# Original Research

# Trace element status and zinc homeostasis differ in breast and formula-fed piglets

**Martin JJ Ronis[1,2], Isabelle R Miousse[1,3], Andrew Z Mason[4], Neha Sharma[1], Michael L Blackburn[1] and Thomas M Badger[1,2]**

[1]Arkansas Children's Nutrition Center, Little Rock, AR 72202, USA; [2]Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA; [3]Department of Environmental and Occupational Health, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA; [4]Department of Biological Sciences, California State University, Long Beach, CA, 90840-9502, USA
Corresponding author: Martin Ronis. Email: ronismartinj@uams.edu

## Abstract

Differences in trace element composition and bioavailability between breast milk and infant formulas may affect metal homeostasis in neonates. However, there is a paucity of controlled studies in this area. Here, piglets were fed soy infant formula (soy), cow's milk formula (milk), or were allowed to suckle from the sow from PND2 to PND21. Serum iron concentrations were higher in formula-fed compared to breastfed piglets ($P < 0.05$). Serum zinc values were higher in milk compared to breastfed or soy groups ($P < 0.05$). Zinc transporter *Zip4* mRNA was elevated in small intestine of the soy compared to breastfed group ($P < 0.05$). Transporter *Znt1* mRNA was greater in small intestine of both formula-fed groups and in liver of the milk compared to the breastfed group ($P < 0.05$). Metallothionein *Mt1* mRNA expression was higher in small intestine and liver of milk compared to breastfed and soy groups ($P < 0.05$). In liver, metallothionein protein levels and protein bound zinc were also highly elevated in the milk compared to other groups ($P < 0.05$). mRNA encoding the hepatic zinc-regulated gene *Gclc* was higher in the milk than soy group ($P < 0.05$). ChIP assay revealed increased binding of the zinc-regulated transcription factor MTF1 to the promoters of hepatic *Mt3* and *Gclc* genes in the milk compared to the soy group. These data provide evidence that trace element status differs in breastfed, milk-fed, and soy-fed piglets and that despite similar levels of dietary supplementation, allows strong causal inference that significant differences in serum zinc after cow's milk formula compared to soy formula consumption result in compensatory changes in expression of zinc transporters, binding proteins, and zinc-regulated genes.

**Keywords:** Trace element, zinc, breastfeeding, infant formula, pig, metallothionein

## Introduction

The composition of infant formulas is constantly being modified as a result of characterization of additional bioactive components of human milk and as nutrient needs of different groups of infants are better established.[1] However, nearly all breastfeeding research is based upon observational studies, which is a major weakness in this field. One group of formula additives is the metal trace elements. There is a paucity of controlled studies on the differences in trace element bioavailability and homeostasis between breast milk, cow's milk formula, and soy milk. Breast milk is low in iron and iron deficiency has been documented in exclusively breastfed infants in several randomized control trials.[2,3] Therefore, iron is now routinely added to infant formulas. We have previously published data using a piglet model of breast versus formula feeding showing that iron supplementation of infant formula leads to changes in iron homeostasis with increased hepatic iron accumulation, dramatic elevation in hepatic expression of hepcidin, a major regulator of iron trafficking and suppression in hepatic expression of the transferrin receptor in formula-fed compared to breastfed piglets at weaning.[4] These data suggest that differences in trace element composition and bioavailability between breast milk and formula can influence metal homeostasis and metal-regulated gene expression in neonates and prompted us to more closely examine trace element status and in particular zinc homeostasis in this model which answers a question of causality (albeit in pigs) in a framework that is ethically impossible to implement in humans.

Low levels of a number of metals in cow's milk and soy including copper, selenium, and manganese have led to supplementation of dairy and soy-based infant formulas with these trace elements to alleviate potential deficiency

of these elements in formula-fed infants.[1,5,6] In addition, zinc is another essential trace metal nutrient that is involved in nearly all aspects of metabolism. Over 50 different enzymes in mammals necessitate zinc directly as a cofactor in the reaction they catalyze.[7] Zinc also has a structural function, as it contributes to the shaping of zinc finger motifs in hundreds of proteins. Severe zinc deficiency in infants causes growth retardation, dermatitis, alopecia, diarrhea, and immune deficiency.[7–9] As a result of the observation that infants fed cow's milk formula had lower zinc status and grew more slowly than breastfed infants and that zinc supplements prevented this, zinc supplementation of infant formulas was recommended by the American Academy of Pediatrics.[10,11]

Consumption of soy, as well as other legumes and cereals, has been associated with low zinc status. Soy products contain phytic acid, which has the ability to chelate zinc in the gut and prevent its absorption.[12,13] In 1984, Lonnerdal *et al.*[14] published a study suggesting lower levels of zinc absorption by human adults from cow's milk formula compared to breast milk and from soy formula compared to breast milk or cow's milk formula at different levels of zinc supplementation. These results were consistent with other studies showing lower zinc absorption in soy formula than cow's-milk formula in animal models.[15] However, despite these findings, the only epidemiological study in humans comparing serum zinc levels in infants consuming regular and soy formulas has shown no sign of deficiency in infants consuming soy formula.[16] Mean serum concentration was low in infants fed soy formula, but not significantly so. However, there are several limitations associated with using serum zinc level to evaluate the adequacy of zinc intake as it does not consider the compensatory ability of cellular zinc to modulate transcription of zinc transporters and binding partners to maintain homeostatic control of the metal.

