A blood spot method for detecting fumonisin-induced changes in putative sphingolipid biomarkers in LM/Bc mice and humans

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Fumonisins (FB) are mycotoxins found in maize. They are hypothesised risk factors for neural tube defects (NTDs) in humans living where maize is a dietary staple. In LM/Bc mice, FB1-treatment of pregnant dams induces NTDs and results in increased levels of sphingoid base 1-phosphates in blood and tissues. The increased level of sphingoid base 1-phosphates in blood is a putative biomarker for FB1 inhibition of ceramide synthase in humans. Collection of blood spots on paper from finger sticks is a relatively non-invasive way to obtain blood for biomarker analysis. The objective of this study was to develop and validate in an animal model, and ultimately in humans, a method to estimate the volume of blood collected as blood spots on absorbent paper so as to allow quantification of the molar concentration of sphingoid base 1-phosphates in blood. To accomplish this objective, blood was collected from unexposed male LM/Bc and FB1-exposed pregnant LM/Bc mice and humans and applied to two types of absorbent paper. The sphingoid base 1-phosphates, absorbance at 270 nm (A270), and total protein content (Bradford) were determined in the acetonitrile:water 5% formic acid extracts from the dried blood spots. The results show that in both mouse and human the A270 of the extracts. In mouse blood spots, as in tissues and embryos, the FB1-induced changes in sphingolipids were correlated with urinary FB1. The half-life of FB1 in the urine was short (<24 h) and the elevation in sphingoid base 1-phosphates in blood was also short, although more persistent than the urinary FB1.

Keywords: sphingolipid; fumonisin; biomarkers; LC-MS; blood spots; maize; corn; sphinganine 1-phosphate

Introduction

Fumonisins (FB) are mycotoxins that cause farm animal diseases (reviewed in Bolger et al. 2001; Bulder et al. 2012) and are hypothesised to be potential environmental risk factors for human diseases including neural tube defects (NTDs), serious birth defects of the brain and spinal cord (Marasas et al. 2004; Gelineau-van Waes et al. 2009), liver cancer and chronic liver disease (Torres et al. 2015), and growth retardation in children (Kimanya et al. 2010; Shirima et al. 2015). Fumonisins are commonly produced by Fusarium verticillioides (formerly known as F. moniliforme); however, they are also produced by F. proliferatum, a few other Fusarium species and some Aspergillus species (reviewed in Månsson et al. 2010; Bulder et al. 2012). More than 30 FB analogues have been isolated and characterised (Rheeder et al. 2002). Of these, fumonisin B1 (FB1) is the most prevalent and believed to be the most toxic. Maize, the most frequently contaminated crop, is a staple food in many regions of the world such as Mexico, Central America, and sub-Saharan Africa, where the incidence of NTDs and FB exposure is also potentially very high (Marasas et al. 2004). For example, in Guatemala, intake of FB (Torres et al. 2007, 2014) frequently exceeds the provisional maximum tolerable daily intake (PMTDI) recommended by the Joint FAO/WHO Expert Committee on Food Additives (Bulder et al. 2012).

The mechanistic basis for the farm and laboratory animal diseases caused by FB1 is inhibition of ceramide synthases, key enzymes in the biosynthesis of sphingolipids (reviewed in Bolger et al. 2001; IARC 2002; Bulder et al. 2012). FB1 disruption of the biochemical pathways for de novo sphingolipid biosynthesis and turnover and subsequent disruption of lipid metabolism have been shown to play an important role in the induction of NTDs in mouse models (Gelineau-van Waes et al. 2005, 2012). Recent studies have shown that FB1 treatment of human neural epithelial progenitor cells in culture results in the inhibition of ceramide synthase and consequent elevation in sphinganine 1-phosphate (Sa 1-P)
The elevation in Sa is a consequence of the inhibition of ceramide synthases, the enzymes responsible for N-acetylation of Sa to dihydroceramide. Free sphingosine (So) also becomes elevated in tissues but to a much lesser extent than Sa since it is not a precursor of ceramide biosynthesis in the de novo biosynthesis pathway (Merrill et al. 2001; Riley et al. 2001). Free Sa that accumulates in tissues of rats treated with FB₁ is phosphorylated to form Sa 1-P (Riley & Voss 2006) and in mice there is a significant increase in the levels of Sa 1-P in the liver, kidney (Bondy et al. 2012), plasma and the blood spot extracts (Gelineau-van Waes et al. 2012).

Although the mechanism of how FB₁ induces NTDs in mice is unclear, studies show that when FB₁ inhibits ceramide synthase, the sphingolipid-dependent signalling pathways that regulate cell growth and cell death are altered (reviewed in Bulder et al. 2012). In addition to the elevated levels of bioactive sphingoid bases and sphingoid base 1-phosphates, tissues from mice exposed to FB₁ have decreased amounts of complex sphingolipids (Voss et al. 2009). Elevation in the intracellular concentrations of the sphingoid bases, sphingoid base metabolites and depletion of total ceramides have been used successfully as mechanism-based biomarkers in many animal studies (reviewed in Bulder et al. 2012) and thus are potential biomarkers of effect (ceramide synthase inhibition) in humans exposed to high levels of FB₁. However, attempts to use elevated levels of Sa and the ratio of Sa to So (Sa/So) as a serum or urinary biomarker for FB exposure and/or evidence of ceramide synthase inhibition in human populations consuming large amounts of maize has met with little or no success (reviewed in Van der Westhuizen et al. 2013). Conversely, the use of urinary FB₁ as an exposure biomarker has been successfully used in several recent studies (reviewed in Van der Westhuizen et al. 2013). For example, we have completed human studies in the USA to determine the kinetics of urinary FB₁ excretion and to develop and validate the use of urinary FB₁ as an exposure biomarker for intake of dietary FB (Riley, Torres, et al. 2012). The urinary exposure biomarker has been used successfully in studies conducted in high- and low-exposure communities in Guatemala (Torres et al. 2014) and in several countries and regions where maize is potentially contaminated with FB and is consumed in large amounts (reviewed in Van der Westhuizen et al. 2013). For example, the results of our study in Guatemala showed that urinary FB₁ levels in women living in areas where FB contamination of the maize was high also had significantly higher urinary FB₁ compared to the women living in areas where the FB contamination levels were significantly lower. The study by Torres et al. (2014) involved 1240 maize-consuming women living in high- and low-exposure communities and was conducted over the course of 1 year with sampling at 3 month intervals. The results showed that urinary FB₁ levels were predictive of the estimated FB intake in a dose-dependent manner and that many women in the high-exposure department (departamentos = 22 first-level subnational country governmental subdivision) exceeded the JECFA PMTDI (Torres et al. 2014).

