzyme intake in cystic fibrosis. *Clin Nutr* 33: 1024–1032, 2014. doi: 10.1016/j.clnu.2013.11.004. [PMCID: PMC4306434] [PubMed: 24268783] [CrossRef: 10.1016/j.clnu.2013.11.004]

9. Featherston WR, Rogers QR, Freedland RA. Relative importance of kidney and liver in synthesis of arginine by the rat. *Am J Physiol* 224: 127–129, 1973. doi: 10.1152/ajplegacy.1973.224.1.127. [PubMed: 4683290] [CrossRef: 10.1152/ajplegacy.1973.224.1.127]

10. Goutal I, Fairand A, Husson A. Expression of the genes of arginine-synthesizing enzymes in the rat kidney during development. *Biol Neonate* 76: 253–260, 1999. doi: 10.1159/000014166. [PubMed: 10473900] [CrossRef: 10.1159/000014166]

11. Hurwitz R, Kretchmer N. Development of arginine-synthesizing enzymes in mouse intestine. *Am J Physiol Gastrointestinal Liver Physiol* 251: G103–G110, 1986. doi: 10.1152/ajpgi.1986.251.1.G103. [PubMed: 3728668] [CrossRef: 10.1152/ajpgi.1986.251.1.G103]

12. Köhler ES, Sankaranarayanan S, van Ginneken CJ, van Dijk P, Vermeulen JLM, Ruijter JM, Lamers WH, Bruder E. The human neonatal small intestine has the potential for arginine synthesis; developmental changes in the expression of arginine-synthesizing and -catabolizing enzymes. *BMC Dev Biol* 8: 107, 2008. doi: 10.1186/1471-213X-8-107. [PMCID:

PMC2621195] [PubMed: 19000307] [CrossRef: 10.1186/1471-213X-8-107]

13. Marini JC. Quantitative analysis of ^{15}N-labeled positional isomers of glutamine and citrulline via electrospray ionization tandem mass spectrometry of their dansyl derivatives. *Rapid Commun Mass Spectrom* 25: 1291–1296, 2011. doi: 10.1002/rcm.5007. [PubMed: 21491530] [CrossRef: 10.1002/rcm.5007]

14. Marini JC, Agarwal U, Robinson JL, Yuan Y, Didelija IC, Stoll B, Burrin DG. The intestinal-renal axis for arginine synthesis is present and functional in the neonatal pig. *Am J Physiol Endocrinol Metab* 313: E233–E242, 2017. doi: 10.1152/ajpendo.00055.2017. [PMCID: PMC5582884] [PubMed: 28611027] [CrossRef: 10.1152/ajpendo.00055.2017]

15. Marini JC, Didelija IC, Fiorotto ML. Extrarenal citrulline disposal in mice with impaired renal function. *Am J Physiol Renal Physiol* 307: F660–F665, 2014. doi: 10.1152/ajprenal.00289.2014. [PMCID: PMC4166726] [PubMed: 25056350] [CrossRef: 10.1152/ajprenal.00289.2014]

16. Marini JC, Stoll B, Didelija IC, Burrin DG. De novo synthesis is the main source of ornithine for citrulline production in neonatal pigs. *Am J Physiol Endocrinol Metab* 303: E1348–E1353, 2012. doi: 10.1152/ajpendo.00399.2012. [PMCID: PMC3774079] [PubMed: 23074237] [CrossRef: 10.1152/ajpendo.00399.2012]

17. Mohammad MA, Didelija IC, Stoll B, Marini JC. Citrulline generation test: what does it measure? *J Parenter Enteral Nutr* 43: 726–733, 2019. doi: 10.1002/jpen.1462. [PMCID: PMC6482105] [PubMed: 30357864] [CrossRef: 10.1002/jpen.1462]

18. Moinard C, Nicolis I, Neveux N, Darquy S, Bénazeth S, Cynober L. Dose-ranging effects of citrulline administration on plasma amino acids and hormonal patterns in healthy subjects: the Citrudose pharmacokinetic study. *Br J Nutr* 99: 855–862, 2008. doi: 10.1017/S0007114507841110. [PubMed: 17953788] [CrossRef: 10.1017/S0007114507841110]

