Antibiotic treatment affects the expression levels of copper transporters and the isotopic composition of copper in the colon of mice

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Copper is a critical enzyme cofactor in the body but also a potent cellular toxin when intracellularly unbound. Thus, there is a delicate balance of intracellular copper, maintained by a series of complex interactions between the metal and specific copper transport and binding proteins. The gastrointestinal (GI) tract is the primary site of copper entry into the body and there has been considerable progress in understanding the intricacies of copper metabolism in this region. The GI tract is also host to diverse bacterial populations, and their role in copper metabolism is not well understood. In this study, we compared the isotopic fractionation of copper in the GI tract of mice with intestinal microbiota significantly depleted by antibiotic treatment to that in mice not receiving such treatment. We demonstrated variability in copper isotopic composition along the length of the gut. A significant difference, $\sim 1.0‰$, in copper isotope abundances was measured in the proximal colon of antibiotic-treated mice. The changes in copper isotopic composition in the colon are accompanied by changes in copper transporters. Both CTR1, a copper importer, and ATP7A, a copper transporter across membranes, were significantly down-regulated in the colon of antibiotic-treated mice. This study demonstrated that isotope abundance measurements of metals can be used as an indicator of changes in metabolic processes in vivo. These measurements revealed a host–microbial interaction in the GI tract involved in the regulation of copper transport.

copper isotopes | intestines | microbiota | CTR1 | ATP7A

Isotopic tools are powerful techniques to monitor metal processing and identify disruptions or malfunctions in normal metal homeostasis. These tools include the application of enriched stable or radioactive isotope tracers (1) and monitoring the shift in natural isotopic composition induced by metabolic processes (2). The latter is a result of the redistribution of isotopes among chemical species, and subsequently different cells and organs of the body. The redistribution of the isotopes is known as isotopic fractionation. The difference in zero-point energies of a bond upon isotopic substitution causes the chemical structure of the binding sites of a molecule to have an energetic advantage to bind one isotope over another.

The power of isotopic tools has been reported for many of the biologically important metals. Zinc isotopes reveal changes in metal handling in the brains in Alzheimer's mouse models, correlated to the formation of amyloid-beta plaques (3). A significant enrichment in the heavier iron isotopes is observed in the serum of iron-deficient patients with chronic kidney disease (4). Calcium isotopes in urine can be used as a proxy for bone loss during extended bed rest (5).

The focus of this study is on the variations in the relative abundances of the Cu stable isotopes, ^{63}Cu and ^{65}Cu. Cu isotopes are systematically redistributed among organs and bodily fluids of mice (6) and the distribution is altered by changing the expression of the ubiquitously expressed cellular prion protein (7). Recent reports have provided theoretical predictions of isotopic fractionation due to the binding sites in Cu-binding proteins (8, 9). These predictions showed good agreement with the isotopic fractionation due to Cu-transporter 1 (CTR1) in yeast cells (10). Breast and colon cancer, hepatocellular carcinoma, Wilson's disease, amyotrophic lateral sclerosis, and liver cirrhosis (11–16) are all known to cause disruptions in Cu metabolism that are reflected in the isotopic composition. Therefore, the investigation of Cu isotope abundances has the proven potential to monitor Cu metabolism in living systems.

The primary site of entry for Cu into the body is in the gastrointestinal (GI) tract. The uptake and absorption mechanisms of Cu in the gut are reasonably well characterized (see refs. 17 and 18 for thorough reviews), although our understanding is not complete. Cu is transported into intestinal epithelial cells (IECs) primarily via CTR1, although other pathways have been suggested, including transfer by divalent metal transporter 1 (DMT1) (19). Once inside the cell, Cu chaperones immediately bind the free Cu and deliver it to Cu-binding proteins and enzymes such as metallothionein and Cu/Zn-superoxide dismutase (SOD1). Cu leaving the IECs is transported by the Cu-binding ATPase ATP7A, either through transmembrane transport or by loading vesicles near the basolateral membrane. The fate of newly

Significance

Isotopic analysis has the ability to detect changes in metal homeostasis in vivo. Using this advanced technique to investigate metal metabolism, we identified a significant change in copper processing in the gut of mice with depleted gut microbiota. In addition, we observed changes in the expression of copper transporters in the colon. The role of intestinal microbiota in copper processing in the gastrointestinal tract was previously unknown. Using the unique ability of isotopic analyses to detect changes in metal processing has enabled the identification of the intestinal microbiota as a significant influence on copper metabolism in the gut.
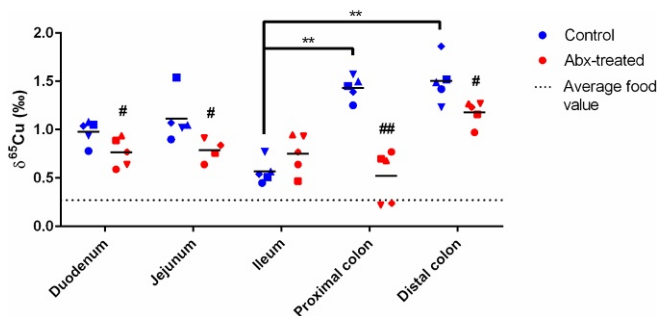
Fig. 1. Regional distribution of Cu isotopes (δ^{65}Cu) in the gut of control and antibiotic (Abx)-treated mice. Each symbol refers to an individual mouse. Different Cu isotope composition is observed comparing ileum versus both colon regions (**$P < 0.01$; Kruskal–Wallis followed by Dunn's multiple comparison test was applied between control samples). Duodenum, jejunum, and distal colon had a reduction in the δ^{65}Cu, but it is in the proximal colon that we observed the greatest reduction in the δ^{65}Cu (#$P < 0.05$, ##$P < 0.01$, Mann–Whitney U test comparing control with Abx-treated mice in each the region). Ileum did not exhibit any changes in Cu isotope composition following antibiotic treatment. $n = 5$ per group.