The long-term objective of the present study is to develop a method using human blood applied to FTA™ Elute Micro Cards that will allow us to accurately estimate the molar concentration of Sa 1-P and Sa 1-P in blood spots collected from our human studies in Guatemala and elsewhere. However, variability in the volume of blood within the collected blood spots makes it difficult to estimate the molar concentration of Sa 1-P and Sa 1-P in the blood precisely. To that end, the specific objectives of this study were to (i) develop a method to estimate the actual volume of blood collected as blood spots using male LM/Bc mouse blood collected on absorbent paper, (ii) validate in pregnant LM/Bc mice that oral exposure to FB₁ induces NTDs and there is a dose-dependent increase in Sa 1-P and Sa 1-P in blood spots, and (iii) test the method using blood spots collected from humans in the USA and Guatemala. The goal is to be able to quantify the mass amount (nanomoles) of Sa 1-P and Sa 1-P normalised to blood volume. The results show that after extraction the ultraviolet absorbance at 270 nm (A₂₇₀) and total protein content of the extracts are closely correlated with the volume of mouse and human blood spotted and the FB₁-induced elevation in So 1-P and Sa 1-P in red blood cells (RBCs) of mice is correlated with urinary FB₁, the liver, kidney and embryo levels of free sphingoid bases, and is rapidly reversible in the absence of FB₁ exposure. Thus, the experimentally determined predictive relationship between blood volume (ml) and the A₂₇₀ of the extracts of the blood spots used for the quantitation by LCMS of Sa 1-P and So 1-P can be used to estimate the molar concentrations of these sphingoid base 1-phosphates in the blood. Based on the mouse studies the results also show that detection of differences in sphingoid base 1-phosphates in blood spots indicative of inhibition of ceramide synthase in human populations exposed to low and high levels of fumonisin is feasible.

Materials and methods

Experimental design and sample collection – mouse studies

Blood collection from male LM/Bc mice for titration of volume in blood spots

Male LM/Bc mice (4–9 months of age) were housed in stainless steel wire-mesh cages kept in an environmentally controlled room with a 12-hour light/dark cycle. Male mice were used because the study did not involve any
**Fumonisin oral gavage dose–response study in pregnant female LM/Bc mice**

In order to determine the dose–response relationship between FB₁ intake, urinary FB₁, and the concentrations of sphingoid base 1-phosphates in maternal blood and tissues (liver and kidney), and also to evaluate the dose–response relationship between sphingolipid metabolites in embryonic tissues and the threshold of oral FB₁ exposure necessary for induction of NTDs, a study was conducted using pregnant LM/Bc mice. Inbred LM/Bc mice were housed in microisolator cages containing autoclaved corn cob bedding, given ad lib access to normal irradiated rodent chow (Harlan-Teklad #8904) and autoclaved water, and maintained on a 12-hour light/dark cycle in climate-controlled AAALAC-approved laboratory animal facilities at Creighton University School of Medicine. Virgin females 60–90 days of age were placed with a male overnight for timed matings. The male was removed to his home cage the following morning, and females exhibiting vaginal plugs were weighed, and placed in a separate cage. The time of conception was considered to be midnight on the evening of the mating. Plugged females were weighed again on the morning of gestational day (GD) 6.5, and if they had gained weight since their plug date, they were placed into a treatment group.

Purified FB₁ (Cayman Chemical, Ann Arbor, MI, USA) was administered to pregnant dams on GD 6.5, 7.5 and 8.5 by daily oral gavage at 5, 10, 15, 25 and 50 mg/kg bw (in a volume of 0.1 ml/10 g bw). Pregnant mice in the control group were given 0.1 ml/10 g bw sterile water by oral gavage. On GD 9.5, pregnant mice were anaesthetised, and blood spots collected from retro-orbital bleeds on Whatman 903® Specimen Collection Paper (4–5 spots/mouse). After drying overnight the cards were stored desiccated at 4°C (or stored at −20°C for prolonged storage). Following collection of blood spots, dams were sacrificed and maternal tissues (liver and kidney) harvested, weighed in 1.5 ml polypropylene snap-top tubes, snap-frozen on dry ice, and stored at −80°C. Urine was collected directly from the bladder at necropsy, and placed into 1.5 ml polypropylene snap-top tubes, flash-frozen on dry ice and stored at −80°C. Individual urine volumes ranged from 0 to 350 µl (one mouse in the 5 mg/kg bw dose group did not have any urine for collection). The uterus was then removed, and the total number of implants recorded. Embryos were separated from the placenta and extraembryonic membranes in ice cold phosphate-buffered saline (PBS), and the phenotype recorded. The head of each embryo was then removed (at the caudal aspect of the rhombencephalon) and placed into a microfuge tube, weighed, snap-frozen on dry ice, and stored frozen at −80°C for liquid chromatography–mass spectrometry (LC-MS) determination of sphingolipid metabolites. All animal procedures were done in accordance with the Public Health Service (PHS) policy on Humane Care and Use of Laboratory Animals, and approved by the Creighton University Institutional Animal Care and Use Committee (IACUC).

**Kinetics of urinary FB₁ elimination and sphingoid base 1-phosphate elevation in blood spots following intraperitoneal FB₁ administration in non-pregnant female LM/Bc mice**

Our previous studies demonstrated that sphingoid base 1-phosphates are elevated in maternal blood spots collected 24 h after the last intraperitoneal injection of FB₁ (20 mg/kg/day on GD 7.5 and 8.5; Gelineau-van Waes et al. 2012). In order to determine how quickly sphingoid base 1-phosphates return to normal levels after withdrawal of FB₁ exposure, non-pregnant female LM/Bc mice (60–90 days of age, n = 3 per time point) were dosed via intraperitoneal (i.p.) injection with pure FB₁ at 20 mg/kg bw for three consecutive days. At designated time points after the final (third) FB₁ injection (4 h, 24 h, 48 h, 72 h or 120 h), mice were anaesthetised and blood spots collected for sphingolipid analysis (retro-orbital bleed) and urine samples collected for FB₁ analysis (from bladder at necropsy) as described above. In order to determine normal baseline levels of sphingoid bases and sphingoid base 1-phosphates, blood was also collected from mice that had not been treated with FB₁. The mice were housed and cared for at Creighton University as previously described for the dose–response study.
Experimental design and sample collection – human studies

Sphingoid base 1-phosphates in blood spots from FB-exposed humans under controlled conditions

Healthy (self-described) volunteers ($n=10$ total) were recruited in Athens, Georgia, USA to participate in studies to develop and validate urinary FB exposure biomarkers and also for developing methods for measuring possible mechanism-based biomarkers using changes in levels of sphingoid base 1-phosphates in blood spots. The study design, demographic information for the volunteers, and results of the study to develop and validate the urinary FB$_1$ exposure biomarker have been reported in detail in Riley, Torres, et al. (2012). Briefly, volunteers were asked to abstain from eating any maize-based foods for 3 days followed by 3 days consuming six tortillas and five biscuits per day followed by 5 days abstaining from maize-based foods. The masa flour and cereal-atol used to prepare the tortillas and biscuits were purchased from local grocery stores. A detailed description of the selection, fumonisin analysis, and cooking of the maize-based foods are provided in Riley, Torres, et al. (2012). The intake of FB$_1$ during the 3 day consuming period was $2.94 \pm 0.55 \, \mu g/kg$ bw/day (Riley, Torres, et al. 2012).