Zinc present in food is taken up in the small intestine by zinc transporters that fall into two families, the Zrt-, Irt-like proteins (ZIP) family, comprising 14 members, whose function is to import zinc inside the cell, and the zinc transporter (ZnT) family, comprising 10 members, whose function is to take zinc from the cytoplasm and excrete it in the extracellular milieu.[7,17] Under low zinc conditions, the expression of these transporters is modulated to increase zinc uptake from diet and minimize losses in urine and feces. In the small intestine, the transporter Zip4 is expressed on the apical membrane and is responsible for the import of dietary zinc into enterocytes. Zip4 is mainly expressed in the liver, the digestive tract, the uterus, and placenta.[7] Mutations in this gene are responsible for the autosomal recessive disorder acrodermatitis enteropathica. Zinc absorption is severely reduced in these patients and the symptoms are similar to dietary zinc deficiency. *Zip4* mRNA expression has been shown to be upregulated as a result of zinc deficiency.[7,17] ZnT1 is expressed on the basolateral membrane of enterocytes and shuttles zinc from the enterocyte to the circulation. It is ubiquitously expressed, with higher levels in the small intestine and placenta. *Znt1* knockout is embryonic lethal in mice. *Znt1* mRNA expression has been shown to be downregulated in zinc restricted

mice and was recently demonstrated to be significantly upregulated in the intestine of weanling pigs in response to high levels of dietary zinc.[7–9,17] Other zinc transporters are mainly located on vesicles and the Golgi for regulation of intracellular pools. In the serum, zinc is bound to the carrier protein albumin and, to a smaller extent, to $\alpha_2$-macroglobulin.[7–9] Inside the cell, zinc is found mainly bound to metallothionein (MT), which is found as four isoforms: MT1-MT4.[7] MTs are regulated by zinc so that in replete conditions, mRNA and protein synthesis is upregulated, allowing sequestration of the additional zinc to form a zinc reserve intracellularly. MT is degraded after dissociation from zinc. Under conditions of zinc deficiency, less MT is produced to ensure that a pool of free zinc ions is biologically available.[7] The transcription of MTs, *Zip4*, and *Znt1* genes is regulated by zinc.[7] Zinc binding to the transcription factor MTF1 results in activation and interaction with metal responsive elements (MRE) sequences located in the promoter region of these genes. Other genes also known to be regulated by zinc through MTF-1 include the catalytic subunit of glutamate-cysteine ligase (*Gclc*), CEBPα, and α-fetoprotein.[7–9,18] As many homeostatic mechanisms exist to compensate for marginal zinc status, direct measurement in tissues of zinc-regulated genes is a more sensitive measure of zinc status than plasma level.[19,20]

For this study, we used our well-established porcine model to reproduce the consumption of infant formulas by human infants. Piglets were fed cows' milk-based formula, soy-based formula, or were left to suckle breast milk from the sow to determine whether neonatal diet influences trace element levels and zinc homeostasis.

## Materials and methods
### Animal experiments

Pig experiments were performed as previously described.[4,21] Briefly, White × Dutch Landrace × Duroc sows were fed a soy-free diet and were artificially inseminated. Piglets were allowed to suckle for 48 h before being randomly distributed between three groups of approximately equal mean weight. Male and female breastfed piglets ($n = 5$/group) (sow) were placed with sows for the duration of the experiment and allowed to breastfeed *ad libitum*. Male and female piglets ($n = 5$/group) were fed cow's milk-based formula (milk) (Similac Advance powder; Ross Products, Abbott Laboratories, Columbus, OH). Male ($n = 6$) and female ($n = 4$) piglets were fed soy-based formula (soy) (Enfamil Prosobee Lipil powder; Mead Johnson Nutritionals, Evansville, IN). Formula-fed piglets were trained to drink from small bowls on a fixed schedule as described previously, to provide 1.047 MJ/kg/day until sacrifice on postnatal day (PND) 21.[22] Formula diets were modified to meet the energy and nutrient recommendations of the National Research Council (NRC) for growing pigs[22] and the formula diet composition has been previously published.[21] Trace element composition of the formula diets and of sow milk based on literature values is shown in Table 1.

All animals were housed in the animal facilities of the Arkansas Children's Hospital Research Institute, an

**Table 1** Trace element content of sow milk and infant formulas (µg/L)

| Element | Sow milk | Cow's milk infant formula | Soy infant formula |
|---------|----------|---------------------------|--------------------|
| Cu | 1400 | 610 | 510 |
| Fe | 1000 | 12,000 | 12,000 |
| Zn | 3000–7000 | 5100 | 5100 |
| Se | 70 | 120 | 120 |
| Mn | 200 | 30 | 170 |

Data for sow milk content of Cu, Zn, and Mn from Park et al.[23] Data for sow milk content of Fe from Hollis.[24] Data for sow milk Se content from Mahan.[25] Formula concentrations of trace elements as previously published.[21]

Association for the Assessment and Accreditation of Laboratory Animal Care-approved animal facility. Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research established and approved by the institutional Animal Care and Use Committee at University of Arkansas for Medical Sciences (Little Rock, AR). Pigs were killed by exsanguination after anesthetization with isoflurane at 0800–1000 h, 6–8 h after the final feeding period. Blood was collected and serum prepared by centrifugation. In addition, liver samples were snap-frozen in liquid nitrogen. Frozen livers were stored at $-70°C$ until use.