absorbed Cu into the body is reviewed elsewhere (20). Cu uptake occurs in all regions of the GI tract, but absorption occurs primarily in the duodenum and the upper jejunum. As Cu concentration must be tightly controlled due to its potential toxicity, Cu regulation includes a complex series of protein signaling steps to limit the amount of Cu that enters the body.

Identifying all components contributing to Cu processing in a living organism is essential to fully understanding the intricacies of the metal metabolism. One important component in the GI tract is the trillions of microorganisms that reside in the gut, living in a mutualistic fashion, beginning at birth (21). Although considered beneficial in health, alterations in intestinal microbiota have been implicated in the pathogenesis of numerous disease states, including obesity, diabetes, and multiple sclerosis (22–24). In the gut, bacterial dysbiosis has been shown to modulate the function of IECs (25), which comprise the epithelial barrier, the single-cell layer of cells that separates the lamina propria from the antigenic and microbial contents of the gut lumen. Alteration in the function of IECs changes their efficiency as a protective barrier and compromises the host.

Although the commensal microbiota in the gut play an important role in regulating intestinal epithelial barrier function, they are not the only contributing factor. Cu, both a required nutrient, but also a potential toxin, has also been shown to affect epithelial barrier function in vitro (26). Although dysregulation of Cu and dysbiosis has been implicated in GI disorders, such as the inflammatory bowel diseases (27, 28), the interplay between the gut commensal bacteria and Cu is not well understood. The source and amount of Cu has been shown to affect the microbial diversity in the gut in pigs and mice (29, 30). While these studies focused on the effect of Cu on the bacterial populations, few studies have investigated the effect of the intestinal microbiota on Cu processing. It has yet to be determined if Cu regulation involves interactions with the GI microbiota.

The GI tract has a unique advantage when studying isotopic composition because the Cu source is known (i.e., food), and the GI tract is the first opportunity for a significant isotopic fractionation. Therefore, to explore the sensitivity of the natural isotopic composition of Cu to changes in the gut microbiome, we characterized the Cu isotope amount ratios in the different regions of the GI tract. We then compared the Cu isotopic composition of control mice with mice subject to broad-spectrum antibiotic treatment, which significantly depletes the commensal microbiota. Cu-binding proteins can cause alterations in isotopic fractionation (10). To explore if Cu isotopic fractionation mea-

surements are reflective of alterations in Cu metabolism, we assessed the protein expression levels of CTR1, ATP7A, and SOD1 in each region of the GI tract.

Results

Cu Isotopic Composition Is Different Throughout Regions of the Gut and It Is Altered After Antibiotic Treatment. The food pellets for the mice had an average isotopic composition (expressed as δ^{65}Cu, defined in *Materials and Methods*) of 0.27‰ (range 0.20 to 0.34‰, $n = 4$). The isotopic composition in the five regions of the gut were enriched in ^{65}Cu compared with the food source (Fig. 1). In the control group we observed the duodenum (δ^{65}Cu average of 0.98‰, range 0.78 to 1.08‰) and jejunum (δ^{65}Cu average of 1.12‰, range 0.90 to 1.54‰) displayed similar Cu isotopic ratios (Kruskal–Wallis test, $P > 0.05$). The lowest ratio, found in the ileum (δ^{65}Cu average of 0.57‰, range 0.45 to 0.77‰), was significantly different (Kruskal–Wallis test, $P < 0.01$) compared with the proximal colon (δ^{65}Cu average of 1.43‰, range 1.25 to 1.57‰) and distal colon (δ^{65}Cu average of 1.50‰, range 1.23 to 1.86‰) but was not significantly different compared with the duodenum or jejunum (Kruskal–Wallis test, $P > 0.05$).