All consenting volunteers were asked to participate in training on the procedure for collecting blood spots at three time points during the study: (i) once before consuming maize-based foods, (ii) once after consuming maize-based foods for three consecutive days, and (iii) once after abstaining from maize-based foods for an additional 5 days. For the purposes of the study described herein, five of the volunteers (gender, two female and three male) provided additional drops of blood at the end of the study, and these samples were used to spot known volumes of blood on FTA™ Elute Micro Cards. The Human Subjects research protocol and Informed Consent form were approved by the University of Georgia Human Subjects Office Institutional Review Board (Project Number 2009-10769-2).

Sphingoid base 1-phosphates in blood spots from FB-exposed humans in two low exposure communities in Guatemala

In order to test our ability to recruit volunteers and to store, ship and process the blood spots collected in Guatemala, two communities (departments of Chimaltenango and Escuintla) were chosen for a small ($n=76$ women and 100 men) pilot study. These two departments were selected based on FB surveys conducted in 2005 and 2007 (Riley, Voss, et al. 2012). It was expected that Chimaltenango would be a low FB exposure community and Escuintla would be a high exposure community. However, when the maize and urine were analysed it was found that both communities had low exposure to FB (Riley, Torres, et al. 2012), a condition which continued over the course of the entire sampling year (Torres et al. 2014). The study design and demographics of the consenting volunteers can be found in Riley, Torres, et al. (2012) and Torres et al. (2014). The Human Subjects research protocol and Informed Consent form were approved by the Comité Institucional de Ética of the Instituto de Nutrición de Centro América y Panamá (Project Number CIE REV003/2010), a US Department of Health, and Human Services Office for Human Research Protections registered Independent Ethics Committee. The Comité Nacional de Ética of the Guatemalan Ministry of Health also gave its ethical approval for this project.

Sample processing, recoveries and stability studies

Chemicals and standards

Acetonitrile (MeCN; Burdick & Jackson, Muskegon, MI, USA) and water (J.T. Baker, Morristown, NJ, USA) were HPLC grade and formic acid ($> 95%$; Sigma-Aldrich, Center Valley, PA, USA) was reagent grade. Sa (d18:0) and So (d18:1) were purchased from Sigma Aldrich (St. Louis, MO, USA). So 1-P, Sa 1-P, C$_{17}$ So 1-P, 1-deoxyxphinganine (deoxySa), and C$_{20}$ So were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Working standards (50 fmol/µl, 20 fmol/µl and 5 fmol/µl) were prepared by dilution from a 300 fmol/µl stock mixture of So, Sa, deoxySa, Sa 1-P, Sa 1-P, C$_{20}$ So and C$_{17}$ So 1-P in 1:1 MeCN:water + 1% formic acid. Specific details for solubilising the sphingoid bases and sphingoid base 1-phosphates can be found in Zitomer and Riley (2011). U-[C$_{13}$]-FB$_1$-solution (25 µg/ml) OEKANAL® was purchased from Sigma-Aldrich Laborchemikalien (Seelze, Germany). Internal standard stock mixture (C$_{17}$ So 1-P and C$_{20}$ So) used to prepare the extraction mix (described below) was prepared from pure C$_{20}$ So and C$_{17}$ So 1-P dissolved in 1:1 MeCN:water + 5% formic acid to a final concentration of 30 pmol/µl.

Processing of mouse urine samples

Frozen urine samples collected from pregnant mice in the oral gavage dose–response mouse study were thawed and U-[C$_{13}$]-FB$_1$ (40 ng total) dissolved in 300 µl of 40% MeCN:60% water with 1.33% formic acid was added directly to 100 µl of each urine sample. The final MeCN, water and formic acid concentration matched the initial proportions of the LC-MS mobile phase (30% solvent A and 70% solvent B). The samples were then diluted 1:1 with mobile phase, filtered and injected directly onto the LC-MS without additional clean-up.

For the kinetics of urinary FB$_1$ elimination study following intraperitoneal FB$_1$ exposure in non-pregnant
LM/Be mice, mouse urine samples were diluted (50- to 250-fold) with the initial LC-MS mobile phase (30% solvent A and 70% solvent B) and then further diluted so that the peak areas for all samples fell within the standard curve. The filtered samples were injected directly onto the LCMS without additional clean-up.

**Extraction of sphingoid bases and sphingoid base 1-phosphates from blood spots and mouse tissues**

Whole blood from both of the human studies was collected on FTA™ Elute Micro Cards (GE Healthcare Biosciences Corp., Piscataway, NJ, USA, Whatman™ Cat No. WB12 0401). Whole blood collected from male mice (for titration of blood volume) was spotted on either FTA™ Elute Micro Cards or Whatman 903® Specimen Collection Paper (GE Healthcare Biosciences Corp., Piscataway, NJ, USA, Whatman™ 10534320). All blood spots were dried at room temperature overnight and stored desiccated at −20°C. A brief description of the rationale for using two types of blood cards in the mouse blood volume titration study is contained in the introductory paragraph of the Online Supplemental Data.

Blood spot discs from human and mouse studies were cut from the FTA™ Elute Micro Cards or Whatman 903® Specimen Collection Paper with a 6 or 8 mm biopsy punch. An 8 mm punch is sufficient to completely remove a 15–17 µl blood spot that has been carefully spotted on the paper. The discs and control blanks (discs without blood) were placed in 2.0 ml microcentrifuge tubes and then cut in half with scissors (this improves the extraction) inside the tubes and stored desiccated at −20°C. Mouse liver and kidney samples and embryo head samples were processed as described in Zitomer and Riley (2011). The polypropylene tubes containing tissue homogenates (20 mg in 100 µl homogenisation buffer) on ice or blood spots equilibrated to room temperature were combined (20 mg in 100 µl homogenisation buffer) on ice or blood for each sample vials for LC-MS analysis as described above.

The recovery of sphingoid bases and sphingoid base 1-phosphates was determined by applying 300 pmol of So, Sa, deoxySa, C_{20} So, C_{17} So 1-P, So 1-P and Sa 1-P onto FTA™ Elute Micro Cards (n = 4) that had been spotted with fresh whole human blood (17 µl) which completely filled the area of an 8 mm biopsy punch. The cards were placed in a desiccator overnight and then extracted and analysed as described above. The percent recovery was based on the areas of the peaks for each analyte in the spiked extracted blood spots corrected for areas in unspiked blood spots divided by the areas in a diluted 300 pmol/ml standard.