19. Morris SM., Jr Regulation of enzymes of urea and arginine synthesis. *Annu Rev Nutr* 12: 81–101, 1992. doi: 10.1146/annurev.nu.12.070192.000501. [PubMed: 1503815] [CrossRef: 10.1146/annurev.nu.12.070192.000501]

20. Morris SM., Jr Arginine metabolism revisited. *J Nutr* 146: 2579S–2586S, 2016. doi: 10.3945/jn.115.226621. [PubMed: 27934648] [CrossRef: 10.3945/jn.115.226621]

21. Robinson JL, Smith VA, Stoll B, Agarwal U, Premkumar MH, Lau P, Cruz SM, Manjarin R, Olutoye O, Burrin DG, Marini JC. Prematurity reduces citrulline-arginine-nitric oxide production and precedes the onset of necrotizing enterocolitis in piglets. *Am J Physiol Gastrointest Liver Physiol* 315: G638–G649, 2018. doi: 10.1152/ajpgi.00198.2018. [PMCID: PMC6415715] [PubMed: 30048597] [CrossRef: 10.1152/ajpgi.00198.2018]

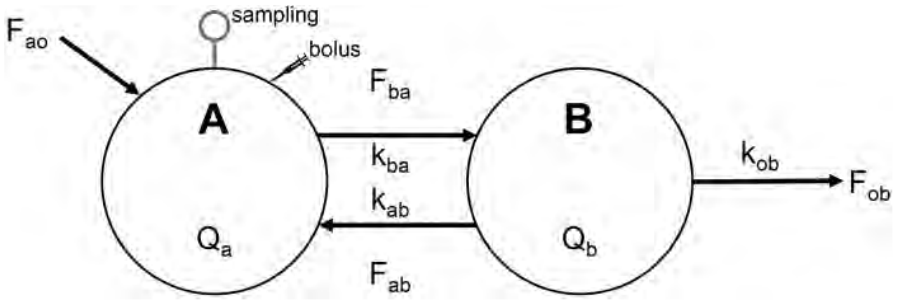
22. Shipley RA, Clark RE. *Tracer Methods for In Vivo Kinetics. Theory and Applications*. New York: Academic, 1972.

23. Windmueller HG, Spaeth AE. Uptake and metabolism of plasma glutamine by the small intestine. *J Biol Chem* 249: 5070–5079, 1974. [PubMed: 4605420]

24. Wu G, Knabe DA. Arginine synthesis in enterocytes of neonatal pigs. *Am J Physiol Regul Integr Comp Physiol* 269: R621–R629, 1995. doi: 10.1152/ajpregu.1995.269.3.R621. [PubMed: 7573565] [CrossRef: 10.1152/ajpregu.1995.269.3.R621]

Figures and Tables

Fig. 1.



Two-compartment model for citrulline metabolism. Citrulline enters *compartment A* where the bolus dose of tracer is administered and samples are collected. Citrulline then exchanges with the second compartment (*compartment B*) from where it is utilized. Q_a and Q_b, are the sizes of *compartments A* and *B* (in mmol/kg body wt), respectively; k_{yx} is the rate of transfer (in min^{-1}) of citrulline to *compartment Y* from *compartment X*; and F_{yx} is the flow rate (in $\mu mol \cdot kg^{-1} \cdot h^{-1}$) of citrulline to *compartment Y* from *compartment X*. F_{ao} represents the entry of citrulline to the system; F_{ob} represents its irreversible loss.

Table 1.