To determine the impact of the gut microbiota on the isotopic composition, we treated mice with a broad-spectrum antibiotic cocktail. The Cu isotopic composition in the different regions of the gut was shifted in antibiotic-treated mice in a region-specific manner (Fig. 1). Lower δ^{65}Cu was observed in the duodenum, jejunum, and distal colon of antibiotic-treated mice (Mann–Whitney U test, $P < 0.05$). Moreover, a more substantial reduction was observed in the proximal colon (Fig. 2; Mann–Whitney U test, $P < 0.01$). The ileum showed no differences between the antibiotic-treated mice and control group (Mann–Whitney U test, $P = 0.16$). The absolute values for the Cu content in the water varied between the groups. We observed a mass fraction of 5 ng/g in the control mice's water and 18 ng/g in the water containing antibiotics. Antibiotic treatment has no effect on body weight, (*SI Appendix*, Fig. S1*A*; two-way ANOVA, interaction $P = 0.99$) but a significant increase in the cecum weight was observed (*SI Appendix*, Fig. S1*B*; Mann–Whitney U test, $P < 0.01$), as previously reported in mice with depleted gut bacteria (31). To ensure antibiotic efficacy, we assessed the bacterial load in the cecum matter of both groups and observed a substantial reduction in the bacterial population in the cecum matter of antibiotic-treated mice (*SI Appendix*, Fig. S1*C*; Student's t test, $P < 0.001$).
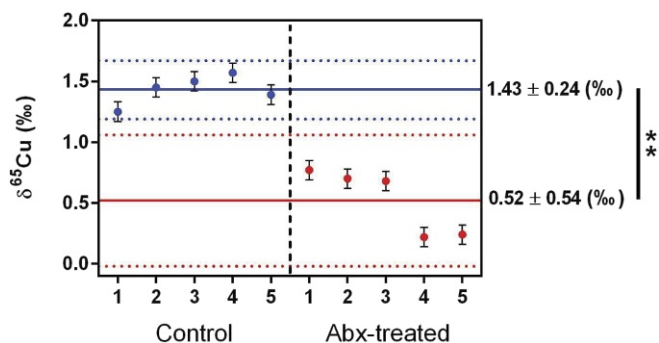

Fig. 2. The δ^{65}Cu values of the proximal colon for each individual mouse in the controls and antibiotic (Abx)-treated group ($n = 5$). Error bars correspond to the expanded uncertainty of the measurement ($k = 2$). Solid blue and red lines correspond to the mean δ^{65}Cu values for the control and Abx-treated groups, respectively. The blue and red dashed lines represent the region enclosing two standard deviations of the mean. Values for the control and Abx-treated groups are statistically different (**$P < 0.01$, Mann–Whitney U test).

Changes in the Expression Levels of Cu Transporters in the Large Intestine Are Detected Along with Alterations in Cu Isotopic Composition. To assess factors that may contribute to the alteration in Cu isotopic composition in the colon observed following antibiotic treatment, the expression levels of Cu transporters and a Cu-binding enzyme in each region of the intestinal tract were measured. No changes in the expression levels were observed in the duodenum, jejunum, and ileum for CTR1, the main Cu importer (Fig. 3; Mann–Whitney U test, P = 0.68, 0.15, and 0.42, respectively), or ATP7A, a Cu transporter across membranes (Fig. 3; Mann–Whitney U test, P = 0.88, 0.84, and 0.15, respectively) when antibiotic-treated mice were compared with the control group. The expression of SOD1, a Cu-binding antioxidant enzyme, was increased in the duodenum (Fig. 3A; Mann–Whitney U test, $P < 0.05$) and in the ileum (Fig. 3C; Mann–Whitney U test, $P < 0.05$) but was not altered in the jejunum (Fig 3B; Mann–Whitney U test, P = 0.22). In the large intestine, CTR1 was down-regulated in both the proximal and distal colon (Fig. 4 A and B; Mann–Whitney U test, $P < 0.05$), as was ATP7A (Fig. 4; Mann–Whitney U test, $P < 0.05$). The expression level of SOD1 did not change in the proximal or distal colon (Fig. 4; Mann–Whitney U test, P = 0.15 and 0.09 respectively).

Discussion

In this study, high-precision Cu isotopic analysis was used to investigate the in vivo influence of reduced intestinal microbiota on Cu processing in the GI tract. The mechanisms by which protein interactions change the distribution of Cu isotopes is described by Tennant et al. (9). Isotopic fractionations occur due to protein interactions when the binding sites of each protein differ in the ligands, geometries, and/or oxidation states. As many protein interactions are happening concurrently in a system (or organ), if the types or proportions of the binding sites in the system are changed, the resulting isotopic fractionation will also change. In this study a reduction of the expression levels of two key Cu transporters, CTR1 and ATP7A, was observed in the proximal and distal colon of mice treated with antibiotics. There was also a decrease in δ^{65}Cu values (Fig. 1) in the proximal colon, and to a lesser extent in the distal colon, of the antibiotic-treated mice. Changes in the expression of CTR1 have been

shown to induce a large isotopic fractionation in yeast cells (10), and here we have observed similar isotopic fractionations associated with changes in Cu transporters in complex mammalian cells. The mechanisms leading to these changes remain to be determined.

The reason for the striking difference between the influence of microbiota on Cu isotopic fractionation in the proximal colon compared with the distal colon remains unclear. Both regions of the large intestine contain large, diverse bacterial populations; however, the composition of the microbiota and their localization within the large intestine are different between the two regions (32). For example, the proximal colon has more mucus-associated bacteria than the distal colon due to the viscosity gradient of the mucus layer in the large intestine (33), which could contribute to the differences observed.

No changes in the expression levels of the Cu transporters and a decrease in δ^{65}Cu values were observed in the duodenum and jejunum. This is different from what was observed in the colon; however, the small and large intestines are markedly different environments. Relatively few bacteria are present in the duodenum and jejunum, and the processes regulating Cu in these regions are different.

