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Assessment of folate and cobalamin concentrations in relation to their dependent intracellular metabolites in serum of pigs between 6 and 26 weeks of age

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Abstract:

Folate (vitamin B₉) and cobalamin (vitamin B₁₂) play an important role in amino acid metabolism, nucleic acid synthesis, and methyl group transfer. Two intracellular enzymes, methionine synthase and methylmalonyl-CoA mutase, are folate and/or cobalamin-dependent, respectively. At the cellular level, lack of folate and cobalamin leads to accumulation of serum homocysteine (HCY) and lack of cobalamin leads to increased methylmalonic acid (MMA) concentrations. Altered serum HCY and MMA concentrations can influence amino acid metabolism and nucleic acid synthesis in pigs. Therefore, we aimed to evaluate serum folate, cobalamin, HCY, and MMA concentrations in postweaning pigs between 6 and 26 weeks of age. Serum samples from 12 pigs collected at week 6, 7, 8, 9, 10, 14, 18, 22, and 26 as part of an unrelated study were analyzed. Serum folate ($p<0.0001$), cobalamin ($p=0.0001$), HCY ($p<0.0001$), and MMA ($p<0.0001$) concentrations differed significantly during the postweaning period between 6 and 26 weeks of age; with significantly higher serum HCY (at weeks 6 and 7 compared to weeks 9, 14, 18, 22, and 26) and MMA concentrations (at weeks 6, 7, and 8 compared to weeks 14, 18, 22, and 26) and an overall decrease of serum MMA concentrations from week 6 to week 14 in the pigs studied. This study suggests age-dependent changes in intracellular folate- and cobalamin-dependent metabolites (i.e., HCY and MMA) in pigs between 6 and 26 weeks of age, possibly reflecting decreased availability of intracellular folate and/or cobalamin for amino acid metabolism, nucleic acid synthesis, and methyl group transfer.

Keywords: Vitamin B₉; Vitamin B₁₂; Homocysteine; Methylmalonic acid; Swine

Introduction

Maintenance of genome stability is important for growth performance throughout the production process in pigs. For this reason, pigs receive a balanced diet according to their age-dependent requirements. Dietary ingredients such as folate (vitamin B₉) and cobalamin (vitamin B₁₂) play a fundamental role in amino acid metabolism, nucleic acid (i.e., RNA and DNA) synthesis, and methyl group transfer (Fenech, 2012) and are considered essential components for genomic stability.

In farm animals, serum folate and cobalamin concentrations are not a direct reflection of the intracellular folate and cobalamin status (Berghoff et al. 2012; Peterson et al. 2013; Grützner et al. 2016). Based on studies conducted in dogs and pigs, decreased folate and cobalamin concentrations are associated with increased homocysteine (HCY) and methylmalonic acid (MMA) concentrations in serum (House and Fletcher, 2003; Simard et al., 2007; Berghoff et al. 2012; Rossi et al. 2013; Grützner et al. 2015 and 2016; Heilmann et al. 2017). Increased serum HCY and MMA concentrations can result in a malfunction of the intracellular methionine synthase and methylmalonyl-CoA mutase, both of which are cobalamin-dependent enzymes, while methionine synthase is also folate dependent. Folate and cobalamin represent essential co-factors for cytosolic methionine synthase, an enzyme that catalyzes the methylation of HCY to methionine. For this reaction to occur, methionine synthase requires the presence of a methyl group and cobalamin as a co-factor. The methyl group is provided through the conversion of 5-methyltetrahydrofolate to tetrahydrofolate, which is the biologically active form of vitamin B₉. 5-tetrahydrofolate is required for purine and pyrimidine biosynthesis and is thus important for the *de novo* synthesis of DNA and RNA. Cobalamin is an essential co-factor for mitochondrial methylmalonyl CoA mutase, an enzyme that catalyzes the conversion of L-methylmalonyl-CoA to succinyl-CoA. In turn, succinyl-

CoA is incorporated into the tricarboxylic acid cycle also known as Krebs cycle, which fuels cellular energy production. According to previous veterinary studies conducted in dogs and pigs, changes in the serum HCY and MMA concentration reflect the intracellular availability of both vitamins (House and Fletcher, 2003; Simard et al., 2007; Berghoff et al., 2012; Heilmann et al., 2017; Kook et al. 2018). Thus, measurement of HCY and MMA could assist in the diagnosis of deficiencies and associated diseases and may be valuable biomarkers to evaluate the dietary requirements for folate and cobalamin in pigs.

Absorption of folate in pigs takes place in the proximal small intestine (i.e., duodenum and jejunum) and that for cobalamin in the distal small intestine (i.e., ileum; Halsted, 1980; Trugo et al., 1985; Said, 2011). A malabsorption of both may occur after weaning, which represents a period of adverse health effects in a pig's life. Weaning usually occurs, depending on how the farms are organized, between 3 and 5 weeks of age and the postweaning period lasts 5 to 7 weeks before the pigs enter the finisher unit. After weaning, a malabsorption of micronutrients such as folate and cobalamin is possibly due to increased mucosal permeability (Lallès et al. 2004), disturbed absorption and secretion (Boudry et al., 2004), or an altered inflammatory cytokine pattern (Pié et al., 2004). In addition, microbial activity influences gut immunity and motility (Williams et al., 2001) and dysbalances (Rossi et al., 2011) might affect folate and cobalamin absorption in pigs after weaning. Currently, no large data sets are available that compare serum folate and cobalamin concentrations with serum HCY and MMA concentrations in pigs postweaning. Therefore, we aimed to evaluate serum folate, cobalamin, HCY, and MMA concentrations in postweaning pigs from 6 to 26 weeks of age to identify periods where an inadequate supply or even a lack of folate and/or cobalamin at the cellular level may exist. This information can be used by feed mills and pig producers to adjust the amount of folate and/or cobalamin in the diet to provide sufficient

amount of both vitamins on the cellular level for genomic stability and ultimately for adequate growth performance in clinical healthy or even diseased pigs.