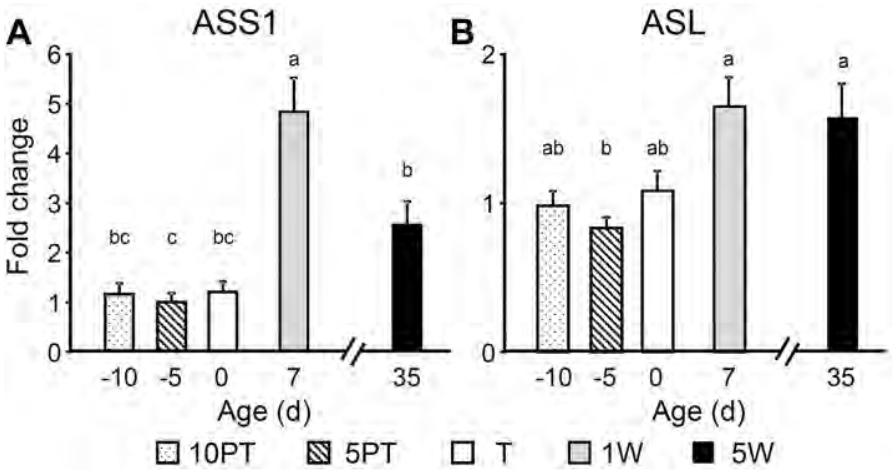
Kidney and body weights of pigs at different ages

	10PT	5PT	T	1W	5W	*P* Value	Lin	Quad
Body weight, g	880 ± 73‡	882 ± 110‡	1440 ± 174‡	3273 ± 281†	7461 ± 518*	<0.001	0.001	0.53
Kidney weight, g	6.9 ± 0.7‡	7.2 ± 0.9‡	10.0 ± 1.3‡	25.8 ± 3.0†	46.4 ± 4.3*	<0.001	0.001	0.86
Kidney/body weight, g/kg	7.7 ± 0.3	8.3 ± 0.7	7.2 ± 0.3	7.7 ± 0.3	6.3 ± 0.4	<0.15	0.046	0.79

Values are means ± SE; *n* = 6–8. Preterm pigs [10 and 5 days preterm (10PT and 5PT, respectively)], term pigs at birth (T), and 1-wk-old (1W) and 5-wk-old (5W) pigs were studied.

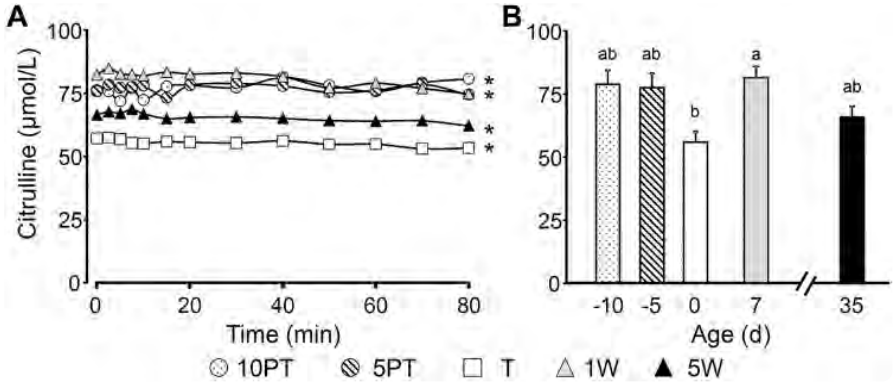
*†‡Values within a row with different symbols differ at $P < 0.05$ (Tukey's post hoc multiple comparisons).

Fig. 2.