The antibiotics used in this study are known to interact with Cu^{2+} and form stable complexes in vitro (34–36). Presumably this metal-binding ability would persist in vivo, although this is difficult to verify experimentally. However, a change in isotopic redistribution and Cu transporter expression would be observed in all regions of the GI tract if the interaction of Cu with these antibiotics was the mechanism in which Cu processing was altered. A decrease in the expression levels of CTR1 and ATP7A is a response typically associated with reduced demand for Cu within the IECs (37–39). If the antibiotics were sequestering Cu from the lumen, then an increase in demand for Cu would likely be observed. This is not supported by the protein expression data. Additionally, an increase in demand for Cu would likely be observed if these antibiotics were sequestering Cu from the lumen. This is not supported by the protein expression data. The increased Cu content in the water containing the antibiotics could account for the apparent decrease in demand for Cu, although if this was the cause for the change in
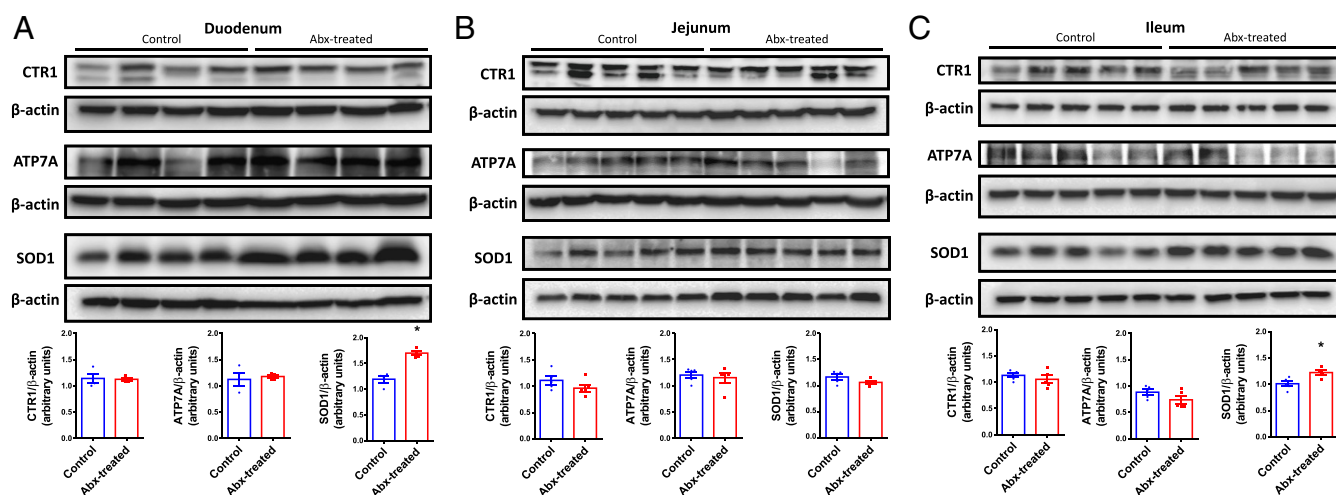
Fig. 3. Treatment with antibiotics does not lead to alterations in the expression of Cu transporters in the small intestine of mice. (*A*) Expression of CTR1, a major Cu importer, and ATP7A, a known Cu exporter, are not altered in the duodenum of antibiotic (Abx)-treated mice. However, the expression of the antioxidant enzyme SOD1 is increased (*$P < 0.05$ with Mann–Whitney U test). (*B*) No alterations were observed in the jejunum regarding CTR1, ATP7A, and SOD1 when comparing control vs Abx-treated mice. (*C*) Ileum samples presented with the same trend observed in the duodenum: no alterations in CTR1 and ATP7A protein levels, with an increase in SOD1 expression (*$P < 0.05$ with Mann–Whitney U test) in the Abx-treated group. Data are presented as mean ± SEM. n = 4 or 5 per group.
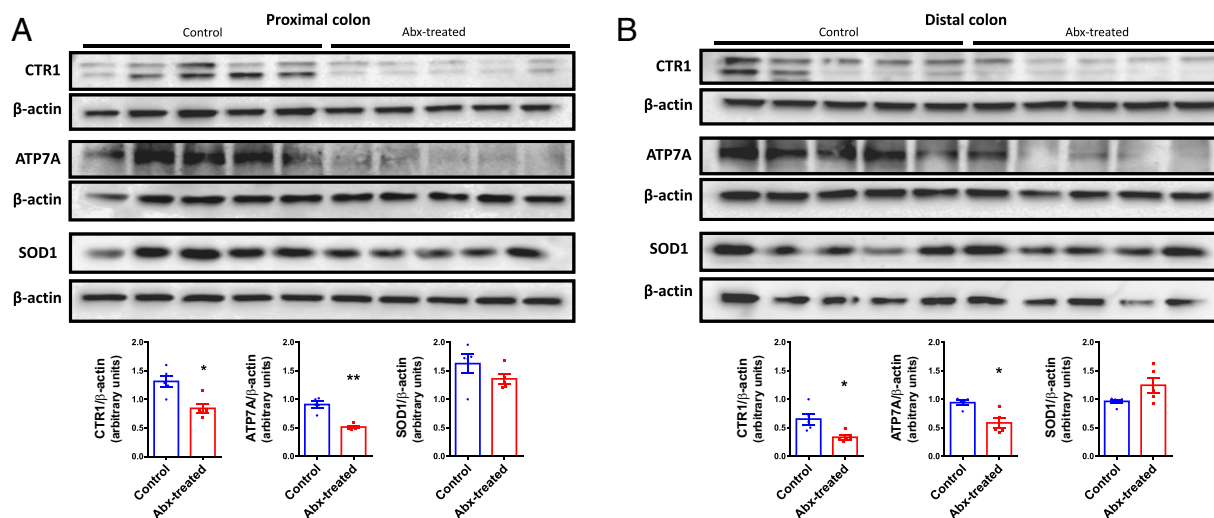
Fig. 4. Depletion of gut microbiota by antibiotics led to a reduction in the expression of Cu transporters in the large intestine of mice. Both proximal (*A*) and distal (*B*) colon of mice treated with antibiotics (Abx) had a reduction in the expression of CTR1 and ATP7A, the major Cu importer and transporter across membranes, respectively, present in the cells (*$P < 0.05$, **$P < 0.01$ with Mann–Whitney *U* test). No alterations were observed regarding SOD1 in both regions. Data are presented as mean $\pm$ SEM. $n = 5$ per group.

