Supplementary Online Content 2

Florian J, Matta MK, DePalma R, et al. Effect of oral ranitidine on urinary excretion of *N*-nitrosodiethylamine (NDMA): a randomized clinical trial. *JAMA*. doi:10.1001/jama.2021.9199

This supplementary material has been provided by the authors to give readers additional information about their work.

eTable 1. NDMA testing results from the drug product used in the study

Testing Date[a]	Relation to Study	NDMA Content (parts per million: ppm)	NDMA Content per 300 mg tablet (ng)
November 30, 2019	Prior to study start	0.021 ppm	6.3 ng
March 11, 2020	Prior to study start	0.025 ppm	7.5 ng
July 28, 2020	After study completion	0.035 ppm	10.5 ng

[a]The 300 mg ranitidine drug product was tested for NDMA content three times over a 20-month period. Testing was performed using publicly available and validated liquid chromatography with high resolution mass spectrometer methods, which are available on FDA's website.[1] The two tests before the clinical study and the one test after the study were all substantially below the FDA's acceptable daily intake limit of 96 ng NDMA per day.

eTable 2. Listing of participants, treatment groups, and diets where all items were not consumed on treatment days

Participant, Period	Meal	Remaining Items
Ranitidine with noncured-meats diet		
13, Period 1	Lunch	Tomato, lettuce, carrots
07, Period 1	Lunch	Carrots, bread crust
Placebo with noncured-meats diet		
13, Period 2	Lunch	Tomato, lettuce, carrots
07, Period 2	Lunch	Carrots, bread crust, pieces of roast beef
10, Period 1	Dinner	Half salad
Ranitidine with cured-meats diet		
04, Period 3	Lunch	Half lettuce, tomato, bread
13, Period 4	Lunch	Tomato, coleslaw, sandwich, V8 juice
07, Period 3	Lunch	Coleslaw, quarter hoagie
14, Period 4	Lunch	Half hoagie (all parts)
09, Period 3	Dinner	Half corned beef
10, Period 4	Breakfast	Bread crust
10, Period 4	Lunch	Quarter hoagie (all parts)
10, Period 4	Dinner	Corned beef
Placebo with cured-meats diet		
13, Period 3	Lunch	Tomato, coleslaw, and half hoagie (all parts)
07, Period 4	Lunch	Coleslaw, hoagie (all parts)
09, Period 4	Lunch	Quarter hoagie (all parts)
09, Period 4	Dinner	Corned beef and half cabbage
10, Period 3	Dinner	Corned beef
01, Period 3	Lunch	Coleslaw and half hoagie (all parts)

eTable 3. Samples below the lower limit of quantification by analyte and biospecimen

Analyte/ biospecimen	Measure	Noncured-meats diet		Cured-meats diet	
		Ranitidine	Placebo	Ranitidine	Placebo
NDMA/urine[a]	# samples below LLOQ (%)	122 (79)	138 (85)	93 (64)	89 (63)
	# of participants with all samples below LLOQ (%)	9 (50)	7 (39)	3 (18)	3 (18)
NDMA/plasma[a]	# samples below LLOQ (%)	209 (89)	211 (91)	192 (87)	197 (89)
	# of participants with all samples below LLOQ (%)	6 (33)	6 (33)	6 (35)	6 (35)
DMA/urine[a]	# samples below LLOQ (%)	0 (0)	0 (0)	0 (0)	1 (1)
	# of participants with all samples below LLOQ (%)	0 (0)	0 (0)	0 (0)	0 (0)
DMA/plasma[a]	# samples below LLOQ (%)	10 (5)	5 (2)	10 (5)	10 (5)
	# of participants with all samples below LLOQ (%)	0 (0)	0 (0)	0 (0)	0 (0)
Ranitidine/urine[a]	# samples below LLOQ (%)	2 (1)	-	0 (0)	-
	# of participants with all samples below LLOQ (%)	0 (0)	-	0 (0)	-
Ranitidine/plasma[a]	# samples below LLOQ (%)	24 (11)[a]	-	24 (12)[b]	-
	# of participants with all samples below LLOQ (%)	0 (0)	-	0 (0)	-

LLOQ: Lower limit of quantification

[a]LLOQ for NDMA urine and plasma was 0.0156 ng/mL, DMA urine and plasma was 0.50 µg/mL, and ranitidine urine and plasma was 15.6 ng/mL

[b]Ranitidine plasma samples below LLOQ were from the 11, 14, and 24 hr samples

eTable 4. Summary table of additional NDMA and DMA 24-hr pharmacokinetic parameters

Outcome	Diet	Ranitidine (Median [IQR]) (n=17)	Placebo (Median [IQR]) (n=17)
NDMA plasma T_{max}, hr	Noncured meats	2 (2 to 4.5)	11 (6.5 to 14)
	Cured meats	4 (2 to 10)	1 (0.5 to 7.5)
DMA plasma T_{max}, hr	Noncured meats	5 (3 to 6)	9 (6 to 11)
	Cured meats	4 (3 to 6)	6 (5 to 11)

IQR: Interquartile range; T_{max}: Time of maximum concentration

eTable 5. Summary table of 24-hr urinary excretion and pharmacokinetic parameters for ranitidine

Exploratory Outcomes	Intervention	Cured-meats diet (Geometric mean [CV%]) (n=17)	Noncured-meats diet	Geometric Mean Ratio [95% CI]	*P* value[a]
Ranitidine 24-hr urinary excretion, mg	Ranitidine	74.1 (39%)	91.7 (29%)	0.81 (0.72 to 0.91)	.001
Ranitidine Maximum plasma concentration, ng/mL	Ranitidine	861 (27%)	1118 (20%)	0.77 (0.65 to 0.91)	.005
Ranitidine plasma T_{max}, hr[b]	Ranitidine	1.5 (1.5 to 2)	1.5 (1.5 to 2)	-	-
Ranitidine $AUC_{0\text{-}last}$, hr·ng/mL	Ranitidine	3736 (29%)	4857 (21%)	0.77 (0.66 to 0.89)	.002

$AUC_{0\text{-}last}$: Area under the curve from time 0 to the last collected plasma sample; CI: Confidence interval; CV: Coefficient of variation; T_{max}: Time of maximum concentration

[a]*P* values are reported as two-sided tests at a 0.05 alpha level as the impact of diet on ranitidine was not known

[b]T_{max} is reported as median [interquartile range (IQR)] with no comparisons

eTable 6. Listing of pre-dose and 24-hour cumulative NDMA urine levels and maximum concentration for participant 12[a]

Period	Treatment	Diet	Pre-dose amount of NDMA in urine (ng)	24-hour cumulative NDMA in urine (ng)	Maximum NDMA plasma concentration (pg/mL)
1	Placebo	Noncured meats	0	29	31.3
2	Ranitidine	Noncured meats	46	512	42.0
3	Ranitidine	Cured meats	69	649	0
4	Placebo	Cured meats	150	746	16.3

[a]Participant 12 was an outlier for urinary excretion of NDMA in 3 of the 4 treatment groups (periods 2, 3 and 4 for this participant). Repeat analysis of the urine samples confirmed the results and review of study records indicated that all urine sample handling and processing steps were followed. The clinical staff noted the participant began menstruating between treatment periods 1 and 2, which continued through the end of the study. The participant's urinalyses at screening and check-in were negative for blood, but at check-out there was 3+ blood in the urine sample. None of the urinalyses were consistent with a urinary tract infection. Studies have noted that red blood cells contain high intracellular nitrite concentrations.[2,3] The *ex vivo* mixing of urine and lysed red blood cells could have released nitrite that facilitated conversion of DMA to NDMA during the relatively brief period before sodium hydroxide was added and the samples were fully frozen.

eTable 7. Comparison of 24-hour urinary excretion of NDMA with participant 12 removed

Outcome	Diet	Ranitidine (Median [IQR]) (n=17)	Placebo (n=17)	Median of Paired Differences [IQR]	*P* value[a]
Sensitivity Analysis – Participant 12 removed					
NDMA 24-hr urinary excretion, ng	Noncured meats	0.3 (0 to 11.6)	7.3 (0 to 17.4)	-6.1 (-12.0 to 28.0)	.76
	Cured meats	10.6 (5.2 to 41.4)	19.7 (8.0 to 31.1)	-1.6 (-18.9 to 10.4)	.55
	Cured meats vs noncured meats comparison on ranitidine			13.1 (5.2 to 24.3)	.009
	Cured meats vs noncured meats comparison on placebo			10.4 (3.1 to 24.4)	.01

IQR: Interquartile range
[a]A 1-sided lower test was used for sensitivity analyses of ranitidine vs. placebo and for the effect of diet on NDMA.

eTable 8. Comparison of 24-hour urinary excretion of NDMA with different LLOQ and limit of detection imputations

Outcome	Diet	Ranitidine (Median [IQR]) (n=17)	Placebo (n=17)	Median of Paired Differences [IQR]	*P* value[a]
Sensitivity Analysis – Urine concentrations < LLOQ were set to LLOQ of 0.0156 ng/mL					
NDMA 24-hr urinary excretion, ng	Noncured meats	39.4 (32.4 to 65.8)	46.0 (41.7 to 63.3)	-0.8 (-10.1 to 14.4)	.41
	Cured meats	44.9 (33.6 to 64.6)	42.8 (35.2 to 71.2)	-1.0 (-8.6 to 14.4)	.57
	Cured meats vs noncured meats comparison on ranitidine			1.7 (-7.5 to 21.2)	.29
	Cured meats vs noncured meats comparison on placebo			-5.2 (-10.8 to 10.5)	.59
Sensitivity Analysis – Urine concentrations < LOD (0.0078 ng/mL) set to 0; other values used as reported					
NDMA 24-hr urinary excretion, ng	Noncured meats	4.7 (2.7 to 35.8)	17.2 (1.9 to 29.0)	0 (-2.7 to 5.2)	.34
	Cured meats	17.0 (9.6 to 52.2)	29.2 (13.7 to 45.2)	0.8 (-10.3 to 12.4)	.50
	Cured meats vs noncured meats comparison on ranitidine			9.9 (2.4 to 27.6)	.004
	Cured meats vs noncured meats comparison on placebo			9.2 (-3.4 to 19.9)	.01
Sensitivity Analysis – Urine concentrations < LOD set to LOD; other values used as reported					
NDMA 24-hr urinary excretion, ng	Noncured meats	26.1 (17.5 to 46.5)	31.0 (21.5 to 50.6)	0.8 (-10.0 to 8.6)	.45
	Cured meats	31.1 (18.8 to 56.0)	39.1 (23.4 to 59.6)	3.9 (-4.9 to 10.7)	.43
	Cured meats vs noncured meats comparison on ranitidine			7.5 (-5.7 to 26.8)	.06
	Cured meats vs noncured meats comparison on placebo			3.4 (-6.2 to 12.8)	.19

IQR: Interquartile range; LLOQ: Lower limit of quantitation; LOD: Limit of detection
[a]A 1-sided lower test was used for sensitivity analyses of ranitidine vs. placebo and for the effect of diet on NDMA.

eTable 9. Incidence and number of adverse events by treatment group

Adverse Event[a]	Lead-In (No Treatment) – (N=18) Incidence (number of events)[b]	Ranitidine + Noncured-meats diet – (N=18) Incidence (number of events)[b]	Placebo + Noncured-meats diet – (N=18) Incidence (number of events)[b]	Ranitidine + Cured-meats diet – (N=17) Incidence (number of events)[b]	Placebo + Cured-meats diet – (N=17) Incidence (number of events)[b]
Abdominal Pain	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)
Diarrhoea	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)
Vaginitis gardnerella	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Vaginal Discharge	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Vessel Puncture Site Inflammation	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)
Vessel Puncture Site Pain	0 (0)	1 (1)	1 (1)	1 (1)	1 (1)

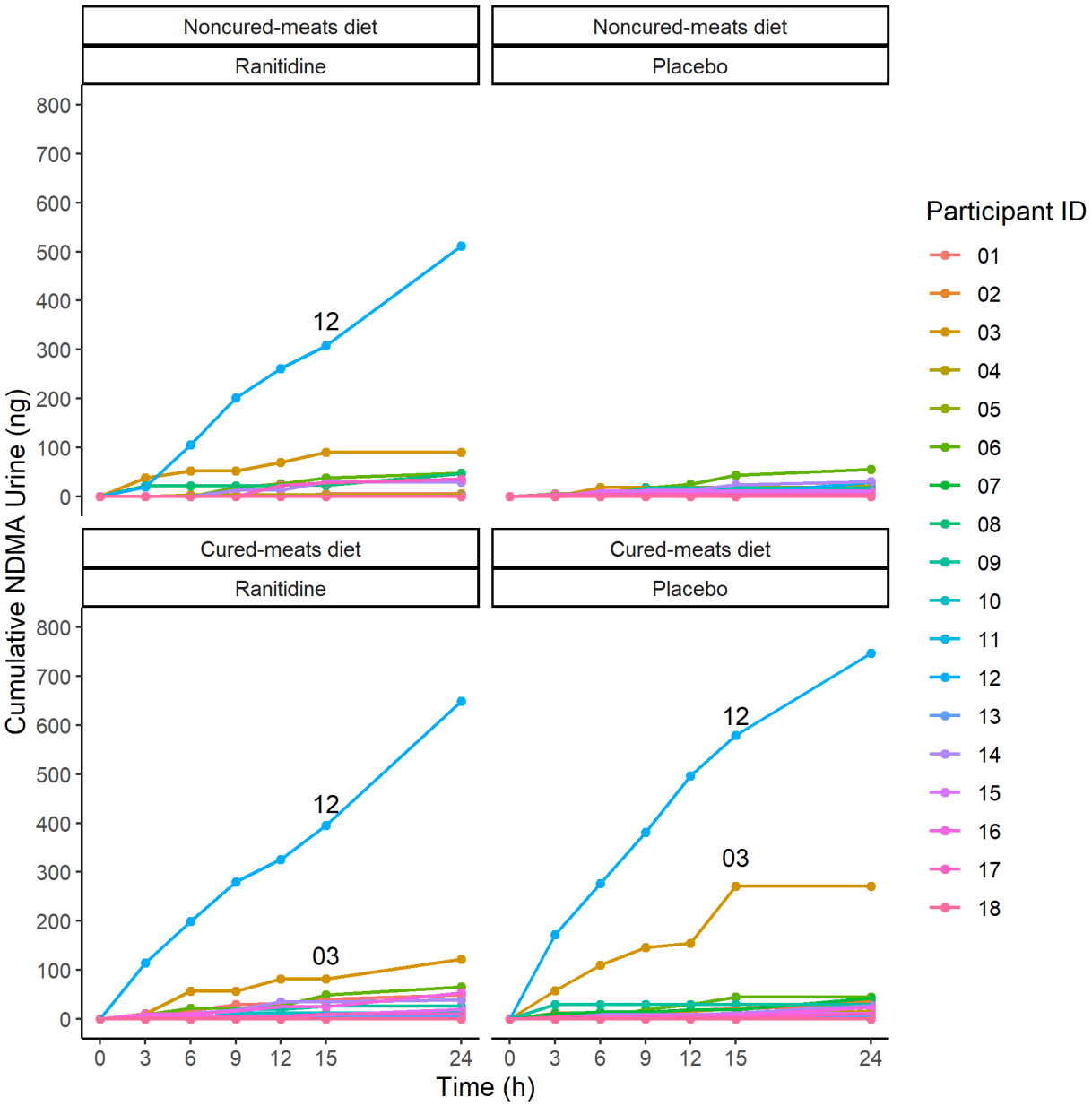
[a]This table reports all Medical Dictionary for Regulatory Activities (MedDRA v.23.0)-defined adverse events during the study
[b]First number stands for incidence and second number in parenthesis stands for number of events.