The stability of sphingoid base 1-phosphates in blood spots collected on FTA™ Elute Micro Cards was determined by spotting multiple cards with blood (17 µl) and extracting and analysing for So 1-P and Sa 1-P periodically over a period of 170 days (t = 0, 14, 21, 61, 93 and 170 days) in storage at −20°C desiccated.

**Liquid chromatography–mass spectrometry (LC-MS) of sphingoid bases, sphingoid base 1-phosphates and fumonisin B₁**

Reversed-phase high-performance liquid chromatography (HPLC) analysis was conducted using a Finnigan Micro AS or Thermo Scientific Dionex Ultimate 3000 autosampler coupled to either a Finnigan Surveyor MS or Thermo Scientific Dionex Ultimate 3000 pump (Thermo Fisher Scientific, Waltham, MA, USA). Separation was accomplished using an Imtakt Cadenza CW-C18 3 µ particle size, 150 mm × 2 mm column (Imtakt USA, Portland, OR, USA). The sample tray in the autosampler was maintained at ambient temperature (≥ 20°C).

For sphingoid bases and sphingoid base 1-phosphates analysis, the flow rate was 0.2 ml/min. Separation was achieved using a 25 min programmed gradient starting at 50% of 97% MeCN/2% water/1% formic acid (solvent A) and 50% of 2% MeCN/97% water/1% formic acid (solvent B). The gradient ran from 50% A, 50% B to 100% A, 0% B at 15 min followed by a 10 min re-equilibration at 50% A, 50% B. (Note: the programmed gradient was sometimes modified so that the initial proportion of Solvent A was as high as 60% and the column temperature was increased to as high as 30°C). The injection volume was 20 µl. The column effluent was directed to a Finnigan LTQ XL linear ion trap mass spectrometer (MS version 2.5.0; Thermo Fisher Scientific, Waltman, MA USA). The MS was operated in the electrospray ionisation (ESI) positive ion mode with an inlet capillary temperature of 210°C, and the sheath gas was nitrogen. For MS/MS of C_{20} So, deoxySa, C_{17} So 1-P, So 1-P and Sa 1-P the collision energy (CID) was 35% and for Sa and So the CID was 20%. The MS tuning parameters for all compounds were based on optimisation for CID. The precursor ion (m/z) and the product ions (m/z) for C_{20} So, So, Sa, deoxySa, C_{17} So 1-P, So 1-P and Sa 1-P were the same as reported previously (Zitomer et al. 2008, 2009).
and Sa 1-P and the second segment (7 to 12 min) scanning for So, Sa, deoxySa, and C_{20} So. On the Imtakt Cadenza column the sphingoid base 1-phosphates elute before the sphingoid bases, which is not the case with other C_{18} reversed-phase columns that we have used previously (Zitomer et al. 2008; Zitomer & Riley 2011). The duration of each segment depended on the gradient, which was sometimes manipulated to optimise the chromatographic separation of the analytes. Quantitation of So and Sa was based on the recovery (area under the peak) of the C_{20} So internal standard and for So 1-P and Sa 1-P recovery of the C_{17} So 1-P internal standard. Both internal standards were included in the extraction mixture at 60 pmol/ml.

For the urinary FB analysis in the oral gavage dose–response study in pregnant mice and the kinetics of urinary elimination study following intraperitoneal dosing in non-pregnant mice, the LC-MS parameters were the same as described for analysis of human urine (Riley, Torres, et al. 2012). However, quantitation in the kinetic study was accomplished based on external standardisation (FB_{1} standard curve 1, 10 and 100 pg/µl).

**UV absorbance and estimation of total protein content of blood spot extracts**

A Beckman Coulter® DU® 730 spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA) was used to conduct full-wavelength UV absorbance scans (A_{350} to A_{240} nm) of filtered blood spot extracts and to determine the maximum UV absorbance at 270 nm. Extracts of the FTAM Elute Micro Cards and Extracts of the Whatman 903® Specimen Collection Paper without blood were used as blanks for baseline correction. The actual UV maximum for the blood extracts fell consistently between 270 and 275 nm. Most proteins have absorption maxima at around 280 nm at neutral pH. The exact wavelength for the absorbance maxima is a function of the relative amounts of aromatic amino acids in the proteins extracted. After further dilution, the total protein content of the same blood spot extracts was estimated using the Coomassie Plus-Bradford Assay Kit (Thermo Scientific, Waltham, MA).

**Model development for estimating blood volume per blood spot from mice and humans**

In order to determine if there was a direct linear relationship between the volume of blood spotted on the card and the levels of sphingoid base 1-phosphates in humans, some volunteers (n = 5) from the study conducted in Athens, GA (controlled conditions) provided additional drops of blood that were used to spot known amounts of blood (2.5, 5, 10 and 15 µl) on FTAM Elute Micro Cards (as described above in the mouse studies). The blood spots were stored, extracted and analysed as described above and the A_{270} of extracts was also determined. The data from these experiments were used to develop a model that would predict the volume of blood in individual blood spots based on the A_{270} in humans.

**Statistics**

Statistical analysis was performed using SigmaPlot® 12.5 (Systat Software, Inc., San Jose, CA, USA). When many groups were compared, one-way analysis of variance was used, followed by post-hoc multiple comparisons. If the data failed normality or were of unequal variance, the Kruskal–Wallis one-way analysis of variance on ranks was used. When only two groups were compared, a Student’s t-test was used. The Mann–Whitney rank sum test was used for comparing two groups if data failed normality or were of unequal variance. The Pearson product moment correlation was used to measure the strength of the association between pairs of variables. All data are expressed as mean ± SD and differences among or between means were considered to be significant if the p-value was < 0.05.

**Results**

Detection of sphingosine 1-phosphate (So 1-P) and sphinganine 1-phosphate (Sa 1-P) in blood spot extracts from FTAM Elute Micro Cards

So 1-P and Sa 1-P were easily detected in extracts of mouse and human blood spots collected on FTAM Elute Micro Cards (Figure 1). Even at the lowest amount spotted (2.5 µl) both sphingoid base 1-phosphates were detected (Figures 2 and 3) and there was a close relationship between the volume of blood spotted and the calculated amounts of So 1-P (Figures 2A and 3A) and Sa 1-P (Figures 2B and 3B) in the extracts of the 8 mm cores. The limits of quantitation for the sphingoid base 1-phosphates in an 8 mm core was approximately
The amount of blood (2.5–15 µl) spotted on the cards had no significant effect on the detection of either So 1-P or Sa 1-P and there was no statistically significant difference in ratio of Sa 1-P/So 1-P (Table 1).