### Trace element analyses

Serum from individual piglets and liver samples pooled from male and female piglets in each diet group were dried to constant weight and microwave (CEM MARS) digested in Teflon bombs using ultrapure, trace-metal grade concentrated nitric acid. The digested samples were evaporated to near dryness and diluted to a known volume with 2% $HNO_3$ prepared in 18 Ω water containing 10 µg/L of rhodium and thulium, which were used as internal standards to correct for changes in instrumental sensitivity during analysis. Each serum sample was analyzed for zinc (Zn), iron (Fe), copper (Cu), molybdenum (Mo), selenium (Se), manganese (Mn), vanadium (V), antimony (Sb), strontium (Sr) nickel (Ni), lead (Pb), cobalt (Co), chromium (Cr), barium (Ba), beryllium (Be), tellurium (Tl), silver (Ag), aluminum (Al), titanium (Ti), and arsenic (As) content using a PE 6100DRC ICP-MS using standard operating conditions and parameters. Accuracy and precision of the preparative and analytical procedures were confirmed by the analysis of replicate samples of certified reference material. Overall accuracy was determined to be better than 5% and precision was greater than 3%. Detection limits, as determined by the values obtained from the analysis of multiple reagent blanks, were typically better than 0.5–5 µg/L for all elements of interest.

Cytosolic extracts from each pooled liver sample were prepared from homogenates by ultracentrifugation for 1 h at 100,000 × g. Filtered (2 µm) extracts containing equivalent amounts of total protein as determined by Coomassie blue were analyzed by directly coupled HPLC ICP-MS as described previously.[26] Samples were fractionated at a flow rate of 1 mL/min on a Showdex DEAE-825, metal-free, biocompatible column (75 × 7.8 mm, Phenomenex) using a stepped gradient of 10 mM Tris (pH 8.2) increasing to 10 mM Tris in 500 mM $NH_4Cl$ (pH 8.2) over a 60-min time period. A metal-scavenger column (7.8 mm ID × 75 mm length), hand-packed with Chelex 100 resin (BioRad), was installed prior to a 100 µL PEEK® sample injector loop to remove metals from the solvent stream. A metal-free, Rheodyne 7125 injector with a 200 µL sample loop installed after the UV/Vis detector and immediately prior to the ICP-MS was used for flow injection analyses (FIA) to quantify the elemental composition of the resolved peaks, to monitor analyte recovery and to evaluate for substitutive exchanges of extraneous metal between the sample and the system hardware and mobile phases. Metals associated with the proteins on the various resolved peaks were quantified using the Perkin–Elmer ChromelinkTM chromatographic application within TotalchromeTM software from the integrated FIA ion intensity response peak from 10 ng of analyte. For comparative purposes, protein injectates were normalized from the integrated area of the total 280 nm absorption profile in the HPLC chromatogram. MT peaks were assigned based upon their 238/280 ratio, which is indicative of the Zn–MT complex.

### Quantitative real-time PCR

RNA was extracted from liver and ileum using Trizol reagent. Crude RNA was purified using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. One microgram was reverse-transcribed using iSCRIPT cDNA synthesis kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. cDNA was diluted 1:5 and 2 µL was used for quantitative real-time PCR, using SYBR Green master mix (Life Technologies, Grand Island, NY) on a ABI 7500 instrument (Life Technologies). The supplied software was used to calculate quantity means from standard curves, and values were normalized to the genes *Rpl27* and *Rps12* using the geNorm software.[27] Primer sequences for genes quantified by real-time quantitative PCR and compared between treatment groups are given in Table 2.

### Hepatic MT protein abundance

MT protein expression was determined in liver homogenates by Western immunoblot analysis using primary monoclonal MT antibody (Clone E9, Dako, Carpinteria, CA) essentially as described by Martin *et al*.[17] Immunoqualtitation by densitometric scanning of the blot was corrected for loading of total protein by normalizing against the signal from membranes stained with amido black as described previously.[28]

### ChIP analysis of MTF-1 binding to hepatic Mt3 and Gclc promoters

A ChIP-It Express Enzymatic Kit and protocol (ActiveMotif, Carlsbad, CA) was used; 75 mg of pooled frozen liver tissue was minced with a razor blade then transferred to a 50-mL

**Table 2** Primer sequences utilized in the current study

| Gene | Forward | Reverse |
| --- | --- | --- |
| *Gclc* | TCCTGAGGATGATGAAGCAGCGAA | TGGAACGTTACCTGGAGACAGCAA |
| *Mt1* | TTGCTCTCTGCTTGGTCTCACCT | GCTCTTCTTGCAGGAGGTGCAT |
| *Rpl27* | AAGTTCATGAAACCCGGGAAGGTG | TCGGTCTGAGGTGCCATCATCAAT |
| *Rps12* | TATGTCAAGTTGGTGGAGGCCCTT | TTGCCATAGTCCTTCACCACCACA |
| *Zip4* | TCATCTGCCTCTGCTCCATCTTTG | AAGGTCTGGATGACGTAGTGGGTA |
| *Znt1* | TCACGCTACCACCATTCAACCTGA | CCGTGTCCCACAACATTGCTTCAA |