eTable 10. Comparison of the current and prior clinical studies on ranitidine and urinary excretion of NDMA

Current Study	Zeng and Mitch 2016[4,5]
Study Design, Settings and Participants	
• Utilized a placebo-controlled, crossover study design.	• Utilized a baseline-controlled, parallel study design.
• Enrolled 18 participants with 17 completers.	• Enrolled 10 participants with 10 completers.
• Each participant was randomized to receive ranitidine 300 mg or placebo over two sets of 2 periods.	• Participants had urine collected over two consecutive days – baseline (*i.e.*, untreated) followed by treatment with ranitidine 150 mg.
• Each period had a specific menu; meals administered on the pre-dose and treatment days with ranitidine and placebo were identical.	• No information provided in the manuscript regarding meals and drinks during the study duration.
• Participants were confined in a phase 1 clinical pharmacology unit for the entire study duration.	• No information provided in the manuscript regarding whether participants were confined for the study duration.
• Ranitidine drug product used in the study had NDMA content confirmed prior to shipment to the clinical site and again when it was returned.	• No information provided regarding NDMA content of the ranitidine product prior to administration in the study.
Urine Sample Collection	
• Each participant collected a series of urine samples using Commode specimen collection containers.	• Each volunteer collected a series of urine samples using Commode specimen collection containers.
• Each voiding was collected in a separate collection container. The protocol included planned voiding times but allowed for unscheduled voids between collections.	• Each voiding was collected in a separate collection container and specific collection times varied among the volunteers.
Urine Sample Storage	
• The *ex vivo* stability was thoroughly evaluated. An optimized sample collection protocol was developed prior to the study initiation.	• Details on *ex vivo* stability and a complete sample handling protocol were not included in the manuscript.
• All urine voids were collected, treated with fixed amounts of sodium hydroxide, and processed according to the urine sample collection protocol (see **Supplement 1**). The samples were stored at −80°C until analysis.	• Immediately after collection, urine samples were preserved with a few sodium hydroxide pellets to halt nitrosation and bacterial growth and stored at −20°C in the dark until analysis.
• Sample storage containers (50 mL volume) were prepared with 240 μL of 5 M sodium hydroxide from a stock solution and placed on ice prior to sample collection. Samples were weighed and up to 40 mL was transferred to the storage container within 5 minutes otherwise a protocol deviation was logged. The 5-minute interval was determined based on *ex vivo* stability experiments to minimize conversion.	• Neither the number of sodium hydroxide pellets used per volume nor how quickly the pellets were added to the urine samples after collection were specified.

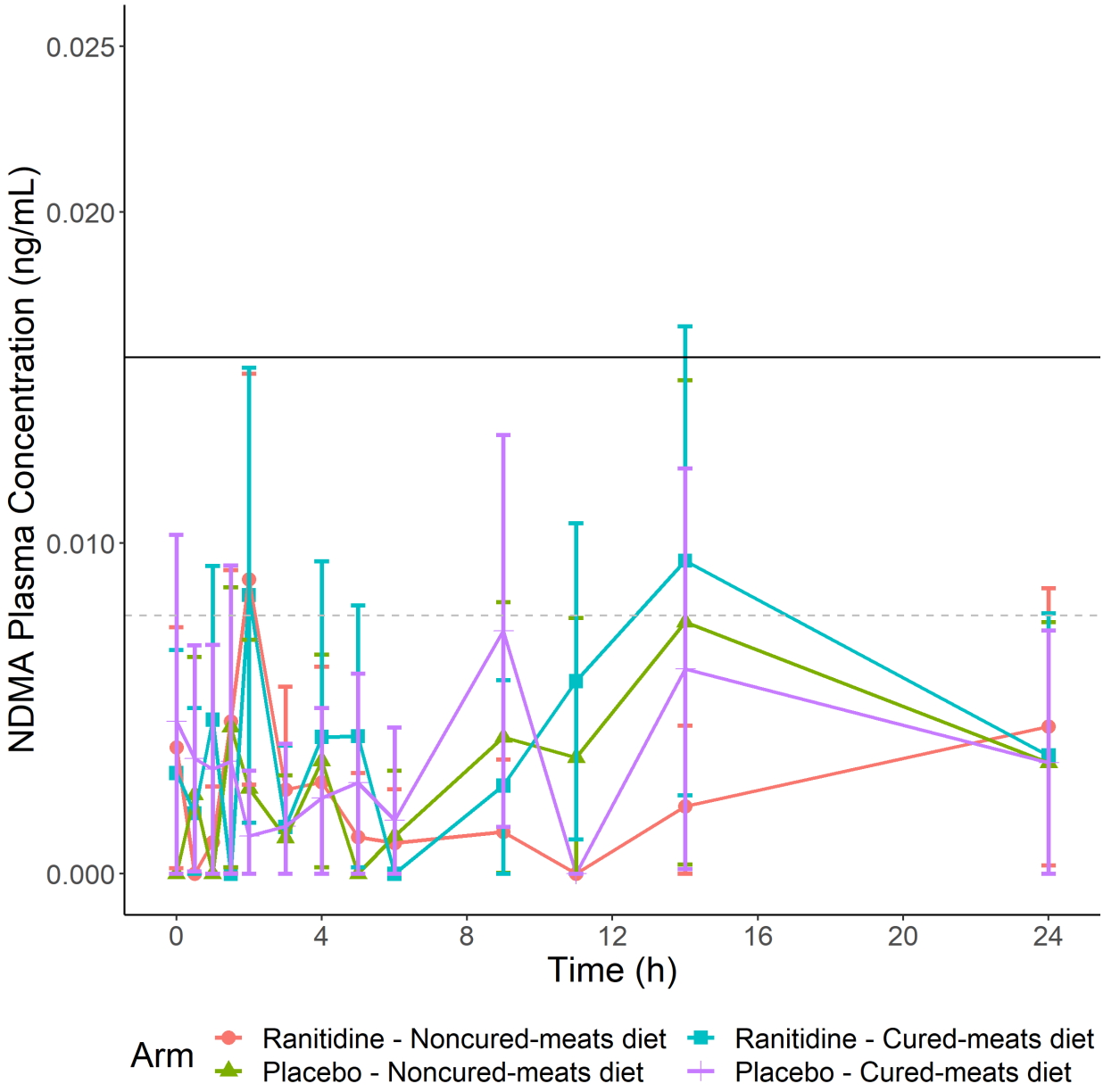
Current Study	Zeng and Mitch 2016[4,5]
Bioanalytical Methods	
• Sample analysis utilized liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) for all analytes (NDMA, DMA, and ranitidine). • Used direct quantitative methods and specific and selective LC-MS/MS detection for determining NDMA concentration.[6]	• NDMA sample analysis utilized gas chromatography/ion trap mass spectrometry (GS/ITMS). • DMA and ranitidine sample analysis utilized liquid chromatography/mass spectrometry (LC-MS/MS). • Used an indirect derivatization method for NDMA analysis (*N,N*-dimethyl-2,3,4,5,6-pentafluorobenzamide quantified).
Study Findings	
• Median (interquartile range [IQR]) 24-hr urinary excretion of NDMA with the noncured-meats diet was 0.6 ng (IQR, 0 to 29.7) for ranitidine compared to 10.5 ng (IQR, 0 to 17.8) for placebo. • With the cured-meats diet, 24-hr urinary excretion of NDMA was 11.9 ng (IQR, 5.6 to 48.6) with ranitidine compared to 23.4 ng (IQR, 8.6 to 36.7) for placebo.	• Following ranitidine intake, the urinary NDMA excreted over 24-hr increased 400-fold from 110 ng to 47,600 ng.
• With the noncured-meats diet, the geometric mean 24-hour urinary excretion of DMA was 38.8 mg (coefficient of variation [CV], 38%) for ranitidine compared to 41.1 mg (CV, 33%) for placebo. • With cured-meats diet, the geometric mean 24-hour urinary excretion of DMA was 40.7 mg (CV, 52%) with ranitidine compared to 43.1 mg (CV, 34%) for placebo.	• 24-hour urinary excretion of DMA was 9.7 ± 2.8 mg prior to ranitidine intake and 24.9 ± 5.2 mg after 150 mg ranitidine. This difference (approximately 13.4 mg higher after ranitidine) would account for 93.4 mg of the ranitidine product on a per mole basis.
• After ranitidine intake, the 24-hour urinary excretion was 29% and 39% of the 300 mg dose when taken with the noncured-meats and cured-meats diet, respectively.	• After ranitidine intake, the 24-h excreted mass of ranitidine accounted for 33±12% of the 150 mg dose.
• The total amount of ranitidine in urine from all sources, when accounting for 50% bioavailability, does not exceed the total ranitidine dose administered.	• The detected amount of ranitidine and DMA in urine accounted for 143.4 mg of the 150 mg ranitidine dose (note that each ranitidine molecule only has one DMA group, which has a lower molar mass than ranitidine). Ranitidine metabolites (ranitidine N-oxide, ranitidine S-oxide, and desmethyl ranitidine) accounted for approximately 9.4% (14.1 mg) of the 150 mg ranitidine dose in urine. These totals exceed the total amount of ranitidine administered. In addition, oral ranitidine only has 50% bioavailability.[7]

eFigure 1. Individual participant cumulative 24-hr urine excretion profiles of NDMA by group



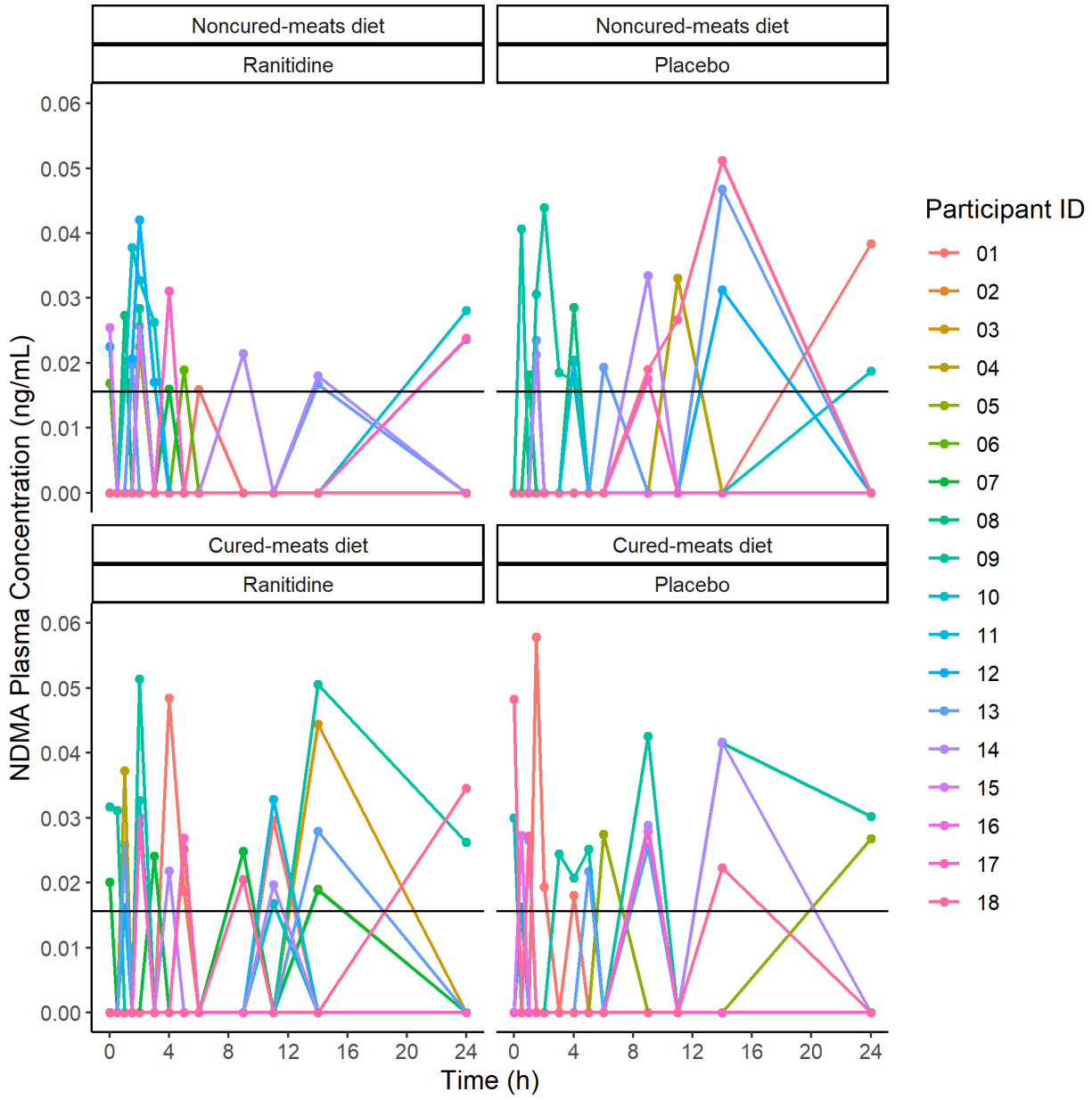
Cumulative NDMA 24-hr urine excretion profiles for each participant by group. Profiles for participant 03 and 12 where cumulative NDMA amounts exceeded 100 ng are labeled on the plot. Additional information on participant 12, including observations from the clinical site, are summarized in eTable 6.