The average ratio of Sa 1-P/So 1-P in the mouse blood extracts (0.45 ± 0.074, n = 32) was significantly higher ($p < 0.001$) than that in the human blood extracts.
Both So and Sa were detected in the mouse blood, but they were not detected in the human blood spots (Figure 1A and B). The percent recovery of So and Sa from human blood spots on FTA™ Elute Micro Cards was 73% ± 10% and 72% ± 6% (n = 4), respectively, and for So 1-P and Sa 1-P it was 61% ± 5% and 74% ± 4% (n = 4), respectively (Online Supplemental Data Table 1S).

After collection, the So 1-P and Sa 1-P in human blood spots collected on FTA™ Elute Micro Cards showed no evidence of significant degradation after 170 days of storage desiccated at −20°C (Online Supplemental Data Figure 1S). The method of sacrifice (isoflurane vs CO₂) of the mice and the addition of EDTA in collection tubes had no significant effect on the levels of Sa 1-P and So 1-P (Introduction in Online Supplemental Data).

Model development using correlation of blood volume with absorbance at 270 nm and total protein

Full-wavelength UV scans of the blood spot extracts from both mouse and humans showed a maximum absorbance at approximately A₂₇₀–A₂₇₅ nm (Figure 1, insets). The absorbance at 270 nm was also closely correlated with the total Bradford protein (A₅₉₅) in the extracts (Figure 2A, inset and 3A, inset) and the volume of blood spotted on the FTA™ Elute Micro Cards (Figure 2B, inset and 3B, inset).

The linear regression for A₂₇₀ versus pipetted volumes (ml) was used to develop models to predict the blood volume that was spotted on the FTA™ Elute Micro Cards. For mouse blood spots (Figure 2B, inset) the regression model (r² = 0.98, p < 0.001) was:

\[
\text{blood volume (ml)} = 0.0168(A_{270}) - 0.00149.
\]

For human blood spots (Figure 3B, inset) the regression model (r² = 0.96, p < 0.001) was:

\[
\text{blood volume (ml)} = 0.0094(A_{270}) - 0.00068.
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A similar predictive model was developed using Whatman 903® Specimen Collection Paper with spotting of mouse blood at 2.5–15 µl (Online Supplemental Data Figure 2S). As with the FTA™ Elute Micro Cards, the absorbance at 270 nm was highly correlated with both total Bradford protein (r² = 0.99) and the blood volume spotted (r² = 0.96).

So 1-P and Sa 1-P levels in human and mouse blood normalised to volume

The model using the A₂₇₀ accurately predicted the volumes of the blood spots for both mouse and human blood spotted on FTA™ Elute Micro Cards (Table 1). The predicted volume for each spot was used to estimate the concentrations of So 1-P and Sa 1-P (Table 1). The calculated concentration of So 1-P was slightly higher in the 2.5 µl blood spot extracts in both mouse and humans; however, the difference was not statistically significant when comparing the 2.5, 5, 10 and 15 µl blood spots (Table 1).
Table 1. The actual volume of blood spotted (ml), calculated concentration (mean pmol/ml ± SD, range (minimum and maximum), and n of sphingosine 1-phosphate (So 1-P), sphinganine 1-phosphate, Sa 1-P/So 1-P ratio, and calculated volume of blood (ml) spotted on FTA™ Elite Micro Cards based on the predictive models from the regression analysis of the absorbance at 270 nm (A270) shown in the insets of Figures 2B and 3B.

| Actual volume spotted (ml)a | Sa 1-P (pmol/ml) | So 1-P (pmol/ml) | Ratio | Predicted ml spotted |
|-----------------------------|------------------|------------------|-------|---------------------|
| LM/Bc mouse                 |                  |                  |       |                     |
| 0.0025 ml                   | 845 ± 447        | 1765 ± 712       | 0.469 ± 0.124 | 0.0024 ± 0.0004 |
|                             | n = 8            | n = 8            | n = 8  | n = 8               |
| 0.0050 ml                   | 708 ± 387        | 1510 ± 684       | 0.457 ± 0.056 | 0.0053 ± 0.0007 |
|                             | n = 8            | n = 8            | n = 8  | n = 8               |
| 0.0100 ml                   | 699 ± 386        | 1523 ± 649       | 0.444 ± 0.048 | 0.0099 ± 0.0008 |
|                             | n = 8            | n = 8            | n = 8  | n = 8               |
| 0.0150 ml                   | 656 ± 344        | 1501 ± 674       | 0.430 ± 0.051 | 0.0148 ± 0.0009 |
|                             | n = 8            | n = 8            | n = 8  | n = 8               |
| Human                       |                  |                  |       |                     |
| 0.0025 ml                   | 662 ± 115        | 2141 ± 404       | 0.312 ± 0.039 | 0.0027 ± 0.0003 |
|                             | n = 5            | n = 5            | n = 5  | n = 5               |
| 0.0050 ml                   | 609 ± 147        | 1816 ± 462       | 0.337 ± 0.007 | 0.0053 ± 0.0007 |
|                             | n = 5            | n = 5            | n = 5  | n = 5               |
| 0.0100 ml                   | 554 ± 120        | 1831 ± 445       | 0.306 ± 0.032 | 0.0097 ± 0.0013 |
|                             | n = 5            | n = 5            | n = 5  | n = 5               |
| 0.0150 ml                   | 558 ± 130        | 1808 ± 326       | 0.307 ± 0.026 | 0.0149 ± 0.0013 |
|                             | n = 5            | n = 5            | n = 5  | n = 5               |

aThe predicted relationship between the spotted blood volume and the absorbance at 270 nm for mouse and human blood spots from Figures 2B and 3B (insets) where blood volume mouse (ml) = 1.68e-2(A270)-1.4988e-3 (df = 31, r² = 0.98) and blood volume human (ml) = 9.406e-3(A270)-6.805e-4 (df = 19, r² = 0.96). The concentration of sphingoid base 1-phosphates were calculated based on the areas of the So 1-P or Sa 1-P under the peak divided by the area of the internal standard (C17: So 1-P) from the LC-MS analysis of the blood extract multiplied by 60 pmol and divided by the predicted volume based on the A270.

So 1-P and Sa 1-P concentrations in blood from FB1-treated mice

Oral gavage of pregnant LM/Bc mice with 10 mg FB1/kg bw/day, for 3 days caused a marked increase in both Sa 1-P and Sa 1-P which was easily observed in the chromatograms from the LC-MS analysis (examples shown in Online Supplemental Data Figure 3S) of the extracts of the blood spots. There was a statistically significant (p < 0.001) positive correlation between the FB1 dose and the calculated blood concentrations of So 1-P (Figure 4A), Sa 1-P (Figure 4B) and the Sa 1-P/So 1-P ratio (Figure 4A, inset). The concentration of So 1-P and Sa 1-P in the blood was calculated using the predictive model developed for mice using the Whatman 903® Specimen Collection Paper (Online Supplemental Data Figure 2S). The mean predicted blood volume based on the A270 of the extracts from the 8 mm cores used for extraction was 0.016 ml ± 0.001 ml (n = 12).