conical tube containing 20 mL Fixation solution (1% formaldehyde in $1 \times$ PBS). The liver was incubated for 10 min at room temperature and stopped with 2.2 mL $10 \times$ Glycine Stop-Fix solution. The liver was washed with 10 mL $1 \times$ PBS and pelleted. A quantity of 2 mL ChIP Lysis Buffer (Santa Cruz Biotechnology, Dallas, TX) $+ 10 \mu$L each PIC and 100 mM PMSF were added to the pellet and placed on ice for 5 min and then transferred to a dounce homogenizer and homogenized on ice with 25 strokes. Homogenates were passed through a 100-μm cell strainer to remove tissue and cell debris, transferred to a 2-mL centrifuge tube, and centrifuged at 2400 rpm for 10 min at 4°C. The pellet was resuspended in 0.35 mL Digestion Buffer $+ 5 \mu$L each of PIC and 100 mM PMSF. Fractionation was carried out using 17 μL Enzymatic Shearing Cocktail with incubation at 37°C for 10 min. Following centrifugation at $15,000 \times$ g for 10 min at 4°C, the supernatant (sheared DNA) was aliquoted and stored at −70°C until used. For immunoprecipitation, 10 μg sheared DNA, 2 5 μL Protein G magnetic beads, 20 μL ChIP Buffer 1, 1 μL PIC, 94 μL $H_2O$, 4 μg MTF-1 goat polyclonal (sc-26844X, Santa Cruz) or normal goat IgG (Santa Cruz), and water to 0.2 mL were mixed in a 1.7-mL siliconized microcentrifuge tube and incubated overnight at 4°C. The beads were washed once with 0.5 mL ChIP Buffer 1 followed by two washes with 0.5 mL ChIP Buffer 2. Beads were resuspended in 50 μL Elution Buffer AM2 followed by the addition of 50 μL of Reverse Cross-linking Buffer. ChIPed samples and 3.5 μg input were reverse cross-linked. The following primers were used for real-time PCR analysis of the MRE of the pig *Mt3* promoter (forward) 5′-GCT GTG CAC TCG GTA GC-3′, and (reverse) 5′-CCA GCT GGG AGC ACA AG-3′ and for end-point PCR analysis of the MRE of pig *Gclc* promoter (forward) 5′-ATT TTC ACA CAG CGC GTT TG-3′, and (reverse) 5′-GAG TGC ATA GGC GTG GTT AGG-3′.

## Statistical analysis

Statistical analysis was performed using Sigma Stat (Systat Software Inc., San Jose, CA). Comparisons between diet groups were made by one-way ANOVA followed by Bonferroni post-hoc analysis or by one-way ANOVA of RANKS followed by Tukey post-hoc analysis where the data were not normally distributed. Differences at $P < 0.05$ were considered significant. Data are mean $\pm$ SEM unless otherwise stated.

## Results

### Body weight gain

Body weights at sacrifice and growth rates of piglets from this experiment between PND2 and PND21 have been previously reported.[4] Body weight and growth rates were greater in male than female piglets ($P < 0.05$) but were not significantly different between the three neonatal diet groups.[4]

### Serum trace element concentrations

As data on trace element concentration and metal homeostasis and the effects of diet composition on this were similar for male and female piglets, in the remaining results, data sets from both sexes were combined to the increase statistical power. With the exception of iron, trace element concentrations in sow milk, cow's milk, and soy-based infant formulas were similar or were lower in the formulas (Table 1). In contrast, formula concentrations of iron were substantially greater than levels reported in sow milk. Consistent with previously reported data demonstrating increased hepatic iron storage, upregulation of hepatic gene expression of the iron-regulated hormone hepcidin and downregulation of the hepatic transferrin receptor in both formula-fed compared to sow-fed piglets,[4] serum iron concentrations were 2- to 4-fold higher in both formula-fed groups compared to the breastfed group ($P < 0.05$) (Table 3). In contrast, serum concentrations of copper tended to be lower in formula-fed groups compared to the breastfed group ($P = 0.07$). Interestingly, despite similar zinc concentrations in sow milk and both formulas, serum zinc concentration was substantially higher in the milk formula-fed group than in the other groups ($P < 0.05$). A similar trend was observed for the element antimony (Sb) ($P = 0.06$) (Table 3). Serum levels of strontium (Sr) were lower in soy formula-fed piglets than breast or cow's milk formula-fed piglet groups ($P < 0.05$). The reverse was found with molybdenum (Mo) where serum concentrations were higher in the soy-formula-fed than breastfed or cow's milk formula-fed groups of piglets ($P < 0.05$) (Table 3). No statistically significant differences were observed between diet groups for any other element measured (data not shown).

### Zinc homeostasis

We analyzed the expression of genes involved in zinc transport and the zinc-binding protein MT in the small intestine.
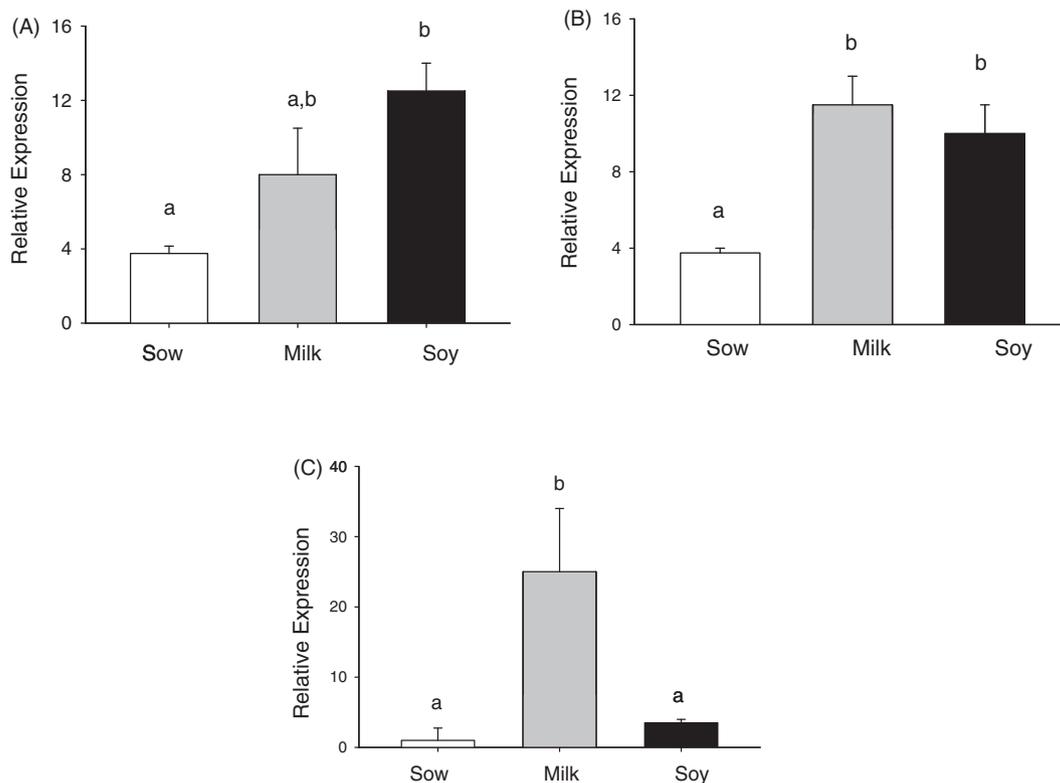
We analyzed expression of zinc transport genes, mRNA and protein expression of MT1, mRNA encoding the zinc-regulated enzyme Gclc, and the binding of MTF-1 to the *Mt3* and *Gclc* promoters in the liver of breastfed and formula-fed piglets. In addition, we utilized LC-ICPMS to assess zinc