Materials and methods

Sample population, collection, and processing of specimens

Based on our preliminary study, where differences within a small sample size (Grützner et al., 2015) were observed, a set of 12 samples were selected to achieve sufficient statistical power to reveal differences with the serum variables in the present study. Archived serum samples from 12 conventional crossbred pigs from blood samples collected at 6, 7, 8, 9, 10, 14, 18, 22, and 26 weeks of age by venipuncture of the jugular vein as part of an unrelated study (Hemann et al., 2012), were used in this study. More specifically, the samples were from a controlled porcine circovirus type 2 (PCV2) challenge trial. In brief, at 6 weeks of age, six pigs were vaccinated with a PCV2 vaccine and the remaining six pigs were left unvaccinated. At 10 weeks of age, nine pigs were challenged with PCV2 by the intramuscular and intranasal routes as previously described (Hemann et al., 2012). The pigs of this particular trial were weaned at three weeks of age and were fed a commercial diet afterwards that met or exceeded all nutrient requirements established by the 1998 National Research Council (NRC). The diet (Heartland CO-OP, Des Moines, Iowa, USA) was free of animal proteins and antibiotics and all the ingredients are described in Table 1, whereas the amount of folate and cobalamin were the same in all three diets (3.72 ppm of folate and 3.81 ppb cobalamin in each diet). During the study period no feed, water, or power failure or climate change was noticed.

All archived serum samples were treated in a similar fashion, stored in polypropylene tubes, and kept frozen at -80°C in a non-defrosting freezer until use (less than one year). Long term stability

studies in humans had revealed that cobalamin and its metabolites are stable in frozen serum samples for several years, except folate (Hustad et al. 2012). Presumably, the same applies for pigs, however studies in pigs are warranted to confirm the stability findings in human serum. According to the literature, decreased serum cobalamin concentrations have been described in human patients or pigs with RNA virus infection (Tang et al., J Nutr 1997; Grützner et al., ESPHM 2018), but not in patients with DNA virus infections. The pigs whose samples were used in this study had been vaccinated and/or challenged with PCV2, a small circularly arranged DNA virus. To rule out any impact of PCV2 on the mineral levels examined a comparison was conducted to see if serum folate, cobalamin, HCY, and MMA concentrations were affected by the DNA virus infection. The PCV2 pigs were only included if the DNA virus infection was not affecting the serum variables (folate, cobalamin, HCY, and MMA).

Sample analyses

Serum folate and cobalamin concentrations were measured using an IMMULITE®2000 assay (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) that was previously analytically validated by Grützner et al. (2016). Serum HCY and MMA concentrations were determined using a gas chromatography-mass spectrometry method as described by Stabler et al. (1987) and Ruaux et al. (2009), respectively.

Additional testing performed

It has previously been reported that altered serum folate and cobalamin concentrations have an effect on amino acid metabolism (e.g., methionine; Giquère et al., 2008; Mou et al., 2018). Therefore, the serum concentrations of six amino acids (i.e., glycine, glutamic acid, methionine,

tryptophan, tyrosine, and lysine) were measured and their relationship with serum folate, cobalamin, HCY, and MMA concentrations in pigs postweaning was evaluated. Serum samples from pigs (n=6) were collected at 6, 10, 14, 22, and 26 weeks of age (handled in the same manner as above) and used to measure serum concentration of these six amino acids by a gas chromatography-mass spectrometry method as described by Lopes et al. (2015).

Data analysis

All data were tested for normality by use of a Shapiro-Wilk W test. The effect of PCV2 challenge on serum folate, cobalamin, HCY, and MMA concentrations was evaluated by comparing postweaning pigs vaccinated against PCV2 between 9 (base line: one-week prior the PCV2 challenge) and 26 weeks of age and those that were not using a MANOVA. Subsequently, a Friedman test for repeated measurements with a Dunn's posttest was performed to compare serum folate, cobalamin, HCY, and MMA concentrations over time. For all variables (i.e., folate, cobalamin, HCY, and MMA) the coefficient of variation in percent (%CV) was calculated. A Spearman rank sum correlation coefficient (ρ) was used to correlate serum folate, cobalamin, HCY, and MMA concentrations. The 95% confidence interval (CI) was calculated for these variables and statistical significance for all tests was set at $p < 0.05$.