Cu transporters we would expect to observe this in all regions of the GI tract. The data within this study suggest that antibiotics are not the dominant factor contributing to the change in Cu handling, although their contribution cannot be completely excluded.

This study investigated in vivo the effect of intestinal microbiota on Cu processing in conventional animals. Previous reports noted an increase in mRNA expression of CTR1 and a decrease in mRNA expression of metallothionein in ileal tissue upon colonization of germ-free animals with *Bacteroides thetaiotaomicron* (40). Our results did not indicate a significant change in Cu handling in the ileum, although our study is not directly comparable to that of Hooper et al. (40), as germ-free and conventional mice display several physiological differences, including properties associated with the mucus layer (41). However, in both the germ-free and conventional mice the presence of bacteria is accompanied with increased expression of CTR1, indicating the presence of bacteria inhibiting the IECs access to extracellular Cu. To further support this notion, an in vitro study determined the bioavailability of Cu to Caco-2 cells after incubation with fecal bacteria and revealed a sharp decrease in the proportion of Cu available for uptake in the cells in the presence of bacteria (42). The microbial composition of fecal bacteria is different from that found in the proximal colon (43), but both bacterial populations, as well as a single strain of bacteria, trigger a physiological response in the Cu metabolism in the GI tract. This suggests that the impact on Cu metabolism by bacteria is not a species-specific response but rather a general response to microbial residence.

In addition to Cu, other transition metals have been shown to interact with the intestinal microbiome. Deschemin et al. (44) showed the expression levels of DMT1 and duodenal cytochrome *B* were decreased and ferroportin was increased upon colonization of germ-free mice with microbiota obtained from their conventionally raised counterparts. Similarly, Gielda and DiRita (45) demonstrated the presence of microbiota increases the expression of host-derived zinc-binding enzymes in chicks to outcompete the microbes for the nutrient. However, unlike iron and zinc, which are essential nutrients of bacteria, most bacteria in the anaerobic environment of the large intestine have limited use for Cu, which is toxic in elevated levels, a property the host exploits. Pathogens and commensal bacteria that

breach the intestinal barrier are engulfed in macrophages which are subsequently filled with extracellular Cu (46). Hence, animals subject to Cu deficiency are known to be more susceptible to infections, which is reversible upon Cu supplementation (47). Commensal bacteria have likely developed strategies to protect against Cu toxicity in the lumen, such as production of sulfide (48) that can precipitate free Cu as CuS out of the luminal fluids. Competition exists for all of the transition metals between the host and the microbe. However, for Cu we speculate that the competition could be driven by the bacteria's need to sequester Cu through metabolites to protect against Cu toxicity, similar to the siderophore yersiniabactin produced by the pathogen *Escherichia coli* (49), hence affecting the host's Cu homeostasis.

The study of changes in Cu isotopic composition in living systems is providing new insights into the role of the metal in biological processes. Understanding Cu homeostasis in the GI tract is not only important due to its critical role as a cofactor in several cellular functions but also due to its role in the innate immune system, intestinal barrier function, and the observed dysregulation of Cu in many GI disorders. In this study the Cu isotopic composition was a vital indicator of the alteration in Cu metabolism in the proximal and distal colon due to changes in the intestinal microbiome.

Conclusions

This study demonstrated that the analysis of natural stable Cu isotope abundance variations is an indispensable tool to detect changes in Cu metabolism in vivo. The measurement of the Cu isotopic composition of specific tissues in the GI tract, based on sound metrological principles, enables the identification of the significant influence of antibiotic treatment on Cu metabolism in the intestinal tract. The shift of the Cu isotopic composition in the large intestine between antibiotic-treated and untreated mice was accompanied by the differential expression of two major Cu-binding transporters found in IECs. The ileum, a region with a relatively high population of commensal bacteria, showed no change in the isotopic composition and no change in the expression levels of CTR1 or ATP7A. The results of this isotopic investigation suggest a critical relationship between the intestinal microbiota (particularly its abundance) and the metabolism of Cu in the large intestine, both of which have been positively identified to affect the health of the GI tract.

Materials and Methods

Mice. All animal experiments were conducted on C57Bl/6 male mice (8 wk old) purchased from The Jackson Laboratory. Mice were housed at the University of Calgary animal facility at 22 ± 2°C on a 12-h light–dark cycle, with free access to food and water. Animal experiments were performed under the guidelines of the Canadian Council on Animal Care and had ethics approval by the Health Sciences Animal Care Committee at the University of Calgary.