**Table 3** Effects of breast versus formula feeding on serum trace metal concentrations in the neonatal pig

| Metal | Breastfed | Milk formula-fed | Soy formula-fed | P Diet effect |
|---|---|---|---|---|
| Cu | $1862 \pm 200$ | $1357 \pm 367$ | $1194 \pm 121$ | 0.07* |
| Fe | $1386 \pm 310^a$ | $3539 \pm 425^b$ | $3994 \pm 595^b$ | < 0.001 |
| Zn | $765 \pm 63^a$ | $1596 \pm 768^b$ | $753 \pm 80^a$ | 0.009* |
| Se | $93 \pm 30$ | $103 \pm 34$ | $110 \pm 36$ | 0.80 |
| V | $63 \pm 8$ | $56 \pm 12$ | $56 \pm 5$ | 0.15 |
| Sr | $20 \pm 3^{a,b}$ | $21 \pm 2^b$ | $16 \pm 2^a$ | 0.03 |
| Mn | $5 \pm 1$ | $6 \pm 4$ | $3 \pm 1$ | 0.11 |
| Mo | $6 \pm 1^a$ | $8 \pm 4^{a,b}$ | $14 \pm 1^b$ | 0.003* |
| Sb | $0.5 \pm 0.3$ | $1.5 \pm 0.8$ | $0.2 \pm 0.2$ | 0.06* |

Data are mean $\pm$ SEM ($\mu$g/L serum) for each metal for $n = 10$ piglets/group. a < b; $P < 0.05$ by one-way ANOVA followed by Bonferroni post-hoc analysis or by *one-way ANOVA of ranks followed by Tukey test post-hoc analysis where the data were not normally distributed.

concentrations in liver proteins and more specifically associated with the MT pool. In the ileum, mRNA encoding *Zip4*, which is considered a crucial factor for zinc uptake,[17] was elevated in soy formula-fed compared to breastfed piglets ($P < 0.05$) and was also somewhat elevated in cow's milk formula-fed piglets compared to breastfed piglets ($P < 0.1$) (Figure 1(A)). Expression of mRNA encoding another zinc transporter *Znt1* was also increased in ileum from both formula-fed groups compared to breastfed piglets ($P < 0.05$) (Figure 1(B)). Expression of mRNA encoding zinc-binding protein *Mt1* was higher in the ileum of cow's milk formula-fed piglets than in either breastfed or soy formula-fed piglets ($P < 0.05$) (Figure 1(C)). Therefore, zinc homeostasis in the ileum was significantly influenced by early diet. In the liver, no significant effects of diet were observed on expression of *Zip4* mRNA. However, hepatic *Znt1* mRNA was expressed at a higher level in cow's milk formula-fed than in breastfed or soy formula-fed piglets ($P < 0.05$) (Figure 2(A) and (B)). Similar to the ileum, mRNA encoding *Mt1* was more highly expressed in the liver of cow's milk formula-fed compared to breastfed or soy formula-fed piglets ($P < 0.05$) (Figure 2(C)). We have previously reported that mRNA expression of the *Mt3* gene is also expressed at much higher levels in the liver of cow's milk formula-fed compared to soy formula-fed piglets.[4] Differences in expression of hepatic MT mRNA were reflected in significant differences in expression of hepatic
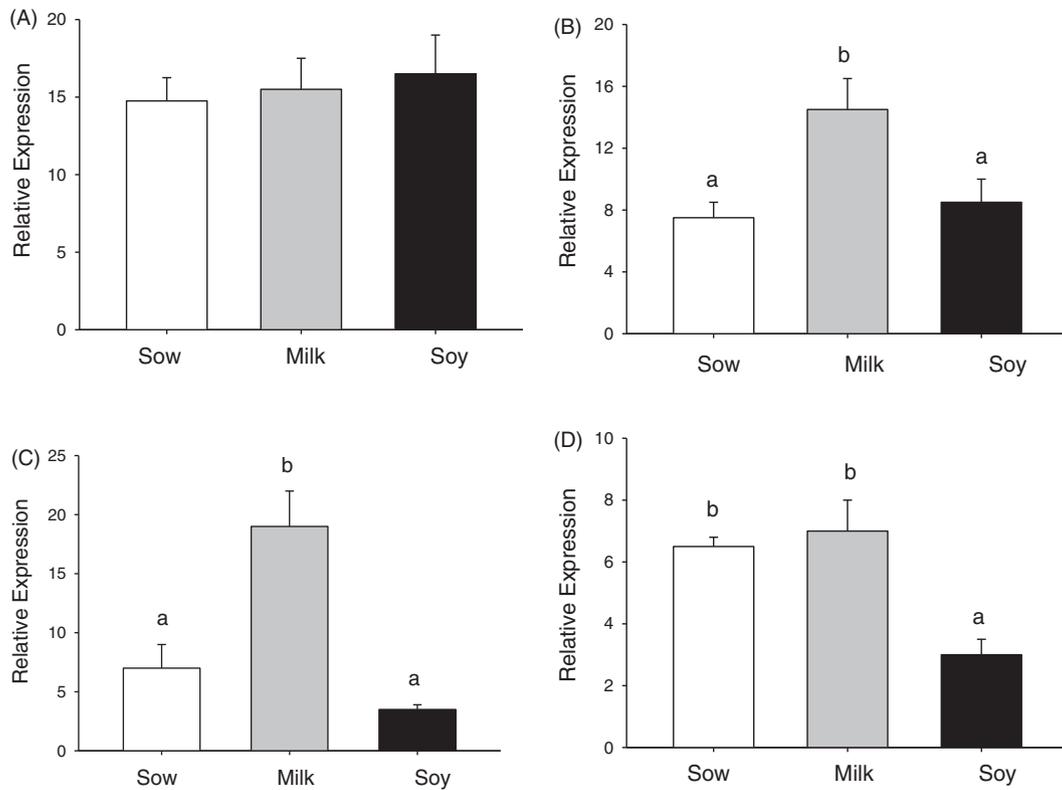