eFigure 2. Arithmetic mean NDMA plasma concentration over time by group



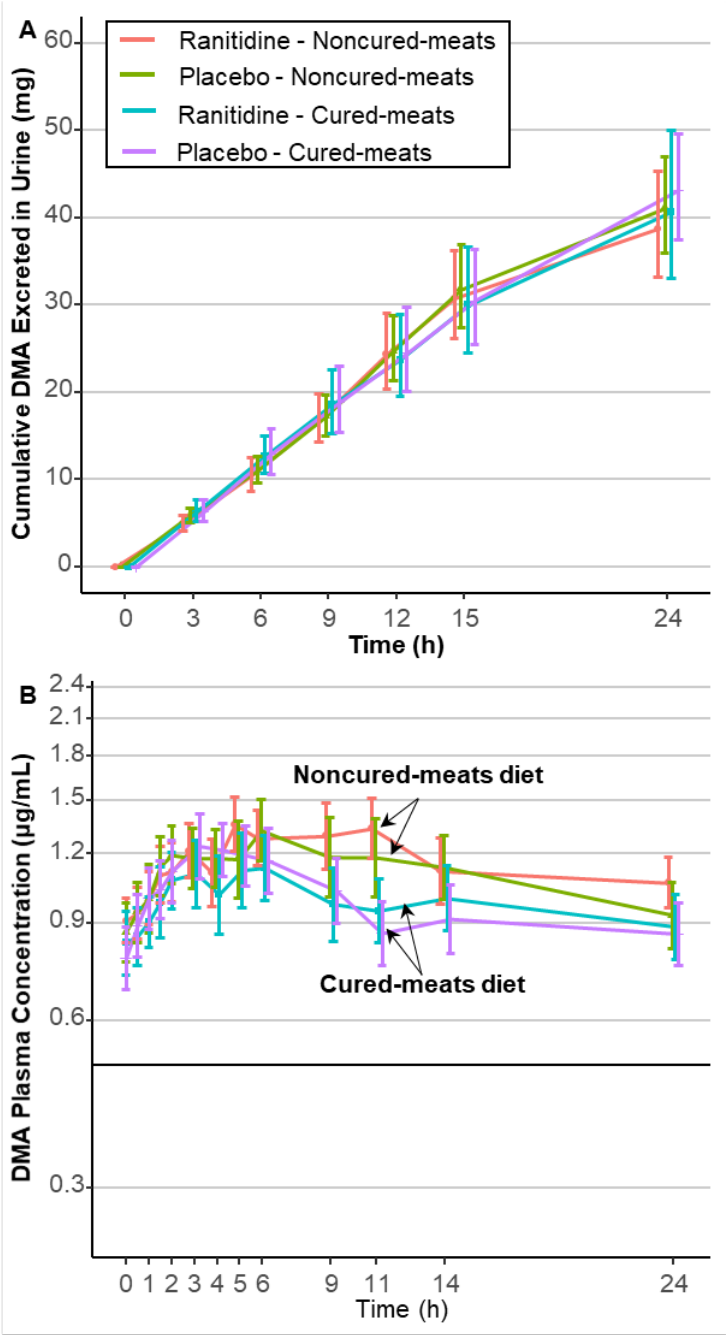
Arithmetic mean (90% confidence interval) NDMA plasma concentration over time by group. The lower limit of quantitation (LLOQ) for NDMA in plasma was 0.0156 ng/mL (horizontal black line). The lower limit of detection for NDMA was 0.0078 ng/mL (horizontal gray dashed line).

eFigure 3. Individual participant NDMA plasma concentration profiles by group



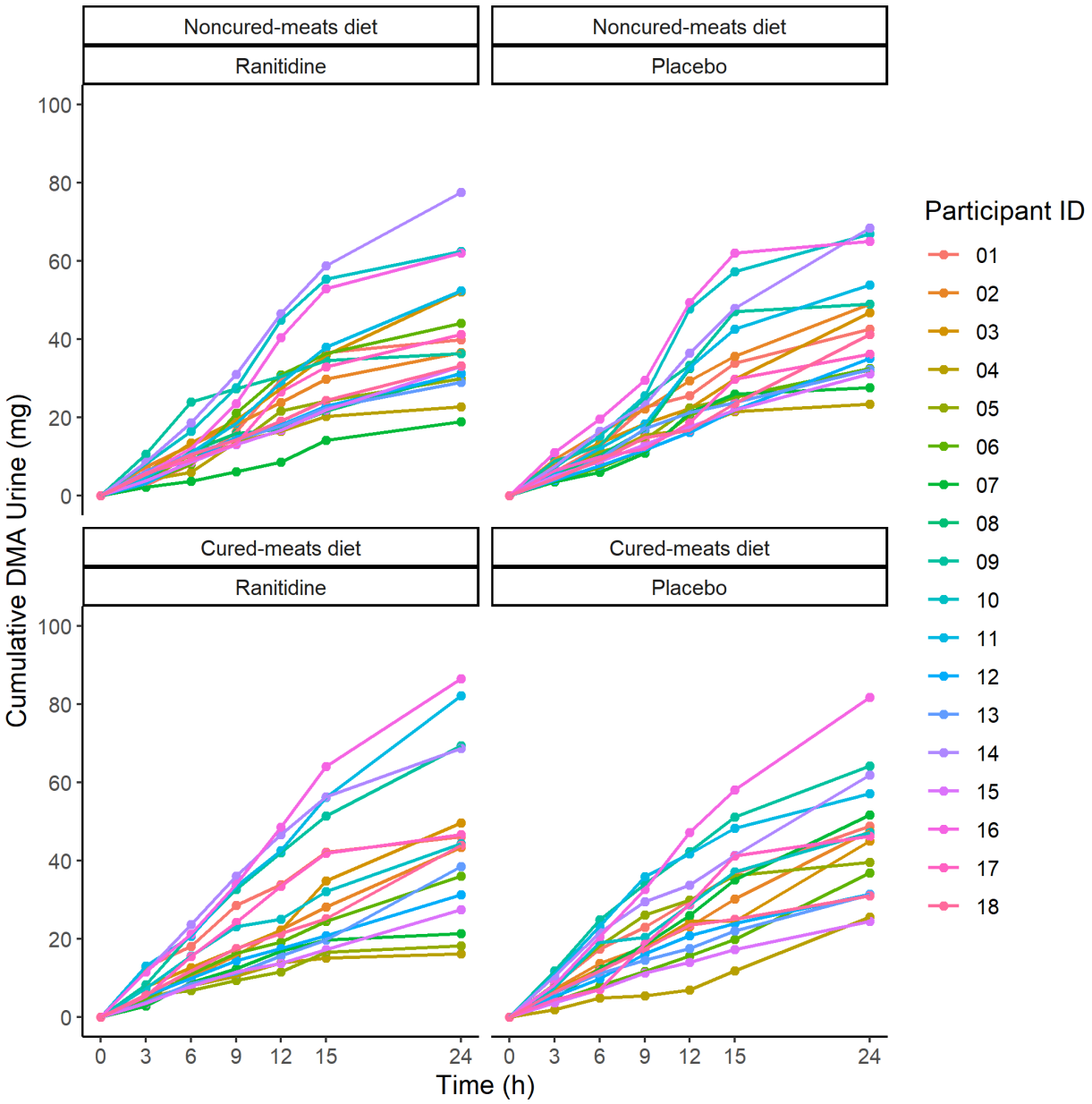
NDMA plasma pharmacokinetic profile for each participant grouped by group. The lower limit of quantitation (LLOQ) for NDMA in plasma was 0.0156 pg/mL (horizontal black line). Values below the LLOQ were set to 0 for plotting.

eFigure 4. Cumulative DMA excreted in urine over 24-hours on a linear scale and DMA plasma concentration on a log scale by group



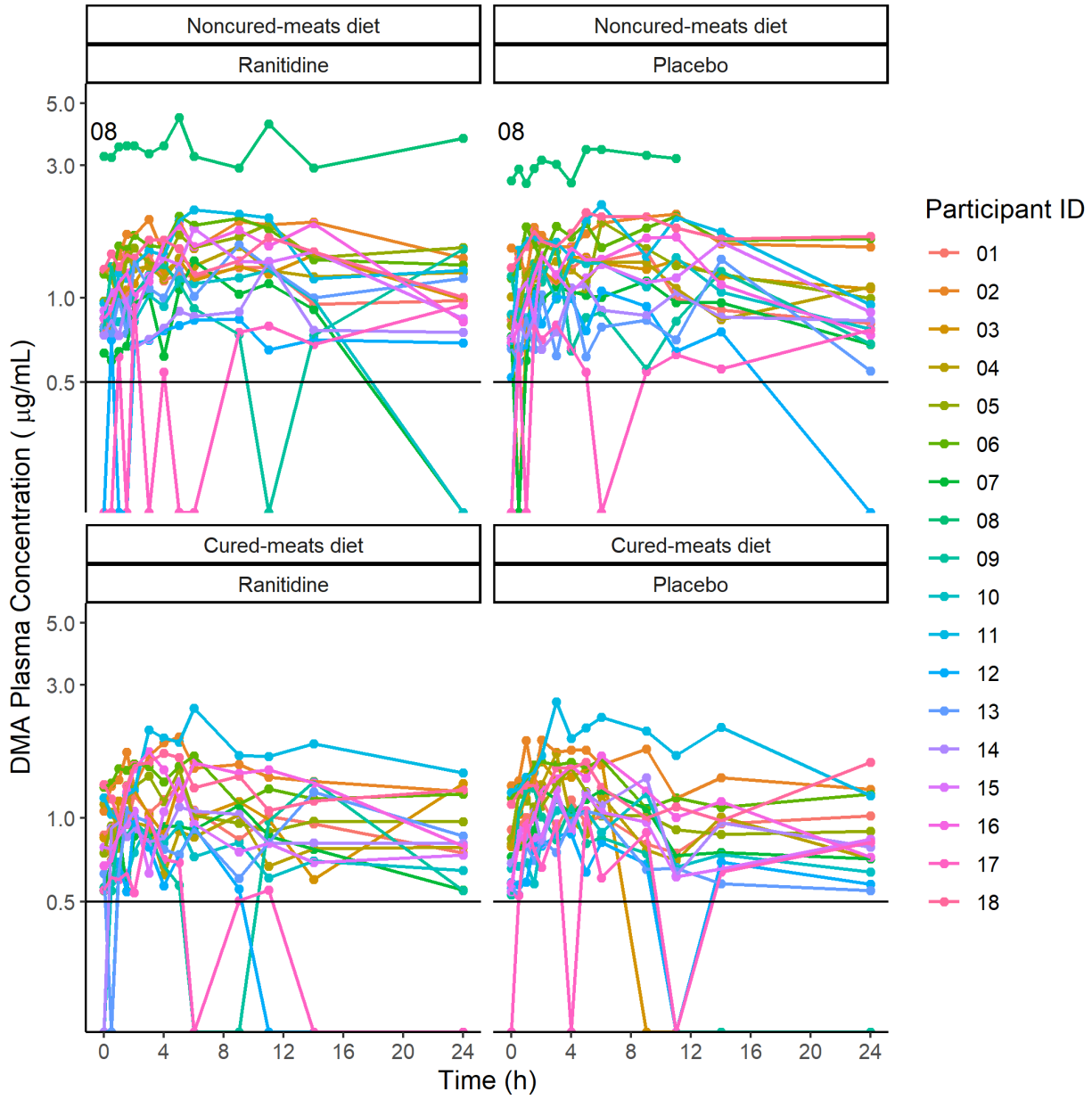
Data presented in panels A and B are the cumulative DMA excreted in urine over 24-hours and the DMA plasma concentration, respectively, for each of the four groups. Each point with accompanying error bars represents the geometric mean with 90% confidence intervals. Each data point in panel A is calculated as the cumulative amount of DMA that was excreted in urine up to that time point. Panel A is shown on a linear scale and panel B is shown on a log-scale. The lower limit of quantification for DMA was 0.500 µg/mL and is shown on panel B as a horizontal black line.

eFigure 5. Individual participant cumulative 24-hr urine excretion profiles of DMA by group