Antibiotic Treatment. After 1-wk habituation in the animal facility, animals were randomly assigned for antibiotic-treated or control groups (n = 4 or 5 per cage). As previously reported (50), a broad-spectrum antibiotic mixture consisting of ampicillin (1 g/L, A9518; Sigma-Aldrich), neomycin (1 g/L, N1876; Sigma-Aldrich), vancomycin (500 mg/L, 94747; Sigma-Aldrich), and metronidazole (1 g/L, M3761; Sigma-Aldrich) was added to the drinking water of the antibiotic-treated group for 4 wk. Due to the large body loss observed in mice treated with metronidazole in drinking water (51), we opted to gradually increase the concentration of metronidazole over time. In this regime, no metronidazole was added in the first day of treatment; on treatment day 2 metronidazole was introduced in a mass concentration of 250 mg/L, on day 6 the concentration was increased to 500 mg/L, and on day 9 the full concentration (1 g/L) was used. Bottles with antibiotic solution were refilled every week. The control group received regular autoclaved water. Body weight was measured three times per week.

Tissue Harvesting. After 4 wk of antibiotic treatment, mice (two separate cohorts of mice were used to ensure the reproducibility of findings) were anesthetized and killed for tissue harvesting. Empty cecum weight was measured as an indirect measurement of the antibiotic cocktail's efficacy. Small and large intestines were removed and divided into the five gut regions: duodenum, jejunum, ileum, and proximal and distal colon. For Cu isotope composition, mucosal and submucosal regions were separated from the muscle using a clean glass slide and the tissues were flash-frozen for future processing. For Western blot, tissue was collected applying the crypt isolation protocol (52). Briefly, gut tissue was removed, opened in the mesenteric border, and rinsed with PBS (Sigma-Aldrich), followed by a 30-min wash in HBSS (Gibco) containing 1 M Hepes (1% vol/vol; Sigma-Aldrich) and Penstrep (1% vol/vol, B4333; Sigma-Aldrich). After washing, tissue was cut in small square pieces and placed in BD Cell Recovery solution (Corning Life Sciences) for 2 h at 4°C. Tissue pieces were then placed in a Petri dish and gently pressed against the dish for mucosal and submucosal release. Released content was collected with HBSS containing 1 M Hepes and Penstrep and centrifuged at 400 × g for 10 min at 4°C. Supernatant was discarded and pellet was dissolved in cell lysis buffer.

Bacterial Quantification. Genomic DNA was extracted from cecal content as previously described (53); 2.8-mm ceramic beads and 0.1-mm glass beads were added to 300 μL of sample in addition to 100 μL of guanidine thiocyanate–EDTA–Sarkosyl and 800 μL of a 200 mM sodium phosphate (NaPO₄) buffer. After bead beat, supernatant were collected and processed in the MagMAX Express 96-Deep Well Magnetic Particle Processor (ThermoFisher) with the multi sample kit (4413022; ThermoFisher). Purified DNA was then submitted to qPCR amplifying the variable region 3 of the 16S rRNA gene with 341F and 518R universal primers. *E. coli* DNA with known concentration was used as standard control.

Isotopic Measurements. Tissues were digested using HNO₃ (ultrahigh purity; Sigma-Aldrich) and 30% hydrogen peroxide (Sigma-Aldrich), and the Cu was isolated using a Cu-specific resin (TrisKem) as described elsewhere (7). The Cu isotopic composition was measured on a Neptune multicollector ICP-MS (Thermo-Fisher Scientific). Instrumental mass bias was corrected for

using elemental external normalization (54) with the nickel isotopic standard SRM-986 (Sigma-Aldrich) added as the dopant. The reference standard used was AE633 (Sigma-Aldrich), recommended by the International Union of Pure and Applied Chemistry (55), as it has an isotopic composition within analytical uncertainty of SRM-976. The Cu isotopic composition is reported as a $\delta^{65}Cu_{AE633}$ value, which is defined as

$$\delta^{65}Cu_{AE633} = \left(\frac{R_{Sam}}{R_{Std}} - 1 \right) * 1{,}000, \qquad [1]$$

where R_{Sam} and R_{Std} are the amount ratios of $^{65}Cu/^{63}Cu$ atoms in the sample and standard, respectively. The $\delta^{65}Cu$ values are reported in units of parts per thousand, or per mil. Expanded uncertainty (k = 2) of the $\delta^{65}Cu$ values was 0.08 ‰.