The urinary FB1 in the mice increased with dose (Figure 5A) and there was a significant positive correlation (r = 0.80, p < 0.002) between the Sa 1-P levels in the blood spots and the urinary FB1 (Figure 5B). The ratio of Sa 1-P/So 1-P was also significantly correlated with the urinary FB1 (Figure 5B, inset). The increased levels of Sa and deoxySa in liver and kidney (Online Supplemental Data Figure 4S) paralleled the increased levels of So 1-P and Sa 1-P seen in the blood spots (Figure 4). The increase in Sa 1-P was also seen in both liver (not shown) and kidney (Online Supplemental Data Figure 4SD, inset), but the levels were two orders of magnitude less than the molar amounts of Sa seen in the liver and kidney (Online Supplemental Data Figure 4SA and B).

A dose-dependent increase in Sa and in the Sa/So ratio was observed in embryonic tissue (heads) harvested from pregnant dams dosed orally with a range (0–50 mg/kg/day) of FB1 concentrations (Online Supplemental Data Figure 5S). However, the Sa/So ratio in embryos exposed to different concentrations of FB1 was only significantly different from control embryos at the 50 mg/kg/day dose (Online Supplemental Data Figure 5S). Although we did not detect a corresponding increase in Sa 1-P or So 1-P in embryonic tissue in this particular study, elevated
sphingosine 1-phosphate (So 1-P) (A), sphinganine 1-phosphate (Sa 1-P) (B) and the Sa 1-P/So 1-P ratio (inset in A) in extracts of mouse blood spots from pregnant LM/Bc mice orally dosed with FB1 at 0, 5, 10, 15, 25 and 50 mg/kg bw/day (GD 6.5 to 8.5). Blood spots were collected on Whatman 903® Specimen Collection Paper. Kruskal–Wallis One-Way ANOVA on Ranks was used to compare various FB treatment groups; the difference among groups (n = 2/group) was not statistically significant (p > 0.05). However, the Pearson product moment correlation (r) between dose and the concentrations of So 1-P, Sa 1-P and Sa 1-P/So 1-P was highly significant (p < 0.001).

Figure 5. Dose–response increase in the estimated (mean ± SD, n = 2 except 5 mg/kg n = 1) in pregnant LM/Bc mice orally dosed with FB1 at 0, 5, 10, 15, 25 and 50 mg/kg bw/day (GD 6.5 to 8.5) (A) and the regression analysis of the relationship between the urinary FB1 and the sphinganine 1-phosphate (Sa 1-P) (B) and the Sa 1-P/So 1-P ratio (inset in B) in extracts of mouse blood spots as shown in Figure 4. The Pearson product moment correlation (r) and p-values are inserted in each panel.

Figure 4. Dose–response increase in the estimated (mean ± SD, n = 2/dose) concentration of sphingosine 1-phosphate (So 1-P) (A), sphinganine 1-phosphate (Sa 1-P) (B) and the Sa 1-P/So 1-P ratio (inset in A) in extracts of mouse blood spots from pregnant LM/Bc mice orally dosed with FB1 at 0, 5, 10, 15, 25 and 50 mg/kg bw/day (GD 6.5 to 8.5). Blood spots were collected on Whatman 903® Specimen Collection Paper. Kruskal–Wallis One-Way ANOVA on Ranks was used to compare various FB treatment groups; the difference among groups (n = 2/group) was not statistically significant (p > 0.05). However, the Pearson product moment correlation (r) between dose and the concentrations of So 1-P, Sa 1-P and Sa 1-P/So 1-P was highly significant (p < 0.001).

dose; however, exencephalic embryos were detected when maternal FB1 exposure were ≥ 10 mg/kg/day. Above this threshold, there appeared to be a dose-dependent upward trend in the number of exencephalic embryos per litter, as well as an increase in the percentage of NTD positive litters (Online Supplemental Data Table 2S). In this study, the apparent threshold for oral FB1 induction of NTDs in LM/Bc mice was approximately 10 mg/kg/day and is similar to a recent report by Liao et al. (2014), in which oral dosing with 12.5 mg/kg FB1 induced NTDs in CD1 mice.
The kinetics of the change in So 1-P and Sa 1-P concentrations and the Sa 1-P/So 1-P ratio in the blood following dosing i.p. with FB

The levels of sphingoid base 1-phosphates in the mouse blood increased for 48 h after i.p. dosing with pure FB and did not return to pre-dosing levels until more than 72 h post-dosing (Figure 6A and B). Conversely, FB was not detectable in the urine at 72 h post-dosing and the half-life was less than 24 h (Figure 6C). The ratio of Sa 1-P/So 1-P paralleled the increase and decrease in sphingoid base 1-phosphates in the blood spots (Figure 6A, inset). The Sa level in the blood spots also paralleled the increase in Sa 1-P in the blood spots, but the molar concentration was 40–50-fold less than that of the Sa 1-P (Figure 6B, inset).

The blood concentrations of So 1-P, Sa 1-P and the Sa 1-P/So 1-P ratio in humans exposed to low levels of fumonisin

There was no significant increase in So 1-P, Sa 1-P, or the Sa 1-P/So 1-P ratio in blood spots, collected on FTA Elute Micro Cards, from humans consuming maize-based foods containing FB at 2.94 ± 0.55 µg/kg bw/day for three consecutive days (Figure 7). There was also no significant correlation ($r = 0.35$, $p = 0.09$) using the Pearson product moment correlation, between urinary FB and the Sa 1-P/So 1-P ratio for all blood and urine samples collected pre-treatment, consuming 72 h, and post-treatment ($n = 25$).

In the Guatemalan women living in the departments of Chimaltenango and Escuintla [departments with low levels of fumonisin contamination in the maize (Riley, Torres, et al. 2012; Torres et al. 2014)] there was no significant correlation using the Pearson product moment correlation between the levels of urine FB and the Sa 1-P/So 1-P ratio in the blood spots ($r = 0.18$, $p = 0.12$, $n = 76$). There was also no correlation between urinary FB and the levels of Sa 1-P or So 1-P (data not shown). The So 1-P and Sa 1-P concentrations in the women from Escuintla were significantly higher ($p < 0.05$) than in the women from Chimaltenango, but the ratio of Sa 1-P/So 1-P was slightly and significantly higher in Chimaltenango (Table 2). The mean urinary FB was also higher in women from Chimaltenango, but the difference was not significant (Table 2). In men from the same departments (Online Supplemental Data Table 3S) the average urinary FB level in the men from Escuintla was significantly higher than the men from Chimaltenango but as in the women, there was no significant correlation using the Pearson product moment correlation between the levels of urinary FB, and the Sa 1-P/So 1-P ratio ($r = -0.16$, $p = 0.11$, $n = 100$) or the concentrations of Sa 1-P or So 1-P in the blood spots (data not shown).