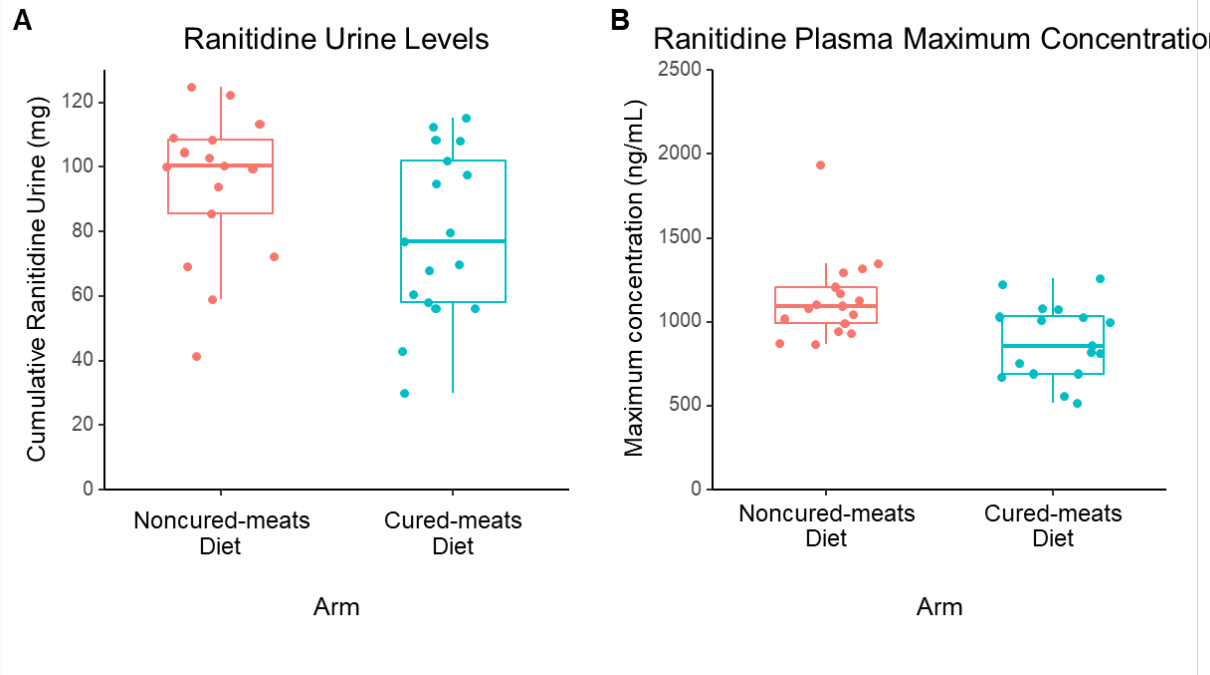
Cumulative DMA 24-hr urine excretion profiles for each participant by group.

eFigure 6. Individual participant DMA plasma concentration profiles by group



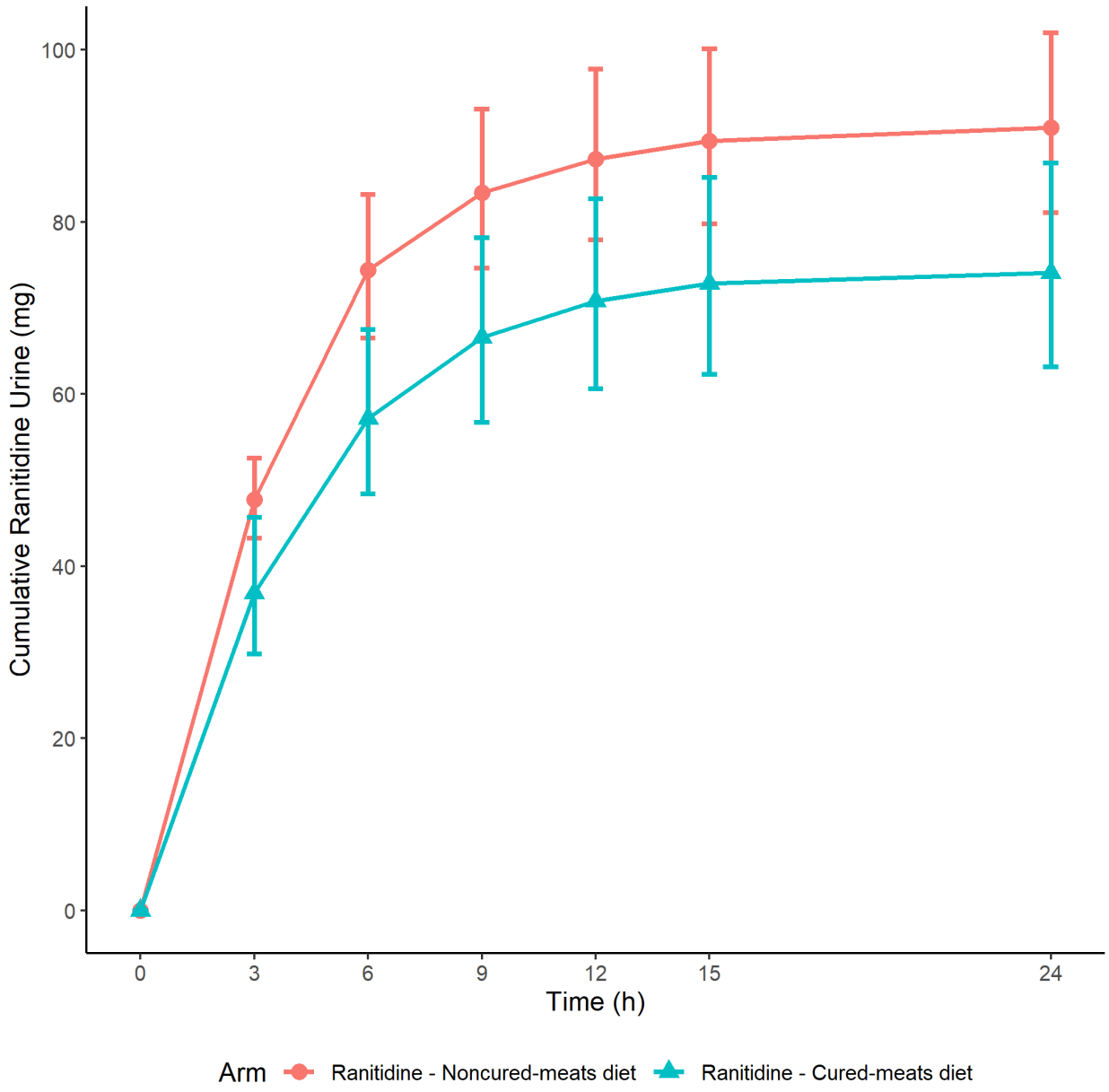
DMA plasma pharmacokinetic profile for each participant by group on a log scale. The lower limit of quantitation (LLOQ) for DMA in plasma was 0.50 µg/mL (horizontal black line). Values below the LLOQ were plotted on the x-axis. Profiles for participant 08, who discontinued during period 2, are labeled on the plot.

eFigure 7. 24-hour urinary excretion and maximum plasma concentration of ranitidine by group



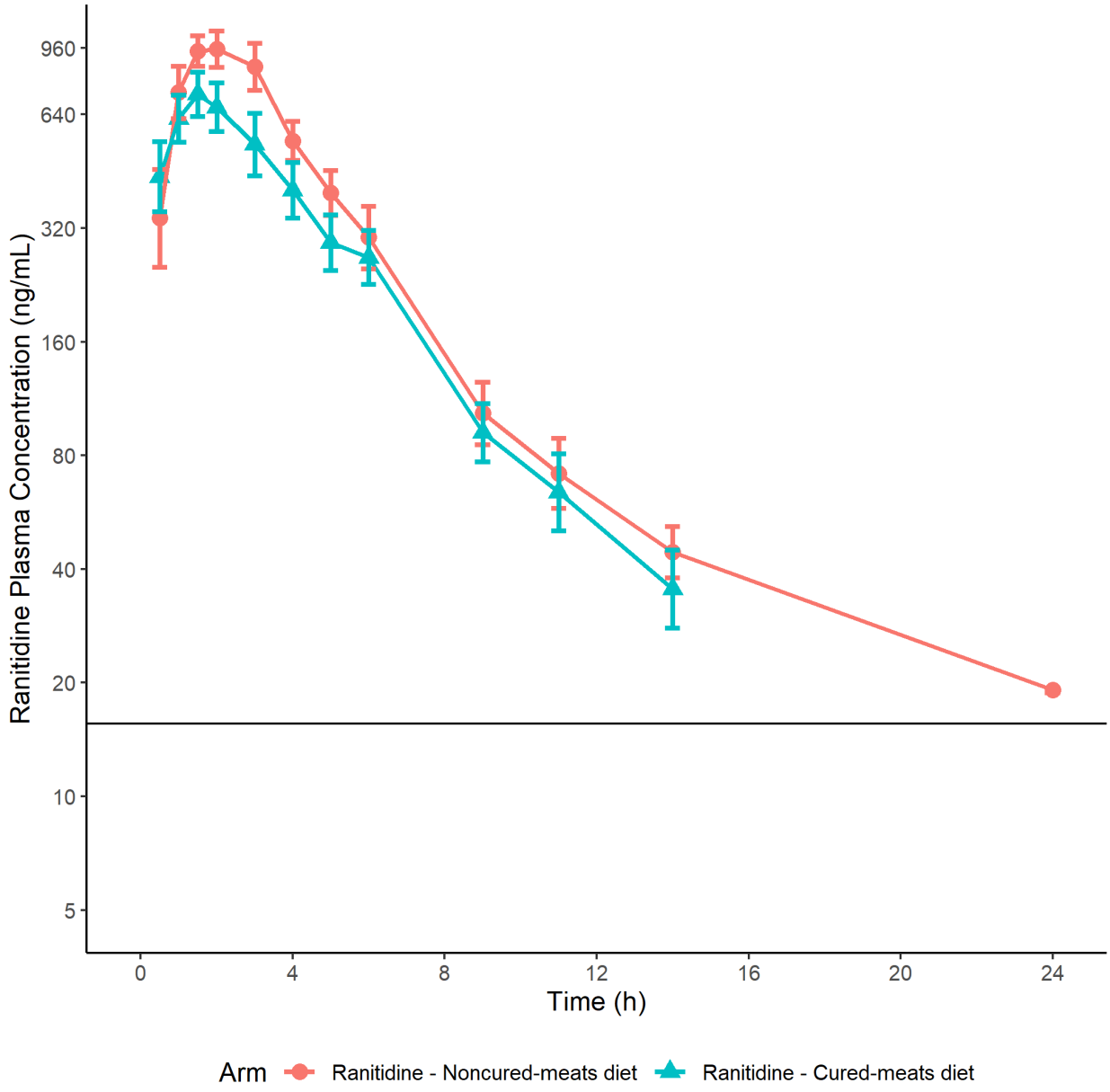
Individual-participant observed data (n=17) and box-and-whisker plot summaries. Panels A and B contain ranitidine 24-hour urinary excretion and maximum plasma concentration data from each of the four groups. The line through the box represents the median. The lower and upper borders of the box represent the 25th and 75th percentile, respectively. The whisker extends from the box border to the last observation within 1.5 times the interquartile range.

eFigure 8. Geometric mean ranitidine 24-hour urinary excretion by diet



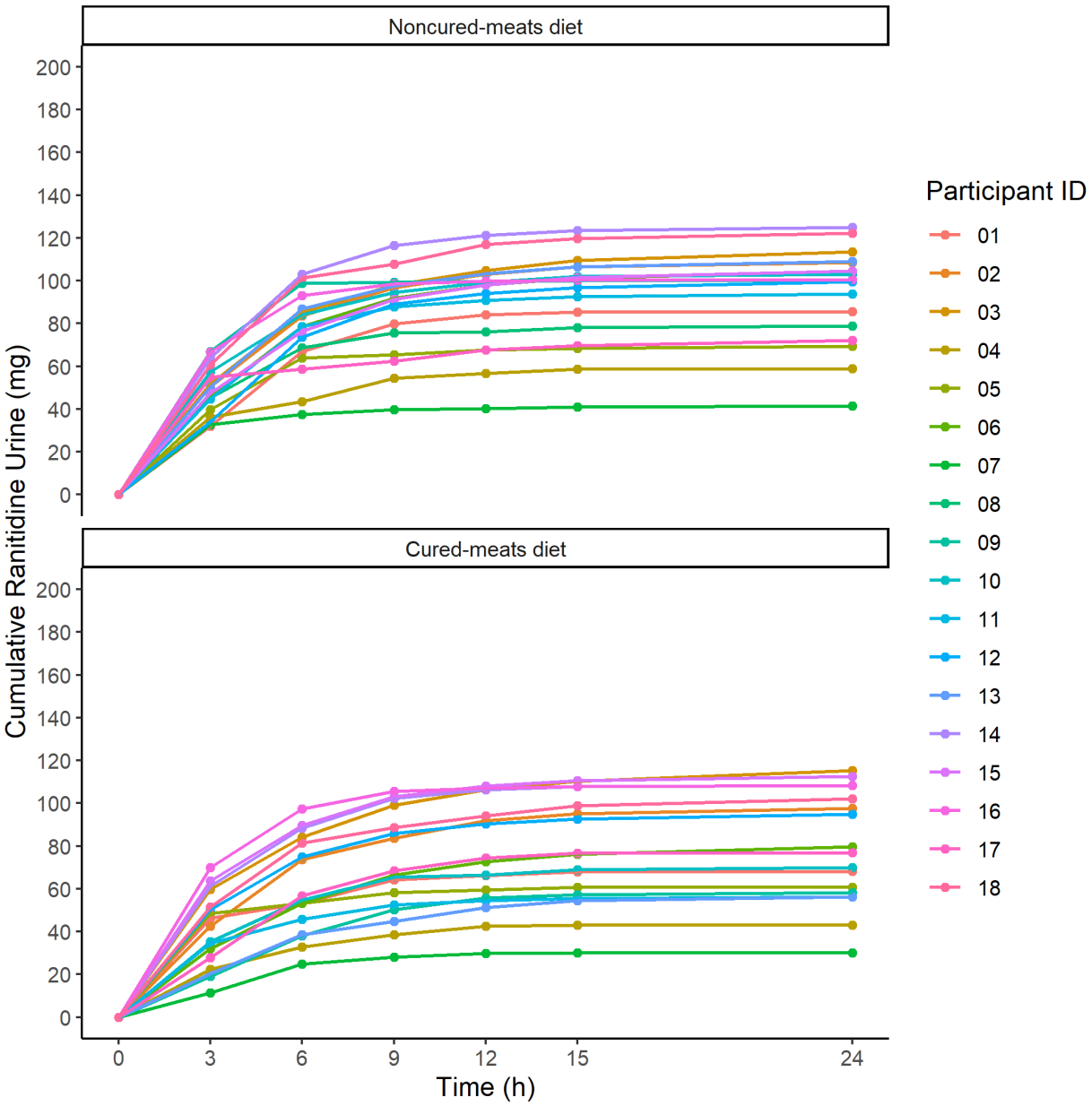
Geometric mean (90% confidence interval) ranitidine 24-urinary excretion over time by diet. Only two of the groups included ranitidine for determining cumulative ranitidine excretion.