**Figure 1** Real-time qPCR measurement of expression of mRNAs encoding zinc transporters and binding proteins in ileum of breastfed and formula-fed piglets on PND21. (A) Zinc transporter *Zip4*; (B) zinc transporter *Znt1*; and (C) zinc-binding protein metallothionein (MT). Sow: breastfed; milk: cow's milk formula-fed; soy: soy formula-fed. Data are mean $\pm$ SEM values for target mRNA from $n = 10$ piglets/group normalized to mRNA for *Rpl27* and *Rps12* using geNorm. One-way ANOVA of RANKS revealed significant diet effects on expression of *Zip4* ($P = 0.003$), *Znt1* ($P < 0.001$), and MT ($P < 0.001$). Means bearing different letters are significantly different at $P < 0.05$, based on Tukey test post-hoc analysis, a < b

**Figure 2**    Real-time qPCR measurement of expression of mRNAs encoding zinc transporters, binding proteins, and zinc-dependent enzymes in liver of breastfed and formula-fed piglets on PND21. (A) Zinc transporter *Zip4*; (B) zinc transporter *Znt1*; (C) zinc-binding protein metallothionein (MT); and (d) zinc-dependent enzyme *Gclc*. Sow: breastfed; milk: cow's milk formula-fed; soy: soy formula-fed. Data are mean ± SEM values for target mRNA from $n = 10$ piglets/group normalized to mRNA for *Rpl27* and *Rps12* using geNorm. One-way ANOVA of RANKS revealed no significant diet effects on expression of *Zip4* ($P = 0.88$), but significant dietary regulation of *Znt1* ($P < 0.001$), MT1 ($P < 0.001$), and Gclc ($P = 0.007$). Means bearing different letters are significantly different at $P < 0.05$, based on Tukey test post-hoc analysis, a < b

MT protein which was higher in cow's milk formula-fed than breastfed piglets ($P < 0.05$) and higher in both these groups compared to the soy formula-fed group ($P < 0.05$) (Figure 3). HPLC-ICPMS analysis of zinc bound to liver proteins revealed three zinc-protein pools (Table 2). Overall the protein-normalized zinc content of liver proteins was four times greater in the cow's milk formula-fed than breastfed or soy formula-fed groups. Absorption at 238 nm, the $\lambda_{max}$ for ZnMT, suggested that peak 2 of the iron chromatogram was composed primarily of MT. Comparison of Zn associated with peak 2 suggested that zinc bound to MT was on average 2- to 3-fold higher in livers from cow's milk formula compared to breastfed or soy formula-fed piglets (Table 4). In addition to MTs, mRNA encoding another zinc-regulated hepatic target gene *Gclc* was expressed at lower levels in piglets fed soy formula compared to cow's milk formula or breastfed piglets (Figure 2(D)). Since MTs and *Gclc* are known to be regulated by zinc via the transcription factor MTF-1, we examined MTF-1 binding to MRE elements in the hepatic *Mt3* and *Gclc* promoters by ChIP assay. The data are shown in Figure 4. For both genes, there was higher MTF-1 promoter binding in the cow's milk formula-fed group compared to the soy formula-fed group.
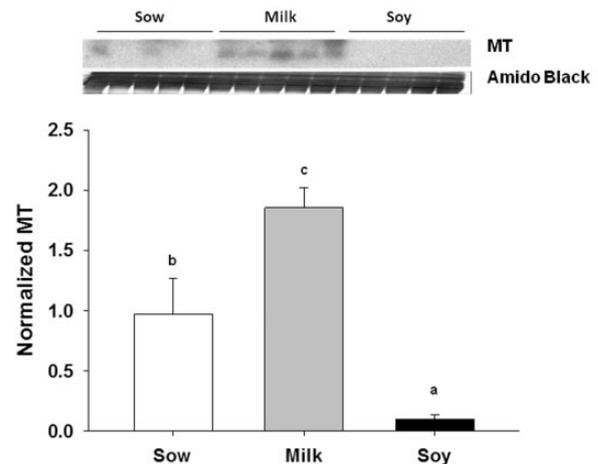