Western Blot. Protein expression levels of CTR1, ATP7A, and SOD1 were assessed via Western blot. Sample proteins were extracted via bead homogenization in cell lysis buffer with protease inhibitors (05 892 970 001; Roche Diagnostics) and phosphatase inhibitors (04 906 837 001; Roche Diagnostics). Protein concentration was measured using the Precision Red reagent (Cytoskeleton) and all samples were equalized. For CTR1 and SOD1 analysis, samples were separated via SDS/PAGE with a 10% gel and blotted in nitrocellulose membranes. Membranes were blocked with 5% weight per volume skim milk (Sigma-Aldrich) and incubated with primary antibodies (anti-CTR1, ab129067, 1:1,000 and anti-SOD1, ab16831, 1:1,000; Abcam), followed by HRP-conjugated secondary antibody (donkey anti-rabbit, 711-035-152, 1:5,000; Jackson ImmunoResearch Laboratories). Membranes were washed three times for 5 min with 0.1% Tween 20 (Sigma-Aldrich) TBS (TBST) after each antibody incubation. For protein band detection, West Phemto (Thermo Fisher Scientific) was applied for CTR1 and standard ECL (Bio-Rad) for SOD1. For ATP7A analysis, samples were separated via SDS/PAGE with an 8% gel and blotted in nitrocellulose membrane. Membrane was then blocked with 5% weight per volume BSA (Sigma-Aldrich) and incubated with primary antibody (anti-ATP7A, ab13995, 1:2,000; Abcam) followed by HRP-conjugated secondary antibody (donkey anti-chicken, 703-035-155, 1:5,000; Jackson ImmunoResearch Laboratories). The membrane was washed eight times for 5 min with TBST after each antibody incubation. Blot images were acquired with a MicroChemi system using Gel Capture Micro-Chemi software (DNR Bio-Imaging Systems) and band densitometry was performed using ImageJ (NIH). β-Actin (anti-β actin, SC-47778, 1:1,000; Santa Cruz Biotechnology), followed by HRP-conjugated secondary antibody (donkey anti-mouse, 715-035-150, 1:5,000; Jackson ImmunoResearch Laboratories), was assessed as a loading control.

Statistical Analysis. Data are presented as mean ± SEM. Kruskal–Wallis test, two-way ANOVA followed by Sidak's test, and Mann–Whitney *U* test were applied as appropriate. All results have an n of 5, unless noted otherwise. GraphPad Prism 6 (GraphPad Software) was applied for all statistical analysis.

1. Larner F, et al. (2013) High precision isotope measurements reveal poor control of copper metabolism in parkinsonism. *Metallomics* 5:125–132.
2. Tanaka Y-k, Hirata T (2018) Stable isotope composition of metal elements in biological samples as tracers for element metabolism. *Anal Sci* 34:645–655.
3. Moynier F, Foriel J, Shaw AS, Le Borgne M (2017) Distribution of Zn isotopes during Alzheimer's disease. *Geochem Perspect Lett* 3:142–150.
4. Anoshkina Y, et al. (2017) Iron isotopic composition of blood serum in anemia of chronic kidney disease. *Metallomics* 9:517–524.
5. Morgan JLL, et al. (2012) Rapidly assessing changes in bone mineral balance using natural stable calcium isotopes. *Proc Natl Acad Sci USA* 109:9989–9994.
6. Balter V, et al. (2013) Contrasting Cu, Fe, and Zn isotopic patterns in organs and body fluids of mice and sheep, with emphasis on cellular fractionation. *Metallomics* 5:1470–1482.
7. Miller KA, et al. (2016) The expression levels of cellular prion protein affect copper isotopic shifts in the organs of mice. *J Anal Spectrom* 31:2015–2022.
8. Fujii T, Moynier F, Abe M, Nemoto K, Albarède F (2013) Copper isotope fractionation between aqueous compounds relevant to low temperature geochemistry and biology. *Geochim Cosmochim Acta* 110:29–44.
9. Tennant A, Rauk A, Wieser ME (2017) Computational modelling of the redistribution of copper isotopes by proteins in the liver. *Metallomics* 9:1809–1819.
10. Cadiou J-L, et al. (2017) Copper transporters are responsible for copper isotopic fractionation in eukaryotic cells. *Sci Rep* 7:44533.
11. Télouk P, et al. (2015) Copper isotope effect in serum of cancer patients. A pilot study. *Metallomics* 7:299–308.
12. Larner F, et al. (2015) Zinc isotopic compositions of breast cancer tissue. *Metallomics* 7:112–117.