eFigure 9. Geometric mean ranitidine plasma concentration over time by diet



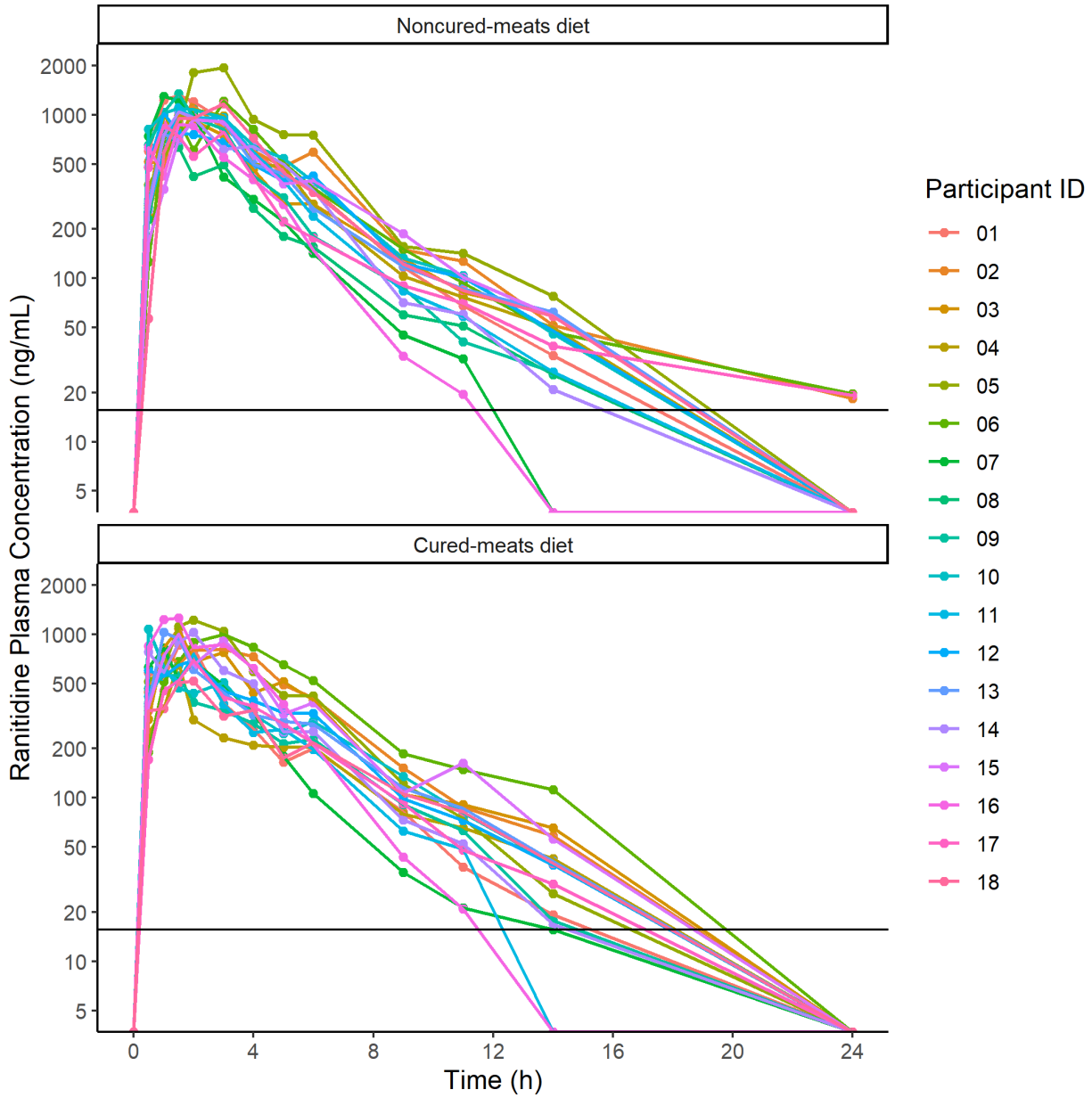
Geometric mean (90% confidence interval) ranitidine plasma concentration over time by diet on a log scale. Only two of the treatment groups included ranitidine for determining plasma concentration. The lower limit of quantitation (LLOQ) for ranitidine in plasma was 15.6 ng/mL (horizontal black line).

eFigure 10. Individual participant cumulative 24-hr urine excretion profiles of ranitidine by diet



Cumulative ranitidine 24-hr urine excretion profiles for each participant grouped by treatment. Only two treatment groups (ranitidine/noncured-meats diet and ranitidine/cured-meats diet) contained ranitidine for calculating urine excretion time course.

eFigure 11. Individual participant ranitidine plasma concentration profiles by diet



Ranitidine plasma pharmacokinetic profile for each participant by diet on a log scale. The lower limit of quantitation (LLOQ) for ranitidine in plasma was 15.6 ng/mL (horizontal black line). Values below the LLOQ were set to 0 ng/mL for plotting.

eMethods 1. Selection of Meals for Study and Full Menu Listing

This randomized, placebo-controlled, crossover study included periods where participants were fed diets containing foods associated with higher or lower nitrite, nitrate, and NDMA content. As nitrites can be produced endogenously from nitrates, considerations were made, when possible, to select items associated with higher or lower nitrate content within each diet. The diets are referred to as noncured meats and cured meats diets.

All participants received identical standardized meals from check-in (day -2) to check out (day 9). The first two periods (noncured-meats diet) included the same standardized set of meals for the pretreatment and treatment days. Likewise, the last two periods (cured-meats diet) had a separate set of standardized meals for the pretreatment and treatment days. The order of periods for the noncured-meats and cured-meats diets was not randomized (i.e., noncured-meats diets were always served in the first two periods) to reduce potential differences in nitrite, nitrate, and NDMA content from needing to repurchase the same fresh ingredients.

In designing noncured-meats and cured-meats diets for this study, the following steps were taken to reduce overall variability:

- To standardize exposure to nitrites, nitrates, and NDMA, participants were introduced to the standardized diet from the date of check-in (day -2). They were only allowed to eat and drink what was given to them and were encouraged to finish all meals. Leftover food was photographed, and details were noted.
- As NDMA and nitrites are found in drinking water, participants were given distilled water to drink throughout the study. The only exception being during the cured-meats diet where participants were given tap water to drink to increase dietary intake of NDMA and nitrites in that group.
- Only organic vegetable and salad ingredients (as nitrate containing fertilizers are not used in organic farming) were used in noncured-meats diet and opposite was true for the cured-meats diet.
- Only cured meats (as nitrates/nitrites are used in the curing process) were used in cured-meats diet and the opposite was true for the noncured-meats diet.
- To maximize the intake of NDMA, nitrites, and nitrates in the cured-meats diet periods, participants were given generous servings of cured meats with every meal and snack.
- For both the cured-meats and noncured-meats diets all the food items were purchased or made together to avoid batch-to-batch variation in nitrites, nitrates, and NDMA in foods.

Food and drinks were chosen for the noncured-meats and cured-meats diet based on the available literature data (Table 1). A complete study menu used to inform foods chosen for the meals is shown below in Table 2.

eMethods 1 - Table 1. Literature references of nitrite, nitrate and NDMA content across study meal items

Item	Nitrites (ug/g)		Ref	Nitrates (ug/g)		Ref	NDMA (ug/kg)		Ref
	Mean	Range		Mean	Range		Mean	Range	
Category: Cured Meat									
Sausage Links	10.4[a,b] 30	0.4-29.3[a,b] <10-61	8 9	77.4[a,b] 56	0.81-540.8[a,b] <5-160	8 9	0.4	0.31-1.5 <0.15-0.4	10 9
Salami	108		11	142.5 98.5	108.9-178.8	12 11		0.04-2.1	10
Pepperoni	0.13[a,b]	0.05-0.36[a,b]	8	51.8[a,b]	1.7-250.9[a,b]	8			
Ham	8.9 34.2 29	 28.7-39.7 <10-43	13 12 9	9 19 50	 10.9-27.1 <5-98	13 12 9	2.3	0.18-0.23 <0.15-3.1	10 9
Bacon	3.8 15.7 7.4[a,b]	 1.2-30.2 3.7-13.0[a,b]	13 12 8	55 23.3 15.4[a,b]	 15.1-31.5 3.4-24.4[a,b]	13 12 8		0.67-0.78	10
Corned Beef	7.8[a,b]	0.27-22.4[a,b]	8	17.4[a,b]	0.25-107.8[a,b]	8			
Hot Dog	0.5 78.6 27.1	 62.2-85	13 12 14	90 69.9 257	 58.6-81.2	13 12 14	2.8		14
Category: Noncured Meat									
Roast Beef	0		15	13.2	10.8- 15.0	15		0-0.48	10
Pork Chop	0		15	11.6	10.2 -14.8	15	0		10
Chicken Thighs	5.4		14	6.6		14	 0.97	0-0.52	10 14
Category: Conventional Vegetables and Fruits (Given with Cured-Meat Diet)									
Spinach	36.3[a] 0.2	0.04-137[a]	8 13	647.3[a] 7410 4849.6 2797	161.5-875.4[a] 4276-5423 65-8000	8 13 12 16		0-6[c]	10
Lettuce	0.33[a]	0.12-0.62[a]	8	206.9[a] 2720 1324 1074	79.4-424.5[a] 1410-4000 56-3660 21-3986	8 17 18 19		0-6[c]	10
Tomatoes	0.3 0.43		13 14	392 36 15 43	 1-144	13 20 21 18		0-6[c]	10
Coleslaw Mix	0.7		13	559		13		0-6[c]	10
V8 Juice	0.09		13	26.17		13			
Cabbage	0.27[a]	0.15–0.41[a]	8	474.9[a] 311 881	255.5–669.7[a] 47-833 112-1864	8 18 19		0-6[c]	10
Oranges (mandarin)	0.2 4.5	 2.1-6.9	13 22	8 132	 20-244	13 22		0-6.2[c]	10
Onion				59 164	 1-638	20 18		0-6[c]	10
Pomegranate Juice	0.06		13	12.93		13			
Strawberries	0		19	94	9-360	19		0-6.2[c]	10
Sport Pepper				41	0-92	23		0-0.42	10
Pickles	7.4	3.6-11.2	22	209 204	180-238 148.6-259.4	23 22		0-0.42	10

Item	Nitrites (ug/g)		Ref	Nitrates (ug/g)		Ref	NDMA (ug/kg)		Ref
	Mean	Range		Mean	Range		Mean	Range	
Category: Organic Vegetables and Fruits (Given with Noncured-Meat Diet)									
Tomato	0.3[d]		13	5 392[d] 43[d]	 1-144[d]	21 13 18		0-6[c,d]	10
Iceberg Lettuce	0.11[a]	0.05-0.27[a]	8	100.1[a] 875[d] 48[d]	57.5-158.5[a] 210-1537[d] 17.8-78.2[d]	8 18 12		0-6[c,d]	10
Carrots	0.39 0.06[d]		24 13	29.4 1[d]		24 13		0-6[c,d]	10
Tomato Soup				8.7		25			
Orange	0.2[d]		13	8[d]		13		0-6.2[c,d]	10
Mustard Greens	0.03[d]		13	1160[d]		13		0-6[c,d]	10
Banana	0.09[d]		13	45[d]		13		0-6.2[c,d]	10
Apple Sauce	0.08[d]		13	3[d]		13			
Kiwi				0[d]		20		0-6.2[c,d]	10
Strawberry				94[d]	9-360[d]	19		0-6.2[c,d]	10
Asparagus				209[d]	1-1459[d]	18		0-6[c,d]	10
Cucumbers	0.2[d]	0-0.8[d]	19	93[d]	4-245[d]	19		0-6[c,d]	10
Category: Other Products									
Cheese[e]	0.82 3.4	 1.9-5.3	14 22	14.2 29.6	 0-66.8	14 22	 2.3	0-0.72	10 14
Nuts[e]	0.33		14	5.7		14	0		14
Whole Milk[e]	0.004		14	0.86		14	0.27		14
Bun[e]	 3.9	0-0.4 0.6-7.2	26 22	 513	7.2-8 298-728	26 22		0-0.98	10
Sub Roll[e]	 3.9	0-0.4 0.6-7.2	26 22	 513	7.2-8 298-728	26 22		0-0.98	10
French Fries[e]	1.7 0.69		13 14	20 37		13 14	0		14
Ketchup[e]	1.3		13	1		13			
Vegetable Oil[e]								0-2.3 1-23	10 27
Almonds[e]	1.7	1.5-1.9	22	285	75-495	22			
Feta Cheese[e]	0		28	3.5		28		0-7	29
Milk 2%	0.04		14	3.5		14	0.85		14
Macaroni & Cheese	0.18		25	4.8		25			
White Rice	2.16		14	16.4		14			
Ice cream (vanilla)	0.12 0.5		25 14	5 2.9		25 14		0-0.28	10
Cheddar Cheese	0		28	3.5		28		9-20	29
Provolone	0		28	3.5		28	9		29
Rice Cake								0-0.8	10
White Bread[f]	 1.6 3.9	0 -0.4 0.6-7.2	26 14 22	 16.4 513	7.2-8 298-728	26 14 22		0-0.98 0.08	10 14
Eggs[f]		0.15-0.18	30		5.1-5.5	30			
Butter[f]	0		14	0		14	0		14
Tap Water[e]					0-3.5	8		0-0.02 0-0.02	31 32
Food Items without Literature References									
Beef Jerky, Relish, Horseradish, Orange Juice, Apple Juice, Mayonnaise[e], Italian Dressing[e], Coleslaw Dressing[e], Vinegar[e], Balsamic Vinaigrette[e], Distilled water									