Serum concentrations of the six amino acids were compared among the different age-groups of pigs (n=6) by using an ANOVA or a non-parametric Kruskal-Wallis test, as applicable. A Spearman rank sum correlation coefficient was used for correlation analyses between concentrations of folate, cobalamin, HCY, and MMA, respectively, and those of each of the six amino acids. The level of statistical significance for the correlation analyses was adjusted according to the number of amino acids that were evaluated, $p < 0.05$ to $p = 0.0085$, using a

Bonferroni correction for multiple statistical comparisons (<http://www.quantitativeskills.com/sisa/calculations/bonfer.htm>, accessed 28th of September, 2018), whereas a trend was defined as a p value between the two cutoffs ($p<0.0085$ and $p<0.05$). A commercially available software package was used to perform all statistical analyses (GraphPad Prism5, GraphPad Software).

Results

Influence of PCV2 infection in pigs on serum folate, cobalamin, HCY, and MMA concentrations (n = 6 pigs per group)

There was no influence of PCV2 vaccination or PCV2 challenge on serum folate ($p=0.4714$), cobalamin ($p=0.8935$), HCY ($p=0.7460$), or MMA ($p=0.4155$) concentrations in postweaning pigs between 6 and 26 weeks of age and a challenge at 10 weeks of age (Figure 1). Therefore, all twelve pigs were used for the remaining parts of the present study.

Serum folate, cobalamin, HCY, and MMA concentrations (n=12)

Serum folate, cobalamin, HCY, and MMA concentrations differed significantly among the nine time points ($p\leq 0.0001$ for all). Results are summarized in Table 2 and Figure 2. For folate the Dunn's posttest demonstrated that the pigs had significantly higher serum folate concentrations at week 6 than at weeks 7 ($p<0.01$), 9 ($p<0.05$), 10 ($p<0.05$), 14 ($p<0.0001$), and 26 ($p<0.0001$). Concentrations of serum cobalamin differed significantly among time points although, the posttest statistics showed no significant difference in serum cobalamin concentrations between the weeks ($p>0.05$; Table 2, Figure 2). For HCY the Dunn's posttest statistics revealed that the pigs had significantly higher serum HCY concentrations at week 6 than at weeks 9 ($p<0.01$), 14 ($p<0.05$), 18 ($p<0.05$), 22 ($p<0.05$), and 26 ($p<0.001$), at week 7 than at weeks 8 ($p<0.05$), 9 ($p<0.001$), 14

($p<0.01$), 18 ($p<0.01$), 22 ($p<0.01$), and 26 ($p<0.01$), at week 9 than at week 10 ($p<0.05$; Table 2, Figure 2). For MMA the posttest statistics showed that the pigs had significantly higher serum MMA concentrations at week 6 than at weeks 14, 18, 22, and 26 ($p<0.001$ for all), at week 7 than at weeks 14 ($p<0.001$), 18 ($p<0.05$), and 26 ($p<0.001$), at week 8 than at weeks 14 ($p<0.001$), 18 ($p<0.01$), 22 ($p<0.05$), and 26 ($p<0.001$), at week 9 than at weeks 14 ($p<0.01$) and 26 ($p<0.05$), and at week 10 than at weeks 14 and 26 ($p<0.05$ for both; Table 2, Figure 2).

As aforementioned all four variables (i.e., folate, cobalamin, HCY, and MMA) revealed a significant difference between the nine time points (for all: $p\leq 0.0001$). However, the calculated coefficient of variation in percent (%CV) for each variable in pigs ($n=12$) between 6 and 26 weeks of age showed a significantly larger %CV for serum MMA concentrations (median: 73.2 %CV, range: 39.5-343.5) when compared to serum folate (median: 43.9 %CV, range: 19.4-82.1; ($p<0.05$), cobalamin (median: 6.4 %CV, range: 0.0-13.3; ($p<0.0001$), and HCY (median: 20.9 %CV, range: 11.8-50.3; ($p<0.001$) concentrations but not between %CV of serum folate, cobalamin, and HCY concentrations (for all three: $p>0.05$).

Serum folate, cobalamin, HCY, and MMA correlations (n=12)

Concentrations of serum folate demonstrated a moderate correlation with serum MMA ($\rho = 0.35$; $p=0.0002$) concentrations, but not with serum cobalamin ($\rho = 0.13$; $p>0.05$) and HCY ($\rho = -0.04$; $p>0.05$) concentrations in postweaning pigs between 6 and 26 weeks of age (Table 3; Figure 3). Serum cobalamin concentrations indicated a moderate correlation with serum MMA ($\rho = -0.49$; $p=0.0138$), whereas no correlation with serum HCY ($\rho = -0.24$; $p>0.05$) concentrations in postweaning pigs between 6 and 26 weeks of age (Table 3; Figure 3). Concentrations of serum

HCY revealed a moderate correlation with serum MMA concentrations ($\rho = 0.49$; $p < 0.0001$) in postweaning pigs between 6 and 26 weeks of age (Table 3; Figure 3).

Serum glycine, glutamic acid, methionine, tryptophan, tyrosine, and lysine concentrations and correlations with folate, cobalamin, HCY, and MMA (n=6)

In postweaning pigs concentrations of serum methionine ($p = 0.0015$; criterion of significant change $p < 0.0085$ based on the Bonferroni correction for all 6 amino acids) and tyrosine ($p = 0.0005$) differed significantly among the five time points, whereas a trend (between $p < 0.0085$ and $p < 0.05$) was observed for concentrations of serum glycine ($p = 0.0215$) and glutamic acid ($p = 0.0173$), and no significant differences were observed for tryptophan and lysine (both $p > 0.05$).