**Figure 3**    Protein expression of zinc-binding protein metallothionein (MT) in the liver of breastfed and formula-fed piglets on PND21. Top panel: Western blot and total protein stain (Amido black). Each lane represents a different piglet in each diet group. Sow: breastfed; milk: cow's milk formula-fed; soy: soy formula-fed. Bottom panel: Immunoquantification of MT protein expression. Data are mean ± SEM densitometric values from $n = 10$ piglets/group normalized to loading of total protein. Overall diet effects were significant at $P < 0.001$. Means bearing different letters are significantly different at $P < 0.05$, based on one-way ANOVA followed by Bonferroni post-hoc analysis, a < b < c

**Table 4** Effects of breast versus formula feeding on hepatic zinc protein concentrations in neonatal piglets

| | Zn/Peak 1[a] | Zn/Peak 2[a] | Zn/Peak 3[a] | Total Zn/Total area[b] |
|---|---|---|---|---|
| Absorption at 238 nm | | | | |
|   Breastfed | 0.37 ± 0.3 | 8.92 ± 2.7 | 0.04 ± 0.001 | 0.74 ± 0.73 |
|   Milk formula-fed | 0.06 ± 0.04 | 32.85 ± 4.3 | 0.53 ± 0.50 | 1.52 ± 0.18 |
|   Soy formula-fed | 0.05 ± 0.05 | 11.00 ± 1.3 | 0.07 ± 0.01 | 0.49 ± 0.02 |
| Absorption at 254 nm | | | | |
|   Breastfed | 0.18 ± 0.2 | 14.4 ± 1.7 | 0.13 ± 0.02 | 1.14 ± 0.5 |
|   Milk formula-fed | 0.03 ± 0.02 | 121.0 ± 26.0 | 0.14 ± 0.01 | 4.15 ± 1.4 |
|   Soy formula-fed | 0.01 ± 0.01 | 19.0 ± 5.0 | 0.17 ± 0.05 | 1.41 ± 0.6 |

Data are mean ± SD for $n = 2$ pooled samples/diet group (one pooled from $n = 4$–5 male and one pooled from $n = 4$–6 female piglets).
[a]Zn value/UV peak area at 238 or 254 nm $\times 10^{-5}$.
[b]Zn value/total UV absorption area at 238 or 254 nm over the entire LC chromatogram.

## Discussion

As far as we are aware, this is the first comprehensive analysis of trace element status and zinc homeostasis in breast milk versus cow's milk formula, and soy formula-fed piglets. The neonatal pig is an excellent model for the human infant since growth, development, metabolism, and endocrine systems are similar between the two species. Moreover, unlike rodents or monkeys, neonatal piglets and infants do not convert the soy isoflavone daidzein into the estrogenic metabolite equol,[29] rendering the neonatal piglet the best animal model to compare the specific effects of soy formula relative to cow's milk formula.

These data are suggestive of changes in trace element/ zinc homeostasis based upon feeding modality, although as a result of the relatively small sample size, one limitation of the current study is that there may be false positives. Nevertheless, the experimental design (controlled study) allows much stronger causal inference than the large-sample observational studies seen in the current breast and formula-feeding literature. The current data on serum iron concentrations are consistent with previously published results demonstrating increased hepatic iron storage and changes in iron-regulated genes associated with formula compared to breastfeeding as a result of iron supplementation of the formulas relative to low levels found in breast milk.[4] In contrast, even though infant formulas are also fortified with copper, serum copper levels tended to be lower in both formula groups compared to the sow-fed piglets. This is consistent with previous data suggesting lower bioavailability of copper when fed as part of infant formulas.[6] Copper is required for normal iron homeostasis and is involved in numerous cellular and physiological activities including blood coagulation, regulation of blood pressure, glucose and cholesterol metabolism, myelination of the brain and spinal cord, and bone mineralization and elemental deficiency is associated with anemia, immune deficiency, and bone loss.[30] However, copper status appears adequate in most populations, including formula-fed infants, suggesting that copper requirements of term infants may be lower than previously suggested.[6] Serum concentrations of molybdenum were significantly higher in soy formula-fed piglets than other groups. Molybdenum is an essential micronutrient which, in a complex with a pterin-scaffold protein MoCo, forms the active center of seven molybdenum-dependent enzymes.[31] Deficiency in molybdenum can be lethal; however, it is only required in ultra-trace amounts and it is unclear what if any health consequences of higher serum values might be. Our data also show lower levels of strontium and antimony in the serum of soy formula-fed piglets compared to those fed sow or cow's milk formula-fed infants and a trend toward higher levels of antimony in cow's milk formula-fed piglets. However, data on the health effects of either element are also limited. Strontium has been suggested to play a role in bone mineralization and has been suggested to prevent dental caries.[32,33] However, there is no information comparing the risk of caries in soy formula-fed compared to cow's milk formula or breastfed children.