Ref: Reference
[a]Values obtained from Chicago region in Bryan et al, 2009[8] as the clinical study site of this study is in West Bend, WI (approximately 75 miles north of the Illinois/Wisconsin border and Chicago region)
[b]Also published by Nunez et al, 2012[16]
[c]General range given in Park et al, 2015[10] for all vegetables and fruits
[d]References do not specify whether the vegetables and fruits used for analysis were organic or conventional
[e]Given with cured-meats diet
[f]Common food items given in both cured-meat and non-cured meat diets

eMethods 1 - Table 2. Full menu listing[a]

Day -2		Noncured-meats diet[b,c]				Cured-meats diet[b,d]			
		Pre-dose Day		Dosing Day		Pre-dose Day		Dosing Day	
Meal items	**Amount**	**Meal items**	**Amount**	**Meal items**	**Amount**	**Meal items**	**Amount**	**Meal items**	**Amount**
Breakfast									
Honey Nut Cheerios	1 oz	Eggs (scrambled)	2	Eggs (scrambled)	2	Eggs (fried)	2	Eggs (fried)	2
2% Milk	8 oz	Kiwi	¼ cup	Kiwi	¼ cup	Vegetable oil	1 tbsp	Vegetable Oil	1 tsp
Apple Juice	6.75 fl oz	Strawberries	¼ cup	Strawberries	¼ cup	Sausage links	4	Sausage Links	4
		White Bread	2 slices	White Bread	2 slices	White Bread	2 slices	White Bread	2 slices
		Butter	1 tbsp	Butter	2 tbsp	Butter	1 tbsp	Butter	1 tbsp
		Orange Juice	8 fl oz	Orange Juice	8 fl oz	Whole Milk	8 oz	Whole Milk	8 fl oz
Lunch									
Flour Tortillas	2 (6 inch)	Macaroni & Cheese	8 oz	Roast Beef	4 oz	Bacon	6 strips	Salami	2 oz
Chicken Breast	4 oz	Apple Juice	6.75 fl oz	White Bread	2 slices	White Bread	2 slices	Pepperoni	2 oz
Monterey Cheese	4 oz	Tomato Soup	8 oz	Tomatoes	2 slices	Tomato	¼ cup	Ham	2 oz
Enchilada Sauce	2 fl oz			Iceberg Lettuce	1 cup	Lettuce	1 cup	Bacon	2 oz
Iceberg Lettuce	1 cup			Horseradish	½ tsp	Spinach	1 ½ cup	Sub Roll	1
Corn	¼ cup			Cheddar Cheese	1 slice	Feta Cheese	¼ cup	Provolone	2 slices
Black Beans	¼ cup			Carrots	½ cup	Almonds	¼ cup	Spinach	1 cup
Tomatoes	¼ cup			Apple Juice	6.75 fl oz	Strawberries	¼ cup	Tomatoes	4 slices
Ranch	1 fl oz					Pomegranate Juice	8 oz	Mayo	1 tbsp
Vegetable oil	1 tsp					Balsamic Dressing	1 fl oz	Coleslaw Mix	4 oz
Lemon Lime Soda	8 fl oz							Coleslaw Dressing	1 tbsp
								V8 juice	8 fl oz
Dinner									
Meat balls	5 (1 oz)	Chicken Thighs	6 oz	Pork Chop	5 oz	Hot Dog (Jumbo/Large)	1	Corned Beef	8 oz
Potatoes	1 cup	White Rice	1 cup	Asparagus	½ cup	Bun	1	Cabbage	1 cup
Gravy	2 fl oz	Milk 2%	8 fl oz	Mustard Greens	1 ½ cup	Onion	1 tbsp	Vinegar	1 tbsp
Milk 2%	8 fl oz			Tomatoes	¼ cup	Relish	1 tbsp	Sugar	1 tbsp
				Cucumbers	¼ cup	Sport Pepper	2	Salt	1 tsp
				Carrots	¼ cup	Tomatoes	2 wedges		
				Apple sauce	1 cup	Pickle	1 spear		
				Milk 2%	8 fl oz	Yellow Mustard	0.2 oz		
				Butter	1 tsp	French Fries	1 oz		
						Ketchup	0.2 oz		
						Salt	½ tsp		

Day -2		Non-cured meats diet				Cured-meats diet			
		Pre-dose Day		Dosing Day		Pre-dose Day		Dosing Day	
Meal items	Amount	Meal items	Amount	Meal items	Amount	Meal items	Amount	Meal items	Amount
Snack									
Chex Mix	1.75 oz	Rice Cake	9 g	Ice cream (vanilla)	4 oz	Beef jerky	3 oz	Spinach	1 cup
Banana	1	Banana	1	Orange	1	Cheese	2 oz	Strawberries	¼ cup
						Nuts	2 oz	Oranges (mandarin)	¼ cup
								Italian Dressing	1 oz
								Beef jerky	¼ cup

fl oz: fluid ounces; g: grams; oz: ounces; tbsp: tablespoon; tsp: teaspoon

[a]Side-by-side listing of menu items from the noncured-meats/organic-vegetables and cured-meats/conventional-vegetables diet (referred to as noncured-meats and cured-meats diets, respectively)

[b]Distilled water was served throughout the study except tap water was served during meals on the cured-meats diet

[c]Organic vegetables and fruits were served with the noncured-meats diet

[d]Conventional vegetables and fruits were served with the cured-meats diet

eMethods 2. Bioanalytical Methods for the Analytes and Biospecimens in the Clinical Study

Sample analysis utilized liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). All the assays were developed with the aim of high sensitivity with reliable quantitation. While optimizing the lower limit of quantitation (LLOQ) of each analyte, various parameters like signal to noise, accuracy, and precision were evaluated. The upper limit of quantitation (ULOQ) was selected based on the concentration at which acceptable linearity range was observed (*i.e.*, concentration vs linear detector response). The details of individual methods and validation parameters are described below. The present study used analytical methods that were validated per FDA's Bioanalytical Method Validation Guidance for Industry.[6]

Bioanalytical Method Conditions for NDMA Urine Method

Validation range: 15.6–2000.0 pg/mL
High Performance Liquid Chromatography (HPLC) instrument: Agilent 1290 Infinity
Mass Spectrometer: AB SCIEX 6500^{+} Qtrap Mass spectrometer

HPLC Conditions
Mobile Phase: 10 mM ammonium formate with 0.1% formic acid in water and acetonitrile (Gradient flow)
Flow rate: 0.4 mL/min
Column: Kinetex Biphenyl 100 Å (2.1 x 50 mm) 5.0 µm
Injection Volume: 20 µL
Retention Time: NDMA: 1.12 minutes
NDMA-d_6: 1.12 minutes
Run Time: 3.5 minutes

Mass Spectrometer conditions
Ionization Source and scan type: Atmospheric Pressure Chemical Ionization (APCI)
Multiple Reaction Monitoring (MRM)
Data acquisition: Analyst 1.7.1

AB SCIEX 6500^{+} Q-TRAP mass spectrometer state file parameters:

Parameter	Positive mode	
	NDMA	**NDMA-d_6**
Q1 mass/Q3 mass (amu)	75.1/43.1	81/46
Collision Energy (V)	19	21
Declustering Potential	90	60
Entrance Potential	10	8
Collision Cell Exit Potential	10	10
Source Temperature (°C):	350	350
Ion Source Gas 1	90	90
Curtain Gas Flow	30	30
Collision Gas Flow	8	8
Nebulizer Current	5	5
Dwell time (ms)	150	150

Sample Preparation: Sample preparation involved the extraction of NDMA from the samples using liquid-liquid extraction (LLE). First, 2.0 mL of surrogate/urine sample (calibration control [CC] or quality control [QC]) and 10 µl of internal standard (100.0 ng/mL of NDMA-d6 in 10mM ammonium formate with 0.1% formic acid) were mixed. After vortexing, 150 µl of extraction additive (1N NaOH) was added. Later, 3 ml of MTBE (methyl tert-butyl ether) was added to each sample followed by 30 minutes of vigorous mixing at 2500 rpm for 30 minutes. After mixing, samples were left to sit for 5 minutes for phase separation. About 2600 uL of supernatant MTBE was transferred to a tube containing 250 ul of MilliQ water. Samples were then evaporated with a vacuum centrifuge at 30°C until the volume condensed to 250 ul (~ 10 minutes). The eluted sample (20 µl) was injected onto the mass spectrometer.

Validation Summary Table for NDMA Urine Method

Description	NDMA
Short description of method	Liquid-Liquid extraction with reverse-phase HPLC with MS/MS detection
Matrix	Human urine and surrogate (water)
Analyte	N-Nitrosodimethylamine (NDMA)
Internal standard (IS)	NDMA-d_6 (deuterated NDMA)
Calibration concentrations	15.6 pg/mL to 2000.0 pg/mL
QC concentrations	Surrogate: 15.6 pg/mL (LLOQ), 30.0 pg/mL (LQC), 800.0 pg/mL (MQC1), and 1600.0 pg/mL (HQC) Urine: 250.0 pg/mL (UQC1) and 1000.0 pg/mL (UQC2)
Selectivity	Interference was observed in some lots of human urine. Thus, water was used as surrogate matrix (surrogate).
Specificity	No interference observed
Lower limit of quantification	15.6 pg/mL Between-run accuracy 101.5% (surrogate) Between-run precision 11.7% (surrogate) Within-run accuracy 100.8%-102.8% (surrogate) Within-run precision 9.0%-14.4% (surrogate)
Between-run accuracy	Surrogate: 99.2%-100.3% Urine: 101.5%-109.4%
Between-run precision	Surrogate: 4.5%-13.8% Urine: 10.3%-12.3%
Within-run accuracy	Batch 1: 91.7%-112.8% (urine); 100.1%-113.8% (surrogate) Batch 2: 102.8%-105.6% (urine); 91.2%-99.5% (surrogate) Batch 3: 100.6%-111.0% (urine); 87.5%-107.5% (surrogate)
Within-run precision	Batch 1: 3.7%-5.8% (urine); 2.7%-9.6% (surrogate) Batch 2: 4.0%-4.4% (urine); 2.5%-9.1% (surrogate) Batch 3: 1.7%-4.9% (urine); 2.0%-5.4% (surrogate)
Matrix effect (IS-normalized)	LQC: 0.89 (5.7 %CV) (surrogate) HQC: 0.88 (1.1 %CV) (surrogate)
Recovery of analyte	70.4% -90.2% (urine)
Recovery of IS	15.7% (urine)
Auto-sampler storage stability	Confirmed up to 24 hours at 10°C nominal Accuracy 109.1% for UQC1 and 102.1% for UQC2 in urine Accuracy 105.7% for LQC and 102.2% for HQC in surrogate
Freeze-thaw stability	Confirmed up to 8 cycles Accuracy 98.2% for UQC1 and 90.2% for UQC2 in urine
Bench top stability	Confirmed up to 24 hours at room temperature Accuracy 101.9% for UQC1 and 114.1% for UQC2 in urine Accuracy 106.5% for LQC and 102.7% for HQC in surrogate
Injector Carryover	Not significant (0.0%)
Long Term Storage Stability	Confirmed up to 211 days at -80°C Accuracy 94.3% for UQC1 and 89.0% for UQC2 in urine

Bioanalytical Method Conditions for NDMA Plasma Method

Validation range:	15.6–2000.0 pg/mL
High Performance Liquid Chromatography (HPLC) instrument:	Agilent 1290 Infinity
Mass Spectrometer:	AB SCIEX 6500^{+} Qtrap Mass spectrometer

HPLC Conditions

Mobile Phase:	10 mM ammonium formate with 0.1% formic acid in water and acetonitrile (Gradient flow)
Flow rate:	0.4 mL/min
Column:	Kinetex Biphenyl 100 Å (2.1 x 50 mm) 5.0 µm
Injection Volume:	20 µL
Retention Time:	NDMA: 1.12 minutes NDMA-d_6: 1.12 minutes
Run Time:	3.5 minutes

Mass Spectrometer conditions

Ionization Source and scan type:	Atmospheric Pressure Chemical Ionization (APCI) Multiple Reaction Monitoring (MRM)
Data acquisition:	Analyst 1.7.1

AB SCIEX 6500^{+} Q-TRAP mass spectrometer state file parameters:

Parameter	Positive mode	
	NDMA	NDMA-d_6
Q1 mass/Q3 mass (amu)	75.1/43.1	81/46
Collision Energy (V)	19	21
Declustering Potential	90	60
Entrance Potential	10	8
Collision Cell Exit Potential	10	10
Source Temperature (°C):	350	350
Ion Source Gas 1	90	90
Curtain Gas Flow	30	30
Collision Gas Flow	8	8
Nebulizer Current	5	5
Dwell time (ms)	150	150

Sample Preparation: Sample preparation involved the extraction of NDMA from the samples using liquid-liquid extraction (LLE). First, 0.5 ml of surrogate/urine sample (CC or QC) and 10 µl of internal standard (100.0 ng/mL of NDMA-d6 in 10mM ammonium formate with 0.1% formic acid) was mixed. After vortexing, 100 µl of extraction additive (1N NaOH) was added. Later, 2 ml of MTBE (methyl tert-butyl ether) was added to each sample followed by 30 minutes of vigorous mixing at 2500 rpm for 30 minutes. After mixing, the samples were left to sit for 5 minutes for phase separation. About 1700 uL of supernatant MTBE was transferred to a tube containing 200 ul of MilliQ water. Samples were then evaporated with a vacuum centrifuge at 30°C until the volume condensed to 200 ul (~ 10 minutes). The eluted sample (20 µl) was injected onto the mass spectrometer.