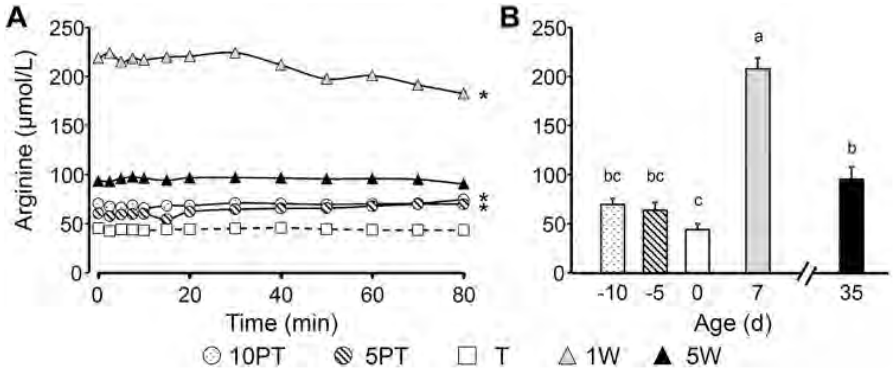
Renal argininosuccinate synthase (ASS1; *A*) and argininosuccinate lyase (ASL; *B*) expression in pigs of different ages. Preterm pigs [10 and 5 days preterm (10PT and 5PT, respectively)], term pigs at birth (T), and 1-wk-old (1W) and 5-wk-old (5W) pigs were studied. Values are means ± SE. Age had an effect ($P < 0.001$) on the expression of ASS1 and ASL. Linear (ASS1 and ASL) and quadratic effects (ASS1) were also detected ($P < 0.05$). [a,b,c]Bars with a different letter differ at $P < 0.05$ (Tukey's post hoc multiple comparisons). $n = 6–8$.

Fig. 3.



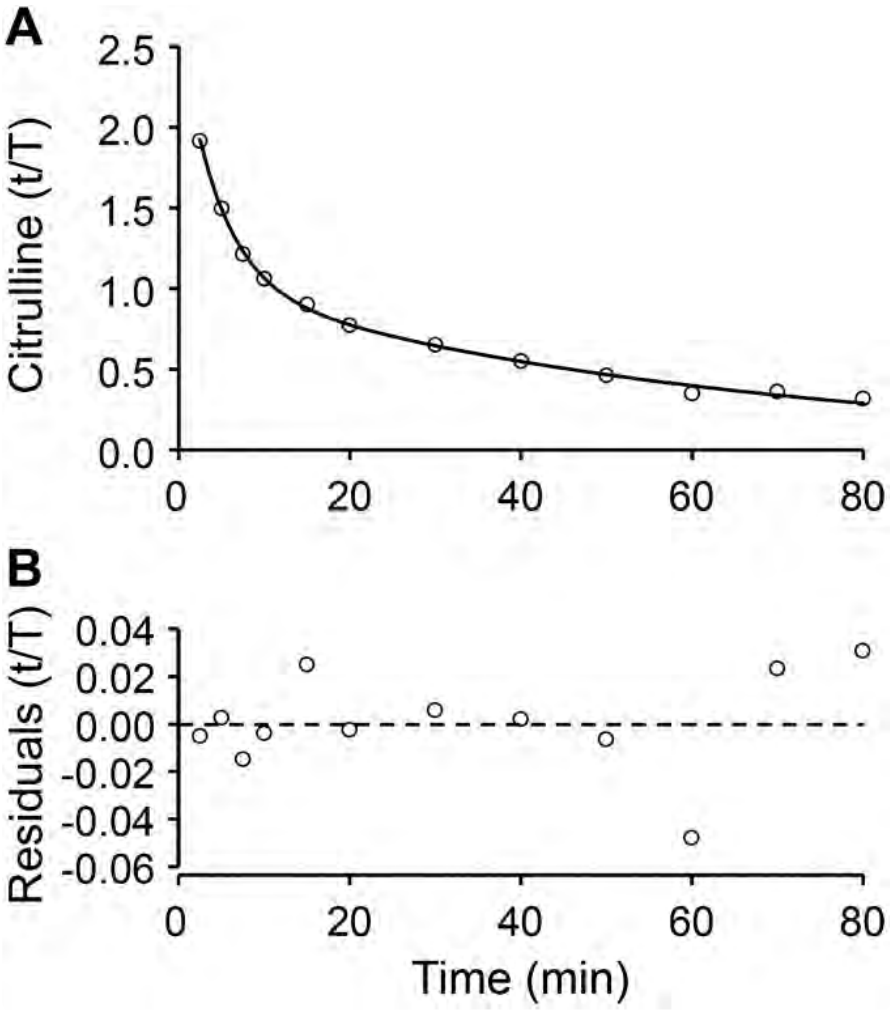
Plasma (endogenous) citrulline concentration in pigs of different ages after the administration of a bolus dose of (ureido) [^{15}N]citrulline (*A*) and average over the 80-min study window (*B*). Preterm pigs [10 and 5 days preterm (10PT and 5PT, respectively)], term pigs at birth (T), and 1-wk-old (1W) and 5-wk-old (5W) pigs were studied. Symbols in *A* represent means (SE not shown for clarity of presentation; pooled SE 7.9 μmol/L) and asteriks denote a linear effect of time ($P < 0.05$). Bars in *B* are means (and SEs). Age had an effect ($P < 0.01$) on plasma citrulline plasma concentration. [a,b]Bars with a different letter differ at $P < 0.05$ (Tukey's post hoc multiple comparisons). $n = 6–8$.