13. Balter V, et al. (2015) Natural variations of copper and sulfur stable isotopes in blood of hepatocellular carcinoma patients. *Proc Natl Acad Sci USA* 112:982–985.
14. Aramendía M, Rello L, Resano M, Frank V (2013) Isotopic analysis of Cu in serum samples for diagnosis of Wilson's disease: A pilot study. *J Anal Spectrom* 28:675–681.
15. Sauzéat L, et al. (2018) Isotopic evidence for disrupted copper metabolism in amyotrophic lateral sclerosis. *iScience* 6:264–271.
16. Costas-Rodríguez M, et al. (2015) Isotopic analysis of cu in blood serum by multi-collector ICP-mass spectrometry: A new approach for the diagnosis and prognosis of liver cirrhosis? *Metallomics* 7:491–498.
17. Van Den Berghe PVE, Klomp LWJ (2009) New developments in the regulation of intestinal copper absorption. *Nutr Rev* 67:658–672.
18. Linder MC, Zerounian NR, Moriya M, Malpe R (2003) Iron and copper homeostasis and intestinal absorption using the caco2 cell model. *Biometals* 16:145–160.
19. Arredondo M, Muñoz P, Mura CV, Núñez MT (2003) DMT1, a physiologically relevant apical Cu1+ transporter of intestinal cells. *Am J Physiol Cell Physiol* 284:C1525–C1530.
20. Linder MC, Hazegh-Azam M (1996) Copper biochemistry and molecular biology. *Am J Clin Nutr* 63:797S–811S.
21. Methé BA, et al. (2012) A framework for human microbiome research. *Nature* 486:215–221.
22. Turnbaugh PJ, et al. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444:1027–1031.
23. Wen L, et al. (2008) Innate immunity and intestinal microbiota in the development of type 1 diabetes. *Nature* 455:1109–1113.
24. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK (2011) Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 108:4615–4622.
25. Wells JM, Rossi O, Meijerink M, van Baarlen P (2011) Epithelial crosstalk at the microbiota–mucosal interface. *Proc Natl Acad Sci USA* 108:4607–4614.
26. Ferruzza S, Sambuy Y, Onetti-Muda A, Nobili F, Laura Scarino M (2002) Copper toxicity to tight junctions in the human intestinal Caco-2 cell line. *Handbook of Copper Pharmacology and Toxicology* (Springer, New York), pp 397–416.
27. Mulder TP, et al. (1991) Decrease in two intestinal copper/zinc containing proteins with antioxidant function in inflammatory bowel disease. *Gut* 32:1146–1150.
28. Alexander S, et al. (2002) Mucosal flora in inflammatory bowel disease. *Gastroenterology* 122:44–54.
29. Højberg O, Canibe N, Damgaard Poulsen H, Skou Hedemann M, Jensen BB (2005) Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. *Appl Environ Microbiol* 71:2267–2277.
30. Zhai Q, et al. (2017) Effects of subchronic oral toxic metal exposure on the intestinal microbiota of mice. *Sci Bull* 62:831–840.
31. Erny D, et al. (2015) Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci* 18:965–977.
32. Donaldson GP, Lee SM, Mazmanian SK (2016) Gut biogeography of the bacterial microbiota. *Nat Rev Microbiol* 14:20–32.
33. Alexander S, et al. (2007) Viscosity gradient within the mucus layer determines the mucosal barrier function and the spatial organization of the intestinal microbiota. *Inflamm Bowel Dis* 13:963–970.
34. Świątek M, et al. (2005) Unusual binding ability of vancomycin towards Cu2+ ions. *Dalton Trans* 23:3808–3813.
35. Patwardhan A, Cowan JA (2011) Influence of charge and structure on the coordination chemistry of copper aminoglycosides. *Dalton Trans* 40:1795–1801.
36. Galván-Tejada N, et al. (2002) Supramolecular structures of metronidazole and its copper (ii), cobalt (ii) and zinc (ii) coordination compounds. *J Inorg Biochem* 91:339–348.
37. Petris MJ, Smith K, Lee J, Thiele DJ (2003) Copper-stimulated endocytosis and degradation of the human copper transporter, hCtr1. *J Biol Chem* 278:9639–9646.
38. Clifford RJ, Maryon EB, Kaplan JH (2016) Dynamic internalization and recycling of a metal ion transporter: Cu homeostasis and CTR1, the human Cu+ uptake system. *J Cell Sci* 129:1711–1721.
39. Chun H, Catterton T, Kim H, Lee J, Kim BE (2017) Organ-specific regulation of ATP7A abundance is coordinated with systemic copper homeostasis. *Sci Rep* 7:12001.
40. Hooper LV, et al. (2001) Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291:881–884.
41. Johansson MEV, et al. (2015) Normalization of host intestinal mucus layers requires long-term microbial colonization. *Cell host microbe* 18:582–592.
42. Yin N, et al. (2017) Investigation of bioaccessibility of Cu, Fe, Mn, and Zn in market vegetables in the colon using PBET combined with SHIME. *Sci Rep* 7:17578.
43. Zoetendal EG, et al. (2002) Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Appl Environ Microbiol* 68:3401–3407.
44. Deschemin J-C, et al. (2016) The microbiota shifts the iron sensing of intestinal cells. *FASEB J* 30:252–261.
45. Gielda LM, J DiRita V (2012) Zinc competition among the intestinal microbiota. *MBio* 3:e00171-12.
46. Djoko KY, Cheryl-lynn YO, Walker MJ, McEwan AG (2015) The role of copper and zinc toxicity in innate immune defense against bacterial pathogens. *J Biol Chem* 290:18954–18961.
47. Lukasewycz OA, et al (1981) Copper deficiency suppresses the immune response of mice. *Science* 213:559–561.
48. Sergio SC, et al. (2014) Arsenic thiolation and the role of sulfate-reducing bacteria from the human intestinal tract. *Environ Health Perspect* 122:817–822.
49. Chaturvedi KS, Hung CS, Crowley JR, Stapleton AE, Henderson JP (2012) The siderophore yersiniabactin binds copper to protect pathogens during infection. *Nat Chem Biol* 8:731–736.
50. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118:229–241.
51. Reikvam DH, et al. (2011) Depletion of murine intestinal microbiota: Effects on gut mucosa and epithelial gene expression. *PLoS One* 6:e17996.
52. Hirota SA, et al. (2010) NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. *Inflamm Bowel Dis* 17:1359–1372.
53. Whelan FJ, et al. (2014) The loss of topography in the microbial communities of the upper respiratory tract in the elderly. *Ann Am Thorac Soc* 11:513–521.
54. Maréchal CN, Télouk P, Albarède F (1999) Precise analysis of copper and zinc isotopic compositions by plasma-source mass spectrometry. *Chem Geol* 156:251–273.
55. Brand WA, Coplen TB, Vogl J, Rosner M, Prohaska T (2014) Assessment of international reference materials for isotope-ratio analysis (IUPAC technical report). *Pure Appl Chem* 86:425–467.

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