Validation Summary Table for NDMA Plasma Method

Description	**NDMA**
Short description of method	Liquid-Liquid extraction with reverse-phase HPLC with MS/MS detection
Matrix	Human plasma and surrogate (5% bovine serum albumin, BSA)
Analyte	N-Nitrosodimethylamine (NDMA)
Internal standard (IS)	NDMA-d_6 (deuterated NDMA)
Calibration concentrations	15.6 pg/mL to 2000.0 pg/mL
QC concentrations	Surrogate: 15.6 pg/mL (LLOQ), 46.8 pg/mL (LQC), 750.0 pg/mL (MQC1), and 1500.0 pg/mL (HQC) Plasma: 120.0 pg/mL (PQC1) and 400.0 pg/mL (PQC2)
Selectivity	Significant interference was observed in human plasma. Thus,5% BSA used as surrogate matrix (surrogate).
Specificity	No interference observed
Lower limit of quantification	15.6 pg/mL Between-run accuracy 109.4% (surrogate) Between-run precision 13.7% (surrogate) Within-run accuracy 105.4%-113.8% (surrogate) Within-run precision 12.4%-15.4% (surrogate)
Between-run accuracy	Surrogate:100.5%-109.4% Plasma: 97.3%-101.9%
Between-run precision	Surrogate: 5.0%-13.7% Plasma: 4.7%-6.1%
Within-run accuracy	Batch 1: 95.9%-96.7% (plasma); 97.6%-109.6% (surrogate) Batch 2: 94.3%-102.7% (plasma); 99.9%-108.8% (surrogate) Batch 3: 102.0%-106.4% (plasma); 103.9%-113.8% (surrogate)
Within-run precision	Batch 1: 3.7%-5.8% (plasma); 2.3%-15.4% (surrogate) Batch 2: 4.0%-4.4% (plasma); 3.7%-12.4% (surrogate) Batch 3: 1.7%-4.9% (plasma); 3.6%-15.2% (surrogate)
Matrix effect (IS-normalized)	LQC: 1.16 (2.4 %CV) (surrogate) HQC: 1.02 (1.7 %CV) (surrogate)
Recovery of analyte	40.0% -49.5% (plasma)
Recovery of IS	43.3% (plasma)
Auto-sampler storage stability	Confirmed up to 24 hours at 10°C nominal Accuracy 109.2% for PQC1 and 97.3% for PQC2 in plasma Accuracy 112.6% for LQC and 95.7% for HQC in surrogate
Freeze-thaw stability	Confirmed up to 8 cycles Accuracy 90.8% for PQC1 and 93.4% for PQC2 in plasma
Bench top stability	Confirmed up to 24 hours at room temperature Accuracy 108.4% for PQC1 and 101.3% for PQC2 in plasma Accuracy 107.7% for LQC and 108.3% for HQC in surrogate
Injector Carryover	Not significant (0.0%)
Long Term Storage Stability	Confirmed up to 211 days at -80°C Accuracy 97.7% for PQC1 and 90.5% for PQC2 in plasma

Bioanalytical Method Conditions for DMA Method (Common Method for Urine and Plasma)

Validation range:	500.0–128000.0 ng/mL
High Performance Liquid Chromatography (HPLC) instrument:	Agilent 1290 Infinity
Mass Spectrometer:	AB SCIEX 6500^{+} Qtrap Mass spectrometer

HPLC Conditions

Mobile Phase:	10 mM ammonium formate with 0.1% formic acid in water and acetonitrile (Gradient flow)
Flow rate:	0.3 mL/min
Column:	Kinetex XB, C18, 2.1*100 A, 100 X 2.1 mm
Injection Volume:	3 µL
Retention Time:	DMA: 0.88 minute DMA-d_3: 0.88 minute
Run Time:	2.4 minutes

Mass Spectrometer conditions

Ionization Source and scan type:	Atmospheric Pressure Chemical Ionization (APCI) Multiple Reaction Monitoring (MRM)
Data acquisition:	Analyst 1.7.1

AB SCIEX 6500^{+} Q-TRAP mass spectrometer state file parameters:

Parameter	Positive mode	
	DMA	**DMA-d_3**
Q1 mass/Q3 mass (amu)	46.2/30.1	49.2/32.2
Collision Energy (V)	26	26
Declustering Potential	125	100
Entrance Potential	4	7
Collision Cell Exit Potential	8	10
Source Temperature (°C):	550	550
Ion Source Gas 1	50	50
Curtain Gas Flow	40	40
Collision Gas Flow	Medium	Medium
Nebulizer Current	5	5
Dwell time (ms)	150	150

Sample Preparation: Sample preparation involved the extraction of DMA from the samples using protein precipitation/sample dilution. An aliquot of 120 µl of acetonitrile containing 0.66 µg/ml of dimethylamine-d3 (internal standard) was added to 96-well protein precipitation plates to which 5 µl of plasma or urine or surrogate sample was added. The samples were shaken at 700 rpm for 5 minutes, followed by the addition of 100 µl of surrogate sample and shaken at 350 rpm for 5 minutes. Later, the samples were centrifuged at 4000 rpm for 10 minutes with a collection plate at the bottom. The sample (3 µl) was injected into the mass spectrometer.

Validation Summary Table for DMA Method

Description	DMA
Short description of method	Protein Precipitation with reverse-phase HPLC with MS/MS detection
Matrix	Human plasma, Human urine and surrogate (0.05% phosphate buffer with 10% *v/v* methanol)
Analyte	Dimethylamine (DMA)
Internal standard (IS)	DMA-d_3 (deuterated DMA)
Calibration concentrations	500.0 ng/mL to 128000.0 ng/mL
QC concentrations	Surrogate: 500.0 ng/mL (LLOQ), 1200.0 ng/mL (LQC), 5000.0 ng/mL (MQC1), 50000.0 ng/mL (MQC2), and 110000.0 ng/mL (HQC) Plasma: 110000.0 ng/mL (PQC) Urine: 110000.0 ng/mL (UQC)
Selectivity	Significant interference was observed in human plasma and urine. Thus, 0.05% phosphate buffer with 10% *v/v* methanol was used as a surrogate matrix (surrogate).
Specificity	No interference observed
Lower limit of quantification	500.0 ng/mL Between-run accuracy 96.4.4% (surrogate) Between-run precision 14.0% (surrogate) Within-run accuracy 91.0%-101.6% (surrogate) Within-run precision 10.9%-16.2% (surrogate)
Between-run accuracy	Surrogate: 94.2%-101.1% Plasma: 103.6% Urine: 107.5%
Between-run precision	Surrogate: 3.2%-14.0% Plasma: 4.6% Urine: 7.7%
Within-run accuracy	Batch 1: 92.4%-102.2% (surrogate); 102.2% (plasma); 98.4% (urine) Batch 2: 91.0%-109.5% (surrogate); 106.3% (plasma); 114.5% (urine) Batch 3: 87.4%-115.2% (surrogate); 102.3% (plasma); 109.6% (urine)
Within-run precision	Batch 1: 0.9%-16.2% (surrogate); 2.9% (plasma); 1.5% (urine) Batch 2: 2.1%-14.2% (surrogate); 5.9% (plasma); 4.0% (urine) Batch 3: 1.9%-10.9% (surrogate); 4.1% (plasma); 6.2% (urine)
Matrix effect (IS-normalized)	LQC: 1.01 (2.1 %CV) HQC: 0.98 (2.0 %CV)
Recovery of analyte	99.7% -113.0%
Recovery of IS	107.8%
Auto-sampler storage stability	Confirmed up to 24 hours at 10°C nominal Accuracy 105.1% for UQC in urine Accuracy 104.8% for PQC in plasma Accuracy 90.1% for LQC and 95.3% for HQC in surrogate
Freeze-thaw stability	Confirmed up to 8 cycles Accuracy 101.1% for UQC in urine Accuracy 107.1% for PQC in plasma
Bench top stability	Confirmed up to 24 hours at room temperature Accuracy 102.5% for UQC in urine Accuracy 99.1% for PQC in plasma Accuracy 103.9% for LQC and 101.8% for HQC in surrogate
Injector Carryover	Not significant (0.0%)
Long Term Storage Stability	Confirmed up to 234 days at -80°C Accuracy 104.3% for PQC and 106.8% for UQC

Bioanalytical Method Conditions for Ranitidine Urine Method

Validation range:	15.6–4000.0 ng/mL
High Performance Liquid Chromatography (HPLC) instrument:	Agilent 1290 Infinity
Mass Spectrometer:	AB SCIEX 6500^{+} Qtrap Mass spectrometer

HPLC Conditions

Mobile Phase:	10 mM ammonium formate with 0.1% formic acid in water and acetonitrile (Gradient flow)
Flow rate:	0.3 mL/min
Column:	Acquity UPLC BEH, Shield, RP18, 2.1*50mm, 1.7µm
Injection Volume:	20 µL
Retention Time:	Ranitidine: 1.75 minute Ranitidine-d_6: 1.75 minute
Run Time:	2.5 minutes

Mass Spectrometer conditions

Ionization Source and scan type:	Atmospheric Pressure Chemical Ionization (APCI) Multiple Reaction Monitoring (MRM)
Data acquisition:	Analyst 1.7.1

AB SCIEX 6500^{+} Q-TRAP mass spectrometer state file parameters:

Parameter	Positive mode	
	Ranitidine	**Ranitidine-d_6**
Q1 mass/Q3 mass (amu)	315.1/176.1	321.1/176.1
Collision Energy (V)	20	25
Declustering Potential	100	100
Entrance Potential	10	4
Collision Cell Exit Potential	10	10
Source Temperature (°C):	500	500
Ion Source Gas 1	50	50
Curtain Gas Flow	30	30
Collision Gas Flow	Medium	Medium
Nebulizer Current	5	5
Dwell time (ms)	100	100

Sample Preparation: Sample preparation involved the extraction of ranitidine from the samples using protein precipitation/sample dilution. An aliquot of 200 µl of acetonitrile containing 0.2 µg/ml of ranitidine-d6 (internal standard) was added to 96-well protein precipitation plates and 20µl of the urine sample was aliquoted to each well. The samples were shaken at 700 rpm for 5 minutes. Later, the samples were centrifuged at 4000 rpm for 10 minutes with a bottom collection plate containing 40 µl of water in each well. The plate was shaken for 5 minutes at 700 rpm and the sample (3 µl) was injected into the mass spectrometer.

Validation Summary Table for Ranitidine Urine Method

Description	**Ranitidine**
Short description of method	Protein precipitation with reverse-phase HPLC with MS/MS detection
Matrix	Human urine
Analyte	Ranitidine
Internal standard (IS)	Ranitidine-d_6
Calibration concentrations	15.6 ng/mL to 4000.0 ng/mL
QC concentrations	15.6 ng/mL (LLOQ), 50.0 ng/mL (LQC), 200.0 ng/mL (MQC1), 1600.0 (MQC2) ng/mL and 3200.0 ng/mL (HQC)
Selectivity	No significant interference was observed (less than 20% LLOQ response)
Specificity	No significant interference observed (less than 20% LLOQ response)
Lower limit of quantification	15.6 ng/mL Between-run accuracy 104.5% Between-run precision 5.1% Within-run accuracy 99.4%-108.8% Within-run precision 3.2%-4.0%
Between-run accuracy	97.4%-104.5%
Between-run precision	2.0%-5.1%
Within-run accuracy	Batch 1: 95.8%-108.2% Batch 2: 96.4%-108.9% Batch 3: 99.4%-110.4%
Within-run precision	Batch 1: 1.5%-3.2% Batch 2: 0.7%-3.8% Batch 3: 1.2%-3.5%
Matrix effect (IS-normalized)	LQC: 1.01 (2.1 %CV) HQC: 0.98 (2.0 %CV)
Recovery of analyte	93.7% -95.0%
Recovery of IS	84.1%
Auto-sampler storage stability	Confirmed up to 36 hours at 10°C nominal Accuracy 109.2% for LQC and 105.7% for HQC
Freeze-thaw stability	Confirmed up to 8 cycles Accuracy 103.9% for LQC and 98.3% for HQC
Bench top stability	Confirmed up to 24 hours at room temperature Accuracy 101.8% for LQC and 100.7% for HQC
Injector Carryover	Not significant (0.0%)
Long Term Storage Stability	Confirmed up to 235 days at -80°C Accuracy 97.5% for LQC and 100.7% for HQC

Bioanalytical Method Conditions for Ranitidine Plasma Method

Validation range:	15.6–4000.0 ng/mL
High Performance Liquid Chromatography (HPLC) instrument:	Agilent 1290 Infinity
Mass Spectrometer:	AB SCIEX 6500^{+} Qtrap Mass spectrometer

HPLC Conditions

Mobile Phase:	10 mM ammonium formate with 0.1% formic acid in water and acetonitrile (Gradient flow)
Flow rate:	0.3 mL/min
Column:	Acquity UPLC BEH, Shield, RP18, 2.1*50mm, 1.7µm
Injection Volume:	20 µL
Retention Time:	Ranitidine: 1.75 minute Ranitidine-d_6: 1.75 minute
Run Time:	2.5 minutes

Mass Spectrometer conditions

Ionization Source and scan type:	Atmospheric Pressure Chemical Ionization (APCI) Multiple Reaction Monitoring (MRM)
Data acquisition:	Analyst 1.7.1

AB SCIEX 6500^{+} Q-TRAP mass spectrometer state file parameters:

Parameter	Positive mode	
	Ranitidine	**Ranitidine-d_6**
Q1 mass/Q3 mass (amu)	315.1/176.1	321.1/176.1
Collision Energy (V)	20	25
Declustering Potential	100	100
Entrance Potential	10	4
Collision Cell Exit Potential	10	10
Source Temperature (°C):	500	500
Ion Source Gas 1	50	50
Curtain Gas Flow	30	30
Collision Gas Flow	Medium	Medium
Nebulizer Current	5	5
Dwell time (ms)	100	100

Sample Preparation: Sample preparation involved the extraction of ranitidine from the samples using protein precipitation. An aliquot of 200 µl of acetonitrile containing 0.2 µg/ml of ranitidine-d6 (internal standard) was added to 96-well protein precipitation plates and 20µl of plasma sample was aliquoted to each well. The samples were shaken at 700 rpm for 5 minutes. Later, the samples were centrifuged at 4000 rpm for 10 minutes with a bottom collection plate and the sample (3 µl) was injected into the mass spectrometer.