The So 1-P (A) and Sa 1-P (B) (mean nmol/ml ± SD) in blood spot extracts from Whatman 903 Specimen Collection Paper and urinary FB (ng/ml) (C) from non-pregnant female LM/Bc mice ($n = 3/time point$) dosed i.p. with FB at 20 mg/kg bw for three consecutive days and blood collected at 4–120 h after the third dosing. No FB was detected (ND) in the urine of control mice (Pre-Dose). Inset in (A) are the ratios of Sa 1-P/So 1-P and the concentrations of Sa are inset in (B) for the same blood spots.
Discussion

Human FB₁ intake assessment and biomonitoring of mechanism-based biological effects (i.e. disruption of sphingolipid metabolism) are a necessary first step to determine if FB₁ exposure contributes to any human disease including the high incidence of NTDs in maize consumers in Guatemala (Marasas et al. 2004; Gelineau-van Waes et al. 2009). A method to quantify urinary FB₁ as an exposure biomarker has been validated and used successfully in Guatemalan women (Riley, Torres, et al. 2012; Torres et al. 2014). Studies are in progress to determine if there is a correlation between urinary FB₁ and the levels of sphingoid base 1-phosphates in the blood of these same women (Riley, Voss, et al. 2012).

The present study was undertaken to develop and validate a method using blood spots to accurately estimate the molar concentration of So 1-P and Sa 1-P in blood. The use of the ratio of Sa/So (Riley, Wang, et al. 1994) and more recently Sa 1-P/So 1-P (Riley & Voss 2006; Voss et al. 2011) has proven very useful in animal studies for assessing FB₁ disruption of sphingolipid metabolism (Bolger et al. 2001; Bulder et al. 2012). In studies with farm and laboratory animals the FB-induced elevation in Sa and Sa 1-P can be easily detected in tissues such as liver and kidney before or at the onset of clinical signs of disease (Riley et al. 2001; Riley & Voss 2006; Voss et al. 2011). In studies with rats, evidence for disruption of sphingolipid metabolism in tissues precedes and is observed at lower dosages in organ tissues (liver and kidney) as compared to serum (Riley, Hinton, et al. 1994; Riley & Voss 2006). In human studies it would be unrealistic to sample liver and kidney for analysis as is done in the animal studies. For this reason, in humans, serum and urine are the most likely sources of biological material for analysis to detect changes indicative of FB₁-induced ceramide synthase inhibition. However, FB₁-induced elevation of the Sa concentration and the Sa/So ratio has not been demonstrated convincingly in any of the human studies using serum or urine (Van der Westhuizen et al. 2013). An alternative to using the Sa level and Sa/So ratio is to use the level of Sa 1-P and the ratio of Sa 1-P/So 1-P in blood spots from finger sticks collected at the same time as urine samples for urinary FB₁ analysis.

The method development and validation studies with humans in the USA and Guatemala, respectively, did not find any statistical correlation between the levels of urinary FB₁ and Sa 1-P or the Sa 1-P/So 1-P ratio. This is not surprising given the low levels of FB₁ intake and, for the study conducted in the USA, the short duration of exposure and the small number of participants. The overall estimated mean and median total FB (FB₁ + FB₂ + FB₃) intake in Chimaltenango and Escuintla (mean 0.41 µg/kg bw/day) (Riley, Torres, et al. 2012) was much less than the JECFA PMTDI of 2 µg/kg bw/day (Bolger et al. 2001; Bulder et al. 2012). In the method-development study conducted in the USA the mean estimated FB₁ intake was 2.9 µg/kg bw/day. Assuming that FB₁ intake is 70% of the total FB intake then the average intake would be approximately twice the...
Table 2. The calculated concentration (mean pmol/ml ± SD), range (minimum and maximum), and n of sphingosine 1-phosphate (Sa 1-P), sphinganine 1-phosphate, the Ratio (Sa 1-P/So 1-P), urinary fumonisin B₁ (UBF₁), and the predicted volume of blood (ml) collected from women in two low fumonisin exposure communities (Chimaltenango and Escuintla) and spotted on FTA™ Elute Micro Cards. The predictive model was from the regression analysis of the absorbance at 270 nm (A$_{270}$) and the actual volume of blood spotted (ml) shown in the insets of Figure 3B.

| Department       | Sa 1-P pmol/ml | So 1-P pmol/ml | Ratio | UBF₁ | Predicted ml spotted |
|------------------|---------------|----------------|-------|------|----------------------|
| Chimaltenango    | 519 ± 201     | 1591 ± 543     | 0.32 ± 0.04* | 0.27 ± 0.43 | 0.011 ± 0.002       |
|                  | 278–1050      | 961–3479       | 0.23–0.42 | 0.0–1.49 | 0.008–0.016         |
| n = 39           | n = 39        | n = 39         | n = 39 | n = 39 |                      |
| Escuintla        | 657 ± 257*    | 2172 ± 807*    | 0.30 ± 0.03 | 0.14 ± 0.13 | 0.009 ± 0.002*     |
|                  | 331–1736      | 1295–5360      | 0.24–0.38 | 0.0–0.47 | 0.005–0.020         |
| n = 37           | n = 37        | n = 37         | n = 37 | n = 37 |                      |

*The predicted relationship between the spotted blood volume and the absorbance at 270 nm for human blood spots from Figures 3B (inset) where blood volume human (ml) = 9.406e-3(A$_{270}$)–6.805e-4 (df = 19, r$^2$ = 0.96). The concentration of sphingoid base 1-phosphates was calculated based on the areas of the Sa 1-P or So 1-P under the peak divided by the area of the internal standard (C$_{17}$ So 1-P) from the LC-MS analysis of the extract multiplied by 60 pmol and divided by the predicted volume based on the A$_{270}$. An * indicates a significant difference between departments based on a t-test or Rank Sum Test.

UBF₁ data from Torres et al. (2014).

JECFA PMTDI (2 µg/kg bw/day). A brief excursion above the JECFA PMTDI would seem unlikely to be sufficient to disrupt sphingolipid metabolism in humans to the extent that it would be detectable as an increase in Sa or So 1-P unless humans are exquisitely sensitive to FB-induced inhibition of ceramide synthase. The original JECFA PMTDI (Bolger et al. 2001) was calculated from the NOAEL in the most sensitive species and target (male rat kidney) and included a 100-fold safety factor. We expect that elevated Sa 1-P and the Sa 1-P/So 1-P ratio in blood spots will only be observed in high-exposure populations where daily consumption of contaminated maize leads to persistent high exposures (Riley, Voss, et al. 2012).