A significant positive correlation was found between serum glutamic acid and cobalamin concentrations ($\rho = -0.61$; $p = 0.0060$; Table 5) as well as serum MMA concentrations ($\rho = 0.60$; $p = 0.0007$; Table 5). A trend for a positive correlation was observed between concentrations of serum glycine and cobalamin ($\rho = 0.53$; $p = 0.0197$; Table 5) as well as serum tyrosine and MMA ($\rho = 0.44$; $p = 0.0163$; Table 5). A trend ($p < 0.05$) for a negative correlation was found between concentrations of serum tyrosine and HCY ($\rho = -0.42$; $p = 0.0249$) as well as serum glycine and MMA ($\rho = -0.37$; $p = 0.0454$; Table 5).

Discussion

To the best of our knowledge this is the first study that investigates the serum concentrations of folate and cobalamin as well as their dependent metabolites (i.e., HCY and MMA) in serum samples from pigs between the ages of 6 to 26 weeks. The results of this study showed age-dependent changes in serum HCY and MMA concentrations in postweaning pigs, possibly

reflecting decreased availability of intracellular folate and cobalamin for amino acid metabolism (Giquère et al., 2008; Mou et al., 2018), nucleic acid synthesis (Fenech, 2012), and methyl group transfer (Toohey, 2017). The correlation analysis between serum folate and cobalamin concentrations in relation to their dependent intracellular metabolites (i.e., HCY and MMA) might suggest that cobalamin and folate, on the cellular level, affects more the intracellular methylmalonyl-CoA mutase (e.g., MMA) than the intracellular methionine synthase (e.g., HCY) in postweaning pigs between 6 and 26 weeks of age. Whether the weaning age affects the intracellular folate and/or cobalamin-dependent enzymes in pigs or a supplementation of one or both vitamins might be beneficial in pigs after weaning requires further investigations. Serum biomarkers can be affected through physiological (e.g., lactation, gestation, and weaning; Wierzechoslawski et al., 2018; van Riet et al., 2015; Grützner et al. 2016) and pathologic (e.g., viral and bacterial infections; Koene et al., 2012; Grützner et al., 2015) conditions. According to the literature, decreased serum cobalamin and/or increased folate concentrations have been described in human patients and pigs with RNA virus infections (Tang et al., J Nutr 1997; Grützner et al., ESPHM 2018), but not in patients with DNA virus infections. This study showed that pigs with a PCV2 (i.e., DNA virus) infection have no significant changes in serum folate, cobalamin, HCY, or MMA concentrations between 10 and 26 weeks of age when compared to PCV2-vaccinated pigs, which would be similar to human subjects with DNA virus infections. Challenge trials in pigs can be quite helpful to investigate how biomarkers respond to certain pathologic conditions (e.g., PCV2 infection). Therefore, studies that evaluate the differences in serum folate, cobalamin, HCY, and MMA in pigs with other DNA virus infections (e.g., Pseudorabies virus, African swine fever virus, swine pox virus, and porcine parvovirus) or RNA virus infections (e.g., influenza A

virus, classical swine fever virus, porcine epidemic diarrhea virus, and virus of foot-and-mouth disease) are warranted to confirm this hypothesis.

The absorption of folate and cobalamin occurs primary in the proximal and distal small intestine, respectively (Trugo et al., 1985; Halsted, 1980; Said, 2011), which could help to localize where potentially malabsorption of these two vitamins takes place. By observing serum folate concentrations in pigs between 6 and 26 weeks of age it would appear to be rather impossible to identify a certain pattern for serum folate concentration in that study period. Although the concentrations of serum folate changed in the pigs studied here, a correlation with MMA, but not with cobalamin and HCY and the six amino acids was observed. The results of this study indicate that serum folate concentrations undergo no relevant age-dependent changes in pigs between 6 and 26 weeks of age. However, this could be different by investigating tetrahydrofolate (THF) and N⁵-methyltetrahydrofolate (5-MF) in addition to the total plasma folate concentration, as conducted by Natsuhori et al. (1996). This research group found significant changes of THF and 5-MF in sows during gestation and suckling piglets. The total plasma folate concentration decreased during nursery after weaning. In contrast, increased serum folate concentrations have been reported in pigs 12 days after weaning (Grützner et al., 2016). A reason for the increase of serum folate concentrations could be due to increased preweaning absorption (Lalles et al., 2004), disturbed absorption after weaning (Boudry et al., 2004), and postweaning small intestinal dysbiosis (Williams 2001, Rossi et al., 2011) in pigs after weaning. Taking the results of the three studies (Natsuhori et al. 1996, Grützner et al., 2016 and the present study) into account it would be warranted to investigate folate (plasma / serum, THF, and 5-MF) in pigs around weaning (e.g., three to four weeks after weaning and different weaning ages) because these studies suggest that

this period revealed the largest changes in folate and cobalamin concentrations as well as their dependent metabolites (i.e., HCY and MMA).