Fig. 4.



Plasma (endogenous) arginine concentration in pigs of different ages after the administration of a bolus dose of (ureido)[^{15}N]citrulline (*A*) and average over the 80-min study window (*B*). Preterm pigs [10 and 5 days preterm (10PT and 5PT, respectively)], term pigs at birth (T), and 1-wk-old (1W) and 5-wk-old (5W) pigs were studied. Symbols in *A* represent means (SE not shown for clarity of presentation; pooled SE 11.7 μmol/L) and asterisks denote a linear effect of time ($P < 0.05$). Bars in *B* are means (and SEs). Age had an effect ($P < 0.001$) on plasma arginine concentration. Linear ($P < 0.004$) and quadratic effects ($P < 0.001$) were also detected. [a,b,c]Bars with a different letter differ at $P < 0.05$ (Tukey's post hoc multiple comparisons). $n = 6–8$.

Fig. 5.



Citrulline enrichment curve (t/T) of a representative 10-day preterm pig. Individual data points and a fitted biexponential curve [$Y_t = 1.59 \times e^{(-0.22 \times t)} + 1.04 \times e^{(-0.016 \times t)}$, $R^2 = 0.998$; A] and residuals (B) are shown.

Table 2.

Parameters of the biexponential function fitter to the citrulline enrichment data in pigs of different ages

	10PT	5PT	T	1W	5W	*P* Value
M_1, t/T	2.11 ± 0.22*†	2.49 ± 0.59*	2.74 ± 0.24*	1.14 ± 0.09†	2.74 ± 0.26*	<0.002
g_1, min^{-1}	0.28 ± 0.02	0.32 ± 0.03	0.29 ± 0.02	0.28 ± 0.02	0.28 ± 0.01	<0.59
M_2, t/T	1.00 ± 0.08†	0.94 ± 0.09†	1.35 ± 0.11*†	0.98 ± 0.08†	1.51 ± 0.13*	<0.003
g_2, min^{-1}	0.016 ± 0.001†	0.018 ± 0.001†	0.020 ± 0.001†	0.029 ± 0.001*	0.023 ± 0.001*†	<0.001
$M_1 + M_2$, t/T	3.11 ± 0.27*†	3.43 ± 0.66*†	4.10 ± 0.30*	2.11 ± 0.17†	4.24 ± 0.39*	<0.002
p-R^2	0.997 (0.995–0.999)	0.997 (0.994–0.999)	0.998 (0.997–0.999)	0.998 (0.997–0.999)	0.998 (0.997–0.999)	

Values are means ± SE; n = 6–8. Preterm pigs [10 and 5 days preterm (10PT and 5PT, respectively)], term pigs at birth (T), and 1-wk-old (1W) and 5-wk-old (5W) pigs were studied.

*Values within a row with different symbols differ at $P < 0.05$ (Tukey's post hoc multiple comparisons).

†Values within a row with different symbols differ at $P < 0.05$ (Tukey's post hoc multiple comparisons).

Table 3.