Validation Summary Table for Ranitidine Plasma Method

Description	**Ranitidine**
Short description of method	Protein precipitation with reverse-phase HPLC with MS/MS detection
Matrix	Human Plasma
Analyte	Ranitidine
Internal standard (IS)	Ranitidine-d_6
Calibration concentrations	15.6 ng/mL to 4000.0 ng/mL
QC concentrations	15.6 ng/mL (LLOQ), 50.0 ng/mL (LQC), 200.0 ng/mL (MQC1), 1600.0 (MQC2) ng/mL and 3200.0 ng/mL (HQC)
Selectivity	No significant interference was observed (less than 20% LLOQ response)
Specificity	No significant interference observed (less than 20% LLOQ response)
Lower limit of quantification	15.6 ng/mL Between-run accuracy 107.7% Between-run precision 14.4% Within-run accuracy 106.0%-109.0% Within-run precision 3.0%-5.1%
Between-run accuracy	100.1%-108.8%
Between-run precision	2.0%-3.1%
Within-run accuracy	98.8%-111.0%
Within-run precision	0.9%-3.2%
Matrix effect (IS-normalized)	LQC: 1.06 (1.4 %CV) HQC: 1.02 (0.9 %CV)
Recovery of analyte	75.8% -86.2%
Recovery of IS	79.9%
Auto-sampler storage stability	Confirmed up to 36 hours at 10°C nominal Accuracy 107.9% for LQC and 101.8% for HQC
Freeze-thaw stability	Confirmed up to 8 cycles Accuracy 96.7% for LQC and 95.2% for HQC
Bench top stability	Confirmed up to 24 hours at room temperature Accuracy 103.4% for LQC and 97.8% for HQC
Injector Carryover	Not significant (0.0%)
Long Term Storage Stability	Confirmed up to 234 days at -80°C Accuracy 100.5% for LQC and 91.2% for HQC

Ex vivo Stability Evaluation

Ex vivo stability Evaluation

The potential for *ex vivo* conversion of ranitidine to NDMA was evaluated by performing a series of *in vitro* experiments prior to the execution of the clinical study. The details of the experimental design and results are summarized below.

Experimental design

The *ex vivo* stability of ranitidine in urine was evaluated at room temperature (24^0C) and at 37^0C in the presence and absence of externally spiked nitrites (10 mM) and nitrates (100 ug/mL). During the stability assessment, the urine samples were spiked with ranitidine (300 ug/mL), ranitidine N-oxide (40 ug/mL), ranitidine S-oxide (16 ug/mL) and desmethyl ranitidine (10 ug/mL) prior to the incubations. Stabilities were evaluated at three different pH values (4.5, 6.0 and 8.0). The urine samples were incubated, and measurements were collected from the samples at different timepoints (from 0 minutes to 240 minutes). NDMA in samples was quantified by liquid chromatography and tandem mass spectrometric methods and compared against the 0-minute levels. The percent conversion was considered significant if the conversion was greater than 20%.

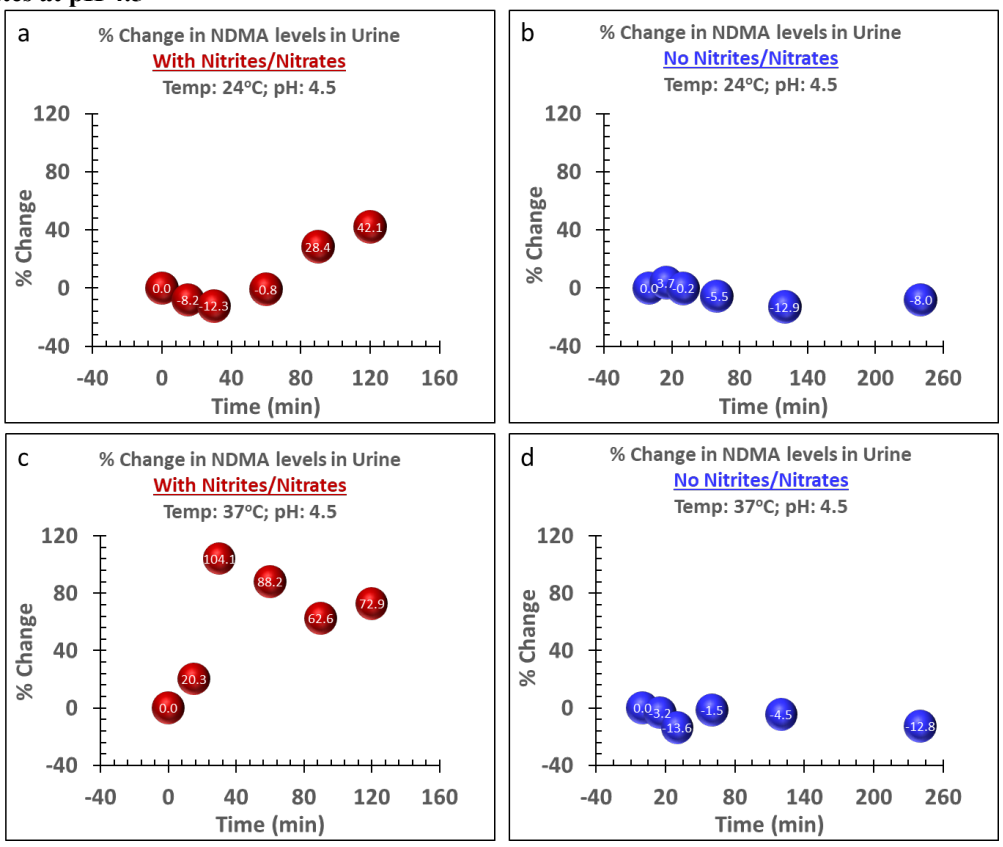
Results

The conversion (formation of NDMA) was increased by higher temperature (37^0C) and lower pH (4.0) and consistently increased with time. The conversion was minimized by adjusting the pH to alkaline conditions (pH 8.0). The summary of results is shown in Figures 1 to 3.

Conclusions

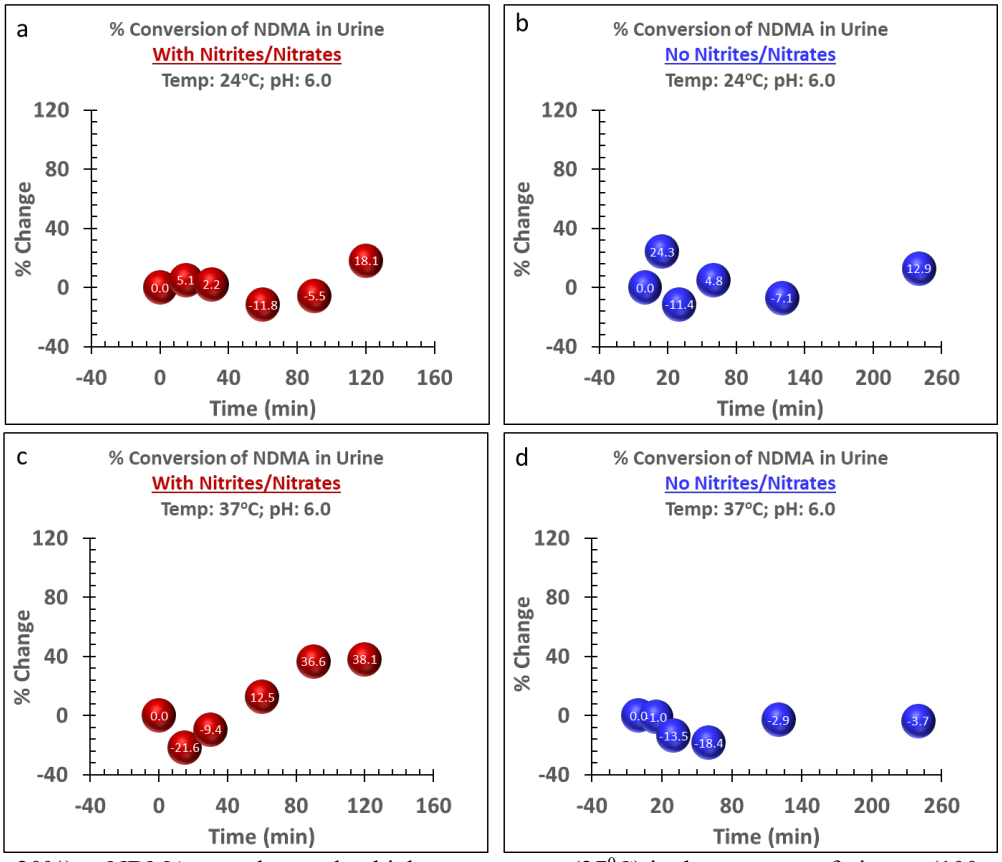
Ex vivo conversion of ranitidine and ranitidine metabolites to NDMA was minimized by adjusting the pH to alkaline conditions (pH 8.0) and maintaining lower temperatures.

Figure 1: Formation of NDMA in urine in the presence of ranitidine and ranitidine metabolites with and without nitrites/nitrates at pH 4.5



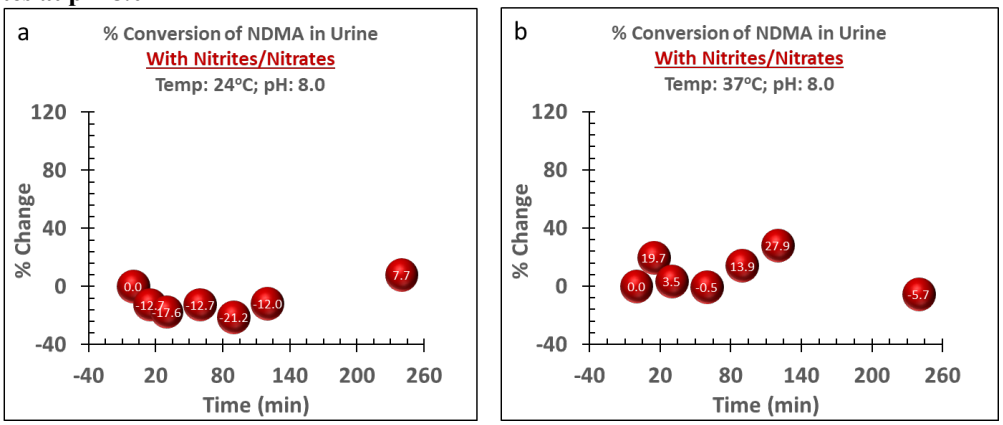
Conversion (>20%) to NDMA was observed at both tested temperatures in the presence of nitrates (100 ug/mL) and nitrites (10 mM) (a and c). No conversion (<20%) to NDMA was observed in the absence of nitrates and nitrates (b and d).

Figure 2: Formation of NDMA in urine in the presence of ranitidine and ranitidine metabolites with and without nitrites/nitrates at pH 6.0



Conversion (> 20%) to NDMA was observed at high temperatures (37^{0}C) in the presence of nitrates (100 ug/mL) and nitrites (10 mM) (c). No conversion (< 20%) to NDMA was observed in the absence of nitrates and nitrates (b and d) and at room temperature in the presence of nitrites (100 ug/mL) and nitrates (10 mM) (a).

Figure 3: Formation of NDMA in urine in the presence of ranitidine and ranitidine metabolites with nitrites/nitrates at pH 8.0



No conversion (< 20%) to NDMA was observed at both tested temperatures in the presence of nitrites and nitrates (a & b).

eDictionary. Data Dictionary for Participant Data Listings

A full listing of participant data, including demographics and concentrations/amount by analyte and biospecimen at each time point are included with the study datasets. Column names and definitions are provided below. Separate datasets are provided for the plasma and urine data.

Variable	Definition
SubjectID	Participant ID
Period	One of 1, 2, 3, or 4, corresponding to study period when data was collected
Time	Nominal sampling time (unit = hours). Unscheduled voids are NA
SampleTimeC	Sample time as character. Unscheduled urine samples are labeled as 'Unscheduled'
Analyte	Measured analyte. One of NDMA, DMA, or ranitidine
Units	Units for the reported 'Concentration' variable. Units are ng/mL for ranitidine and DMA and pg/mL for NDMA (note – these are sample concentrations – need amounts for urine analyses).
Concentration	Reported concentration with values below lower limit of quantification set to zero. (units are defined in Units)
ConcentrationC	Character concentration from bioanalytical readout
Specimen	Denotes whether the sample was from plasma or urine
EarlyTerm	Flag for if the sample was an early termination collection
sample_ct	Count of sample by participant by period – this is the ordering to use for urine samples since there were unscheduled voids
Treatment	One of Ranitidine or Placebo, corresponding to treatment received during the periods
Meal	One of L or H, corresponding to if participant was administered a noncured-meats/organic-vegetables or cured-meats/conventional-vegetables diet, respectively
Sequence	One of ABCD, ABDC, BACD, or BADC, corresponding to sequence the participant was randomized to
Age	Age of the participant (unit = years)
Sex	Sex of the participant (one of 'Female' or 'Male')
Ethnicity	Ethnicity of the participant (one of 'Hispanic or Latino' or 'Not Hispanic or Latino')
Race	Race of the participant (one of 'Asian', 'Black or African American', or 'White')
Weight	Weight of the participant (unit = kg)
TimeAccum	For urine datasets – for grouping unscheduled voids as part of the next planned collection interval
UrineVolume	For urine datasets – volume of the urine void (mL)
Amount	For urine datasets – total amount of analyte in the urine void (ng for NDMA; mg for DMA and ranitidine)
AmountUse	For urine datasets - same as Amount but with time=0 set to 0 (do not include in 24-hr total)
AmountUnits	For urine datasets - units are mg for amount of ranitidine and DMA in urine and ng for amount of NDMA in urine
AccumAmount	For urine datasets – cumulative amount of analyte in the urine since start of the treatment period (ng for NDMA; mg for DMA and ranitidine)

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