The detection limit of the cobalamin immunoassay in veterinary (Berghoff et al., 2012; Grützner et al., 2013) as well as human medicine (Green et al., 2017) poses a problem for the interpretation of serum cobalamin concentrations since a lot of concentrations are below the detection limit as shown in figures one and two. However, it appears that pigs between 6 and 14 weeks of age have lower serum cobalamin concentrations than pigs that are 14 to 26 weeks of age. The lower serum cobalamin concentrations in week 6, 7, and 8 reflect the higher serum concentrations of MMA, which contribute to the hypothesis that decreased cobalamin concentrations are associated with increased MMA concentrations (Berghoff et al. 2012; Grützner et al. 2015), whereas real values (i.e., concentration below detection limit, all = 150 ng/L) for cobalamin are often lacking. Furthermore, it appears that pigs between 14 and 26 weeks of age have a more stable serum cobalamin concentrations and environmental factors and disease conditions such as diarrhea or dietary restriction of cobalamin might be able to affect cobalamin absorption in pigs during that period (Grützner 2016). Overall, the significant correlation between both B vitamins (i.e., folate and cobalamin) and MMA concentrations demonstrates an association between the B vitamins and the mitochondrial methylmalonyl-CoA enzyme as well as their dependent metabolite (i.e., MMA), which has been reported in other studies (Berghoff et al. 2012; Grützner et al. 2015).

HCY and MMA, the two intracellular folate and/or cobalamin dependent metabolites that were studied, exhibited changes between the nine time points in postweaning pigs. Increased serum HCY concentrations were observed in postweaning pigs (fed diet Natur's Match 1.3) in weeks 6, 7, and 10 with increased concentrations in week 6 and 7 possibly being related to the decreased cobalamin concentrations. However, the significant change at week ten raises questions because

the pigs were not moved, received the same feed, and no feed, water, or power failure or climate change was noticed. Even the PCV2 infection showed no effect on the serum HCY concentrations. Hyperhomocysteinemia can result from folate, cobalamin, or pyridoxine (vitamin B₆; Iqbal et al., 2009; Acharya et al., 2008) deficiency. However, this cause of HCY increases seems unlikely because it only occurred at one specific time point. A possible explanation could be that increased HCY concentrations in serum and saliva can occur due to stress in patients and pigs, respectively (Stoney and Engebretson, 2000; Tecles et al., 2017). At 10 weeks of age, pigs were inoculated intranasal and intramuscular routes and blood was collected afterwards. According to Tecles et al. (2017) the HCY concentration increases significantly in saliva between 5 to 15 minutes after stressing a pig, for example by using a nasal snare, which may mean distress due to capturing and restrain of the animals. In the pigs studied, a nasal snare was used to capture and to restrain the pigs during inoculation as well as blood collection, which could explain the increased HCY concentrations in pigs at week 10 and not prior (week 9, blood draw – one intervention) or afterwards (week 14, blood draw – one intervention) in the pigs that were studied. In contrast, serum MMA concentrations were increased 5-10 fold in postweaning pigs (fed diet Natur's Match 1.3) at weeks 6 and 7 when compared to the remaining time points. In addition, an overall descending serum MMA concentration was observed from week 6 to week 14, which might have mirrored the serum cobalamin concentrations in the pigs studied. Correlation analyses with regard to serum MMA concentrations revealed a relationship between the intracellular folate- and cobalamin-dependent enzymes (i.e., cytosolic methionine synthase and mitochondrial methylmalonyl-CoA) as reported elsewhere (Berghoff et al. 2012 and 2012b; Grützner et al. 2015). The elevated concentrations of both HCY and MMA could be due to the emerging changes after weaning. After weaning the gastrointestinal tract of pigs undergoes morphologic changes, such as

villous atrophy and crypt hypertrophy, which can last up to twelve days (Hampson, 1986) and could influence the absorption of micronutrients such as cobalamin, but less likely folate, because of increasing serum folate concentrations after weaning (Grützner et al. 2016). Furthermore, a malabsorption of cobalamin in pigs after weaning could potentially occur either through increased mucosal permeability (Lallès et al., 2004), disturbed absorption and secretion (Boudry et al., 2004), altered inflammatory cytokine pattern (Pié et al., 2004), or microbial activity, which influences gut immunity and motility (Williams et al., 2001; Rossi et al., 2011). Piglets receive folate and cobalamin from sow's milk (Ford et al., 1975; Trugo, 1988) whereas after the morphologic changes of the gastrointestinal tract, cobalamin malabsorption could occur. However, cobalamin could be mobilized from the body reserves (e.g., liver and kidney) for a certain period of time. To our knowledge the period until the cobalamin reserves are depleted has not been determined for pigs. Taking that into account it could be possible that a lack of cobalamin occurs to the aforementioned increased mucosal permeability, disturbed absorption and secretion, altered inflammatory cytokine pattern or microbial activity, will arise two or three weeks after weaning in pigs, which could explain the increased serum HCY and MMA concentrations in pigs between 6 and 8 weeks of age, which in turn could lead to a decreased availability of intracellular cobalamin for amino acid metabolism, nucleic acid synthesis, and methyl group transfer. Studies are underway that evaluate serum folate, cobalamin, HCY, and MMA concentrations between different weaning stages in pigs between 3 to 9 weeks of age.

Assays for the measurement of serum cobalamin and HCY are available for pigs, however, those measurements do not accurately determine the cobalamin status in pigs (House and Fletcher, 2003). Serum cobalamin is not a direct reflection of the intracellular cobalamin status and HCY, a product of the malfunctioning of the intracellular enzyme methionine synthase, has more than one cofactor

(folate, cobalamin, or pyridoxine). In contrast, MMA a product of the malfunctioning off the intracellular enzyme methylmalonic acid-CoA mutase, which is a cobalamin dependent enzyme. The results of this study suggest that the measurement of MMA, a cobalamin-dependent metabolite, in serum holds promise to improve the determination of the cobalamin status in both, healthy and diseased pigs.