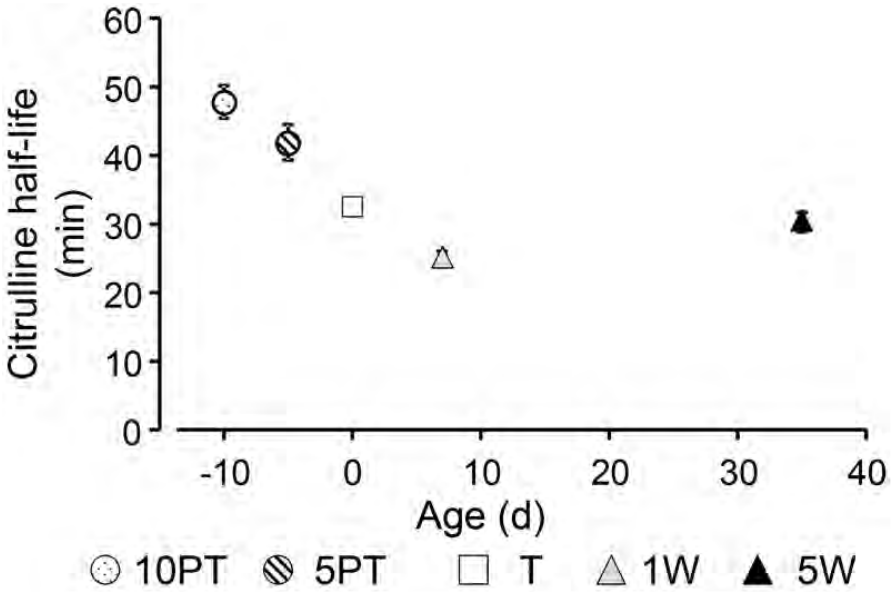
Areas under the enrichment curve, rate of appearance of citrulline, fractional contribution of plasma citrulline to plasma arginine, and citrulline mean retention time and clearance rate in pigs of different ages

	10PT	5PT	T	1W	5W	*P* Value	Lin	Quad
Dose, μmol/kg	50.1 ± 0.5	51.7 ± 1.2	48.3 ± 0.4	50.4 ± 0.3	50.0 ± 0.2			
AUC, $t \cdot T^{-1} \cdot min^{-1}$								
$AUC_{Cit0-\infty}$	75.5 ± 4.2*	62.1 ± 4.8*†	72.3 ± 4.3*	38.4 ± 1.4†	76.6 ± 8.8*	<0.001	0.66	0.004
$AUC_{Cit0-80}$	54.5 ± 3.6*†	47.5 ± 3.7*†	61.1 ± 3.9*	34.7 ± 1.6†	65.3 ± 6.8*	<0.001	0.16	0.025
$AUC_{Arg0-80}$	5.2 ± 0.2†	6.2 ± 0.4†	8.4 ± 0.9*	3.1 ± 0.1‡	5.4 ± 0.2†	<0.001	0.17	0.37
$AUC_{Cit0-80}/AUC_{Cit0-\infty}$, %	73.7 ± 1.8§	76.0 ± 2.2‡§	84.4 ± 0.9†‡	90.3 ± 1.0*	86.1 ± 1.3*†	<0.001	0.001	0.001
RaCit, $\mu mol \cdot kg^{-1} \cdot h^{-1}$	41 ± 2.1†	51 ± 4.2†	48 ± 2.4†	80 ± 3.3[a]	42 ± 4.0†	<0.001	0.99	0.001
Arg from Cit, %	9.6 ± 0.3†	13.3 ± 0.8*	13.9 ± 1.5*	9.0 ± 0.4†	8.6 ± 0.8†	<0.001	0.017	0.36
Clearance, $L \cdot kg^{-1} \cdot h^{-1}$	0.59 ± 0.02†	0.72 ± 0.03†	0.73 ± 0.03†	1.04 ± 0.02*	0.62 ± 0.03†	<0.001	0.755	0.001
MRT, min	62.5 ± 3.6*	54.1 ± 3.8*†	41.0 ± 1.4†‡	32.4 ± 1.5‡	38.8 ± 2.1‡	<0.001	0.001	0.001

Values are means ± SE; n = 6–8. Preterm pigs [10 and 5 days preterm (10PT and 5PT, respectively)], term pigs at birth (T), and 1-wk-old (1W) and 5-wk-old (5W) pigs were studied. AUC, area under the enrichment curve; RaCit, rate of appearance of citrulline; Arg from Cit, fractional contribution of plasma citrulline to plasma arginine; MRT, citrulline mean retention time.

