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ANALYSIS OF THE URINARY GLUCOSE-[N, N]-UREIDE CONTENT IN THE STUDY OF THE LACTOSE-[N, N]-UREIDE METABOLISM IN HEALTHY HUMANS

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1 **ANALYSIS OF THE URINARY GLUCOSE-[¹⁵N, ¹⁵N]-UREIDE CONTENT IN THE STUDY OF THE LACTOSE-**
2 **[¹⁵N, ¹⁵N]-UREIDE METABOLISM IN HEALTHY HUMANS**

3
4 **GC-MS ANALYSIS OF GLUCOSE-[¹⁵N, ¹⁵N]-UREIDE**

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6
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14 Abbreviations: GC-MS: gas chromatography – mass spectrometry; OCTT: oro-caecal transit time; TLC:
15 thin layer chromatography

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1 **ABSTRACT**

2 **Background/Objectives:** Lactose-[¹⁵N, ¹⁵N]-ureide is used to study the fate of the colonic urea-
3 nitrogen metabolism. During the passage through the gastrointestinal tract, lactose ureide is
4 hydrolysed to glucose ureide, which is absorbed to a limited extent from the small intestine and is
5 excreted urinarly. In the present study, a procedure has been developed to quantify the urinary
6 excretion of glucose-[¹⁵N, ¹⁵N]-ureide. In addition, urine and faecal samples obtained during a dietary
7 intervention study with the prebiotic lactulose were retrospectively analysed.

8 **Subjects/Methods:** The glucose ureide and lactose ureide content was measured by GC-MS in
9 nineteen healthy volunteers. After consumption of a standard test meal containing 75 mg lactose-
10 [¹⁵N, ¹⁵N]-ureide, 6 healthy volunteers performed a fractionated 24h urine collection to investigate
11 the urinary excretion of glucose-[¹⁵N, ¹⁵N]-ureide. In 13 volunteers, the effect of lactulose
12 administration on the urinary excretion of glucose-[¹⁵N, ¹⁵N]-ureide was analysed.

13 **Results:** The urinary excretion of glucose-[¹⁵N, ¹⁵N]-ureide reached its maximum level in the 3-6h
14 urine collection and decreased in the 6-9h urine. The label was still detectable in the 9-24h urine
15 collection. The cumulative excretion of ¹⁵N-labelled glucose ureide after 24h amounted 12.91 %. No
16 significant differences in glucose-[¹