Comparison of six amino acids between groups of postweaning pigs revealed differences for methionine and tyrosine. For serum cobalamin, HCY, and MMA concentrations a significant correlation or a trend were observed for three of the six amino acids (i.e., glutamic acid, and glycine as well as tyrosine; respectively). The results of this study suggest that concentrations of serum cobalamin, HCY, and MMA might have an influence on certain serum amino acid concentrations in pigs between 6 and 26 weeks of age. Serum glutamic acid concentrations revealed an association with serum cobalamin and MMA concentrations, which could refer that cobalamin binding proteins have high levels of glutamic acid in their carbohydrates (Trugo, 1988). On the other hand, both vitamins as well as their dependent metabolites (i.e., HCY and MMA) did not show any association with methionine, which is in contrast to the findings of the study by Giquère et al. (2008). The reason for this finding could be that this part of the study had several limitations. One limitation was the small sample size and a four-week-long period between the sample collections. A larger samples size and maybe a one-week interval for the blood sample collection could have changed the results of the study. Furthermore, the lack of experience with the method by Lopes et al. (2015) regarding the porcine serum samples could also have contributed to the obtained results. Because limited information is available regarding the stability of each amino acid in pig's serum with that method by Lopes et al. (2015). However, to avoid variations in the amino acid concentrations for the comparison in the present study all samples were handled in a similar

fashion. Further prospective studies are warranted to confirm the differences in serum amino acids and folate, cobalamin, HCY, and MMA concentrations and whether supplementation of folate and/or cobalamin might be valuable in postweaning pigs with regard to optimal amino acid maintenance. The studied pigs had unrestricted access to feed, which is a common practice in pig production herds worldwide. Those feeding practices and the point that the pigs were not fasted prior to blood collection could also have significantly affected amino acid concentrations and remaining the study variables. For the present study, a diet with a constant folate and cobalamin concentration was intended to have throughout the study period. It would have been supportive of rechecking the levels of folate and cobalamin in the three different diets to see if no variation of both vitamins as well as the amino acids in the diet have occurred, which also revealed a limitation of the present study. However, taking both, the constant folate and cobalamin concentration in the three different diets and the observation of the significant results for serum HCY and MMA concentrations during the feeding period of one diet into account it seems reasonable to suggest that the diets have no confounded effect on the age of the studied pigs. However, due to the narrow window (two to four weeks after weaning) of the significant changes of serum HCY and MMA concentration additional investigations are warranted to evaluate the dietary requirements of folate and cobalamin in pig-diets and pigs within a short period after weaning and maybe depending on the time of weaning.

Conclusions

The results of this study show considerable age-dependent changes in serum HCY and MMA concentrations in pigs between 6 and 26 weeks of age, possibly reflecting decreased availability of intracellular cobalamin (vitamin B₁₂) for amino acid metabolism, nucleic acid synthesis, and

methyl group transfer. Due to the association of serum MMA concentrations with the remaining studied variables (i.e., folate, cobalamin, and HCY) it seems that postweaning pigs have altered changes of vitamin B9 and B12 intracellularly with a greater or lesser extent to the dependent enzymes (i.e., methylmalonyl-CoA mutase and methionine synthase, respectively). Whether the weaning age affects the intracellular folate and/or cobalamin-dependent enzymes in pigs or a supplementation of one or both vitamins might be beneficial in pigs after weaning, warrants further studies.

Conflict of Interest statement

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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534 **Tables**

535 **Table 1:** The pigs of this particular trial were fed a commercial diet that met or exceeded all nutrient requirements established by the
 536 1998 National Research Council (NRC). Each diet (Natur's Match 1.3 [approx. 4 to 10 weeks of age], Natur's Match 1.15 [approx. 10
 537 to 15 weeks of age], Natur's Match 1.05 [approx. 15 to 26 weeks of age]) from Heartland CO-OP, Des Moines, Iowa, USA) had the
 538 similar amount of folate (3.72 ppm [mg/kg]) and cobalamin (3.81 ppb [µg/kg]). Bushels (BU); hundredweight (CWT); and Pound (LB).