Interestingly, serum concentrations of zinc in piglets at weaning were similar in sow-fed and soy formula-fed groups but significantly higher in the cow's milk formula group. Previous studies of zinc bioavailability related to infant formulas which were conducted in adult humans suggested lower bioavailability in soy formula compared to cow's milk formula (16% versus 31%) as a result of the presence of phytate in soy protein isolates.[14] Our data are consistent with these previous studies. Formula-fed piglets would be expected to consume a larger volume of formula compared to the volume of sow milk consumed by breastfed piglets where developmental weight gains are matched, given the lower caloric density of the formulas.[21] As zinc content of formulas and sow milk are comparable, it might therefore be expected that serum zinc levels would be higher in both formula-fed groups if bioavailability was also comparable.[4,21] Consistent with low bioavailability in the soy group and with previous studies in which the effects of changing dietary zinc content from low to high on expression of zinc transporters and binding proteins were examined in rodents and piglets,[17,34] levels of the intestinal zinc transporter *Zip4* were increased in the ileum but not liver of the soy formula-fed group compared to the other diet groups. Also, levels of the zinc-binding protein MT were significantly lower in both ileum and liver of the soy formula-fed compared to cow's milk formula-fed piglets.

## MTF-1 ChIP of MT3


(A)

## MTF-1 ChIP of Gclc


(B)

## MTF-1 ChIP of Gclc


(C)
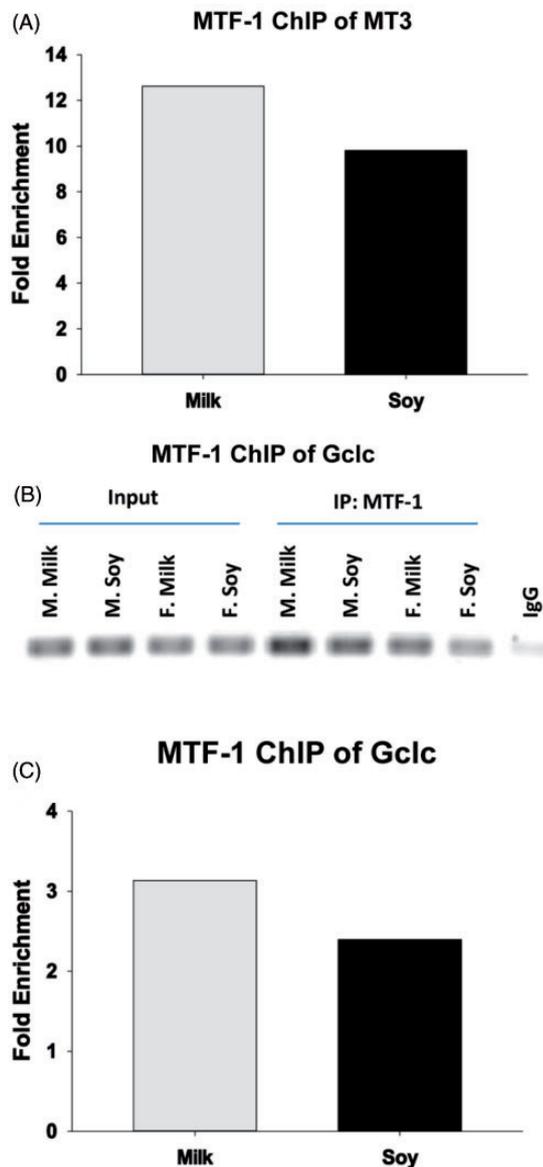
**Figure 4**   ChIP analysis of binding of the zinc-dependent transcription factor MTF-1 to the MRE element on the promoters of the *Mt3* and *Gclc* genes in the liver of formula-fed piglets on PND21. Milk: cow's milk formula-fed; soy: soy formula-fed. (A) Real-time qPCR quantitation of MTF-1 binding to the MT3 promoter. (B) RT-qPCR analysis of MTF-1 binding to the *Gclc* promoter illustrating ChIP specificity and increased MTF-1 binding in milk versus soy groups in both male (M) and female (F) piglets. (C) Real-time qPCR quantitation of MTF-1 binding to the *Gclc* promoter. Data in (A) and (C) are derived from the combined analysis of chromatin purified from livers pooled from $n = 5$–6 male and $n = 4$–5 female piglets/diet group

ICP-MS data from pooled livers also suggested that zinc content of liver proteins and MT in particular were lower in soy formula-fed compared to cow's milk formula-fed piglets. Both hepatic MTs and *Gclc* expression are regulated by zinc via MTF-1.[7–9,18] Consistent with low zinc status and reduced MT and *Gclc* mRNA expression, we observed reduced MTF-1 binding to the MRE promoter response elements of both genes in the soy formula-fed compared to cow's milk formula-fed group. However, despite evidence of lower bioavailability in soy formula, zinc levels in sow-fed and soy formula-fed piglets were comparable

and there was no evidence of zinc deficiency or reduced growth rates in the soy formula-fed group. Interestingly, expression of the zinc transporter *Znt1* was significantly elevated in the cow's milk formula group compared to the other groups in liver and compared to the sow-fed piglets in the ileum. The *ZnT* family of proteins decreases intracellular zinc concentrations by transporting zinc ions from the cytoplasm either into the extracellular matrix or into cell organelles.[7–9] In addition to the higher expression of MT, this may represent a homeostatic response to elevations in zinc status in the cow's milk formula-fed group compared to the sow or soy formula-fed piglets. Increases in expression of *Znt1* in the jejunum of piglets have previously been reported after feeding high zinc diets.[17]

In conclusion, these data indicate that trace element status differs in breast, cow's milk formula, and soy formula-fed piglets and that despite similar levels of dietary supplementation, significant differences in serum zinc in cow's milk formula compared to soy formula exist and result in compensatory changes in zinc homeostasis.

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