*†‡§Values within a row with different symbols differ at $P < 0.05$ (Tukey's post hoc multiple comparisons).

Fig. 6.



Half-life of citrulline in pigs of different ages. Preterm pigs [10 and 5 days preterm (10PT and 5PT, respectively)], term pigs at birth (T), and 1-wk-old (1W) and 5-wk-old (5W) pigs were studied. Symbols are means ± SE. Linear ($P < 0.001$) and quadratic ($P < 0.001$) effects of age were detected. $n = 6–8$.

Table 4.

Citrulline kinetic compartmental analysis in pigs of different ages

	10PT	5PT	T	1W	5W	*P* Value	Lin	Quad
Pool sizes, mmol/kg								
Q_a	17.7 ± 2.6†‡	18.5 ± 3.8†‡	12.1 ± 0.9‡	24.9 ± 1.9*†	12.3 ± 0.9‡	<0.002	0.15	0.07
Q_b	31.8 ± 2.5*†	36.3 ± 3.5*	22.2 ± 2.0*†	28.2 ± 2.2*†	20.2 ± 1.3†	<0.01	0.008	0.57
Volume of distribution, L/kg								
V_a	0.23 ± 0.02†	0.25 ± 0.04*†	0.22 ± 0.01†	0.31 ± 0.01*	0.19 ± 0.01†	<0.004	0.13	0.004
V_b	0.41 ± 0.01*†	0.47 ± 0.03*	0.40 ± 0.02*†	0.35 ± 0.01†	0.31 ± 0.01†	<0.002	0.003	0.65
Rate constants, min^{-1}								
k_{aa}	0.193 ± 0.018	0.232 ± 0.028	0.199 ± 0.019	0.162 ± 0.013	0.189 ± 0.007	<0.11	0.31	0.22
k_{bb}	0.101 ± 0.004†	0.105 ± 0.004†	0.108 ± 0.007†	0.144 ± 0.012*	0.115 ± 0.004*†	<0.002	0.12	0.001
k_{ba}	0.193 ± 0.018	0.232 ± 0.028	0.199 ± 0.019	0.162 ± 0.013	0.189 ± 0.007	<0.11	0.31	0.22
k_{ab}	0.079 ± 0.004	0.081 ± 0.003	0.077 ± 0.006	0.095 ± 0.010	0.081 ± 0.005	<0.28	0.81	0.15
k_{ob}	0.022 ± 0.001‡	0.024 ± 0.002‡	0.032 ± 0.001†	0.048 ± 0.002*	0.035 ± 0.001†	<0.001	0.001	0.001
Fluxes, $\mu mol \cdot kg^{-1} \cdot h^{-1}$								
F_{ba}	195 ± 20*†	229 ± 25*†	142 ± 13†	235 ± 12*	138 ± 10†	<0.001	0.035	0.29
F_{ab}	154 ± 19*†	178 ± 21*	102 ± 11*†	155 ± 11*†	97 ± 8†	<0.007	0.013	0.997
F_{ob}	41 ± 2.1†	51 ± 4.2†	41 ± 2.4†	80 ± 3.3*	42 ± 4.0†	<0.001	0.99	0.001

Values are means ± SE; n = 6–8. Preterm pigs [10 and 5 days preterm (10PT and 5PT, respectively)], term pigs at birth (T), and 1-wk-old (1W) and 5-wk-old (5W) pigs were studied.

*†‡Values within a row with different symbols differ at $P < 0.05$ (Tukey's post hoc multiple comparisons).