1. Natur's Match 1.3	Units	Amounts	2. Natur's Match 1.15	Units	Amounts	3. Natur's Match 1.05	Units	Amounts
Corn	BU	105600	Corn	BU	344400	Corn	BU	450700
Soybean meal HI Pro-Bulk	CWT	28800	Soybean meal HI Pro-Bulk	CWT	97400	Soybean meal HI Pro-Bulk	CWT	86500
DICAL - Monocal 21% Bulk	CWT	0.1400	DICAL - Monocal 21% Bulk	CWT	0.4200	DICAL - Monocal 21% Bulk	CWT	0.4500
Limestone (Bulk)	CWT	0.1200	Limestone (Bulk)	CWT	0.3700	Limestone (Bulk)	CWT	0.4000
Mixing Slat (Bulk)	CWT	0.0400	Mixing Slat (Bulk)	CWT	0.1300	Mixing Slat (Bulk)	CWT	0.1400
LOL TM PMX 2640100	LB	0.8500	LOL TM PMX 2640100	LB	25500	LOL TM PMX 2640100	LB	27700
LOL Pork Vitamins	LB	0.6000	LOL GF Vit PMX 2080900	LB	18000	LOL GF Vit PMX 2080900	LB	19400
Selenium Premix 0.06%	LB	0.2300	Selenium Premix 0.06%	LB	0.6800	Selenium Premix 0.06%	LB	0.7400
Choline Chloride 60%	LB	0.1800	Choline Chloride 60%	LB	0.5300	Lysine HCL 98% 1# (55#)	LB	52500
Lysine HCL 98% 1# (55#)	LB	15000	Grind & Mix	CWT	300000	Choline Chloride 60%	LB	0.5300
Natur's Match Starterbase	CWT	0.8800	Bagging	CWT	300000	Methionine DL 1#	LB	0.8800
Grind & Mix	CWT	100000				Grind & Mix	CWT	300000
Bagging	CWT	100000				Bagging	CWT	300000

Table 2: Medians (ranges) for serum cobalamin (ng/L), folate (µg/L), homocysteine (µmol/L), and methylmalonic acid (nmol/L) concentrations in twelve conventional crossbred pigs at increasing ages (in weeks).

Week	Folate	Cobalamin	Homocysteine	Methylmalonic acid
6	33.4 (25.5-48.1)	150 (<150-158)	23.9 (19.7-44.4)	2,010 (769-8,304)
7	21.7 (5.5-27.9)	150 (<150-152)	24.4 (19.3-40.8)	734 (480-2,350)
8	28.0 (4.6-45.4)	150 (<150-172)	20.3 (14.8-22.7)	799 (558-1,120)
9	22.5 (6.6-32.8)	all <150	18.4 (11.7-23.3)	647 (456-854)
10	23.3 (4.9-38.0)	150 (<150-165)	23.5 (13.6-42.9)	655 (397-819)
14	14.3 (5.7-24.3)	150 (<150-210)	17.6 (11.6-23.7)	336 (241-400)
18	18.8 (6.6-38.2)	151 (<150-235)	18.2 (14.5-22.2)	363 (264-617)
22	28.1 (7.2-34.2)	154 (<150-244)	18.1 (14.9-21.3)	396 (336-590)
26	14.0 (4.2-28.2)	150 (<150-210)	18.1 (15.6-21.7)	318 (180-480)

Table 3: Correlation of the serum vitamin B₁₂-related biomarkers from all pigs in this study.

Bolded *p* values are significant.

Correlation variables	Rho (ρ)	95% CI	<i>p</i> value
Folate vs. Cobalamin	0.13	-0.29 to 0.51	0.5363
Folate vs. Homocysteine	-0.04	-0.24 to 0.15	0.6505
Folate vs. Methylmalonic acid	0.35	0.17 to 0.51	0.0002
Cobalamin vs. Homocysteine	-0.24	-0.59 to 0.18	0.2449
Cobalamin vs. Methylmalonic acid	-0.49	-0.74 to -0.10	0.0138
Homocysteine vs. Methylmalonic acid	0.49	0.33 to 0.63	<0.0001

Table 4: Medians (ranges) for six serum amino acid concentrations in six vaccinated pigs at different ages (in weeks). Significant differences between the groups of pigs are indicated by different superscripts (a,b,c).

Week	Glycine	Glutamic acid	Methionine	Tryptophan	Tyrosine	Lysine
6	81.8 (67-110) ^c	88.9 (71-155) ^{a,c}	9.7 (5-18) ^c	53.2 (36-70) ^a	32.4 (6-39) ^a	35.0 (18-70) ^a
10	67.8 (62-136) ^{a,c}	85.5 (28-191) ^c	15.7 (10-29) ^{a,c}	59.0 (45-87) ^a	52.4 (25-64) ^{a,b}	26.9 (12-73) ^a
15	115.6 (82-198) ^{b,c}	44.3 (33-60) ^c	7.3 (4-10) ^{b,c}	41.5 (23-87) ^a	20.8 (6-44) ^a	22.1 (20-36) ^a
22	67.8 (64-130) ^c	55.6 (35-132) ^c	16.6 (11-24) ^{a,c}	42.6 (10-66) ^a	55.2 (34-114) ^b	54.7 (32-69) ^a
26	85.1 (77-126) ^c	44.8 (26-60) ^{b,c}	9.6 (7-12) ^c	37.7 (17-55) ^a	15.2 (8-30) ^a	32.8 (20-48) ^a

Table 5: Correlation of the six serum amino acids and vitamin B₁₂-related biomarkers in the six vaccinated pigs. Bolded *p* values were significant based on Bonferroni correction (significance set at: *p*= 0.0085) and italic *p* values have shown a trend.