The rationale for collecting blood spots rather than serum or plasma was that in addition to being less invasive and less expensive, RBCs contain large amounts of So 1-P and Sa 1-P. It has been shown that RBCs are the main source of the sphingoid base 1-phosphates in the blood (reviewed in Thuy et al. 2014). In addition, RBCs cannot synthesise sphingolipids de novo (Hänel et al. 2007; Bode et al. 2010) and lack the ability to dephosphorylate sphingoid base 1-phosphates (Ito et al. 2007). However, RBCs retain the ability to phosphorylate sphingoid bases via sphingosine kinases (SphKs) (Yang et al. 1999). Thus, the So and Sa that is phosphorylated in RBCs is produced in non-RBC tissues. In animals large amounts of So and Sa are produced in the liver and kidney following FB exposure (for example, Online Supplemental Data Figure 4S). The So and Sa that is produced in the liver and kidney following FB exposure is a likely source of substrates for the SphKs in the RBCs of FB-treated mice. It has been proposed that under normal physiological conditions, RBCs serve as reservoirs of the bioactive sphingoid base 1-phosphates for the purpose of buffering the So 1-P concentration in the plasma (Thuy et al. 2014).

Based on the unique functional and metabolic characteristics of So 1-P in blood, and in particular the fact that RBCs cannot make Sa or metabolise Sa 1-P, we hypothesise that blood spots from Guatemalan women living in areas where FB exposure is high will show higher Sa 1-P/So 1-P ratios when compared to populations with low exposure (Riley, Voss, et al. 2012), reflecting the increased Sa produced in cells and tissues as a consequence of FB-induced ceramide synthase inhibition. Consistent with this hypothesis would be elevated concentrations of Sa 1-P in the blood spots as we have seen in mice in this and other studies. The levels of Sa 1-P in the RBC closely parallel the levels of Sa seen in liver and kidney (Supplemental Data Figure 4S) and the urinary FB₁ (Figure 4).

The apparent half-life (visually approximated) for urinary elimination of FB₁ in mice dosed i.p. for 3 days (Figure 6C) and in humans consuming maize-based foods containing FB₁ for 3 days (Riley, Torres, et al. 2012) was < 24 h and < 48 h, respectively. Estimating the half-life for elevated Sa 1-P in blood in the mice was complicated by the fact that the increase in Sa 1-P lagged behind the urinary elimination of FB₁ such that the FB₁-induced increase in Sa 1-P was still apparent 120 h post-dosing (Figure 6B). Whether this is true for humans exposed to FB₁ levels sufficient to elevate Sa 1-P is not known. Nonetheless, in the women (n = 1240) sampled in Guatemala (Torres et al. 2014) greater than 99% of the volunteers reported that they ate maize-based foods every day and 90% of the volunteers had consumed maize based food ≤ 24 h before providing the urine sample (on average 9.2 h). Thus, assuming that FB₁ contamination is relatively constant over time in the high FB₁ exposure areas, once elevated the blood levels of Sa 1-P should be less
variable than the levels of urinary FB$_1$ since there appears to be a lag time between FB$_1$ exposure and the maximal elevation in sphingoid base 1-phosphates in the blood. This could lead to a greater chance of having an elevated Sa 1-P level that does not correlate with an elevated urinary FB$_1$. One way to assess whether or not this is a potential confounder is to sample both urine and blood from the same women over a long period of time. This could prove to be very difficult to accomplish. Alternatively, temporal fluctuations in the FB$_1$ dietary exposure should parallel the fluctuations in the average levels of urinary FB$_1$ in the sampled communities. The blood levels of Sa 1-P and the ratio should show similar trends although there would likely be a lag time in the response.

Based on our 1-year study in Guatemala (Torres et al. 2014) and FB surveys conducted across Guatemala in 2005, 2007 and 2012 (Torres et al. 2007, 2015; Riley, Voss, et al. 2012), maize in the high elevation–cool departments such as Chimaltenango and Sacatepequez are consistently low in FB, whereas, the hot–dry departments such as Jutiapa, Chiquimula and Santa Rosa are consistently high in FB. These are the departments where we are focusing our sampling of blood and urine.

Measuring the ratio of Sa 1-P/So 1-P is a simple way to detect changes in de novo ceramide biosynthesis as would be expected with FB$_1$-induced inhibition of ceramide synthase. This has been done in numerous studies in farm and laboratory animals. Nonetheless, there are likely many physiological processes and conditions that will alter So 1-P levels in humans; however, elevation in Sa in tissues and Sa 1-P in RBCs is most likely dependent on decreased ceramide biosynthesis as would be expected with de novo-induced inhibition of ceramide synthase (the first and rate-limiting step in de novo ceramide biosynthesis) resulting in the accumulation of Sa produced de novo. Thus, an important observation that is consistent with FB inhibition of de novo ceramide biosynthesis is elevated ratios of Sa/So or Sa 1-P/So 1-P that is positively correlated with FB intake based on the levels of urinary FB$_1$ and the demonstration that the increased ratio is due to a disproportionate increase in the molar concentration of Sa or Sa 1-P relative to So or So 1-P, respectively. It is important to note that an increase in the Sa/So or Sa 1-P/So 1-P ratio as a result of a decrease in So or So 1-P is not consistent with FB inhibition of ceramide synthase.

**Conclusion**

In order to quantitate the concentration of Sa 1-P and So 1-P in the blood, both the mass amount of the two metabolites and the volume of blood spotted must be determined. One anticipated complication in quantifying the concentration of So 1-P and Sa 1-P in human blood spots was the lack of control over the precise volume of human blood collected as blood spots in the field. In some cases the spot collected did not saturate the paper or produced a spot that was less than the area of the biopsy punch tool used to cut out the spots for extraction. This variability makes it difficult to accurately estimate the molar concentration of the So 1-P and Sa 1-P in the blood. The current study developed a model using non-treated, LM/Bc mouse blood spot extracts and their UV absorbance at 270 nm ($A_{270}$) to normalise the fumonisin-induced sphingolipid biomarkers by volume. Predictive models were developed and validated using FTA™ Elute Micro Cards and 903™ Specimen Collection Paper spotted with human or mouse blood to determine the volume of blood contained in a blood spot. The predictive model was validated in FB$_1$-exposed mice including oral exposure at levels sufficient to induce NTDs. These models, accurately predicted mouse and human blood volume per spot, and can be used to normalise So 1-P and Sa 1-P levels. This method will be used in human studies sampling a large number of women that consume large amounts of maize-based food on a daily basis and comparing populations where FB contamination is relatively low to populations where contamination is relatively high.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**Supplemental data**

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