Correlation variables	Rho (ρ)	95% CI	<i>p</i> value
Folate vs. Glycine	0.02	-0.35 to 0.39	0.9228
Folate vs. Glutamic acid	0.27	-0.11 to 0.58	0.1542
Folate vs. Methionine	0.05	-0.33 to 0.41	0.7928
Folate vs. Tryptophan	-0.01	-0.37 to 0.37	0.9842
Folate vs. Tyrosine	0.07	-0.31 to 0.43	0.7269
Folate vs. Lysine	-0.15	-0.49 to 0.24	0.4381
Cobalamin vs. Glycine	0.53	0.08 to 0.80	<i>0.0197</i>
Cobalamin vs. Glutamic acid	-0.61	-0.84 to -0.20	0.0060
Cobalamin vs. Methionine	0.20	-0.29 to 0.61	0.4069
Cobalamin vs. Tryptophan	-0.02	-0.48 to 0.45	0.9449
Cobalamin vs. Tyrosine	-0.18	-0.60 to 0.31	0.4536
Cobalamin vs. Lysine	-0.30	-0.67 to 0.19	0.2124
Homocysteine vs. Glycine	-0.09	-0.45 to 0.29	0.6529
Homocysteine vs. Glutamic acid	0.11	-0.27 to 0.47	0.5567
Homocysteine vs. Methionine	-0.20	-0.54 to 0.19	0.2958
Homocysteine vs. Tryptophan	0.08	-0.30 to 0.45	0.6640
Homocysteine vs. Tyrosine	-0.42	-0.68 to -0.05	<i>0.0249</i>
Homocysteine vs. Lysine	-0.12	-0.48 to 0.27	0.5228
Methylmalonic acid vs. Glycine	-0.37	-0.66 to 0.00	<i>0.0454</i>
Methylmalonic acid vs. Glutamic acid	0.60	0.28 to 0.79	0.0007
Methylmalonic acid vs. Methionine	0.28	-0.10 to 0.60	0.1358
Methylmalonic acid vs. Tryptophan	0.34	-0.04 to 0.63	0.0725
Methylmalonic acid vs. Tyrosine	0.44	0.08 to 0.70	<i>0.0163</i>
Methylmalonic acid vs. Lysine	-0.07	-0.44 to 0.31	0.7165

Figure legends

Figure 1: Influence of infection with a DNA virus (PCV2 exposure at 10 weeks of age) on folate, cobalamin, HCY, and MMA concentrations in conventional crossbred pigs. Serum folate (**A**: $p=0.5456$), cobalamin (**B**: $p=0.9052$), HCY (**C**: $p=0.6208$), and MMA (**D**: $p=0.8526$) concentrations showed no significant difference in pigs either vaccinated ($n=6$; black line with circles) or not ($n=6$; dashed line with triangles) against PCV2 at different ages (in weeks). The grey shaded area indicates the detection limit, which is 150 ng/L, of the Immunoassay for cobalamin (**B**). The challenge with the PCV2 was performed at 10 weeks of age (black arrow).

Figure 2: Serum folate, cobalamin, HCY, and MMA concentrations in pigs ($n=12$) between 6 and 26 weeks of age. A) Serum folate concentrations differed significantly among time points ($p<0.0001$; Table 2). Posttest statistics showed that the pigs had significantly higher serum homocysteine concentrations at weeks 6 than at weeks 7, 9, 10, 14, and 26 (for all: $p<0.05$). **B)** Serum cobalamin concentrations differed significantly among time points ($p=0.0001$; Table 2); although, the posttest statistics showed no significant difference in serum cobalamin concentrations between the nine weeks ($p>0.05$). **C)** Serum homocysteine concentrations differed significantly among time points ($p<0.0001$; Table 2). Posttest statistics showed that the pigs had significantly higher serum homocysteine concentrations at weeks 6 than at weeks 9, 14, 18, 22, and 26 (for all: $p<0.05$), at weeks 7 than at weeks 8, 9, 14, 18, 22, and 26 (for all: $p<0.05$), and at weeks 9 than at weeks 10 ($p<0.05$). **D)** Serum methylmalonic acid concentrations differed significantly among time points ($p<0.0001$; Table 2). Posttest statistics showed that the pigs had significantly higher serum methylmalonic acid concentrations at weeks 6 than at weeks 14, 18, 22, and 26 (for all: $p<0.05$), at weeks 7 than at weeks 14, 18, and 26 (for all: $p<0.05$), at weeks 8 than

at weeks 14, 18, 22, and 26 (for all: $p<0.05$), at weeks 9 than at weeks 14 and 26 (for both: $p<0.05$), and at weeks 10 than at weeks 14 and 26 (for both: $p<0.05$). Every solid black lines indicate one of the 12 pigs.

Figure 3: Correlations between MMA and the remaining studied variables (i.e., folate, cobalamin and HCY) in serum of pigs (n=12) between 6 and 26 weeks of age. A) A positive correlation was observed between serum MMA and folate concentrations (ρ : 0.35; $p=0.0002$). B) A negative correlation was found between serum MMA and cobalamin concentrations (ρ : -0.49; $p=0.0138$). C) A positive correlation was observed between serum MMA and HCY concentrations (ρ : 0.49; $p<0.0001$). For each graph the concentrations (dots), 95% confidence interval (dotted lines) and regression line (solid line) are shown. Additional information are shown in table 3.

Figure 4: Serum glycine, glutamic acid, methionine, tryptophan, tyrosine, and lysine concentrations in six vaccinated pigs at different ages (in weeks). The serum concentrations for A) glycine ($p=0.0215$); B) glutamic acid ($p=0.0173$); C) methionine ($p=0.0015$); and E) tyrosine ($p=0.0005$) differed significantly among time points whereas D) tryptophan ($p=0.1895$) and F) lysine ($p=0.1664$) were not significantly different among time-points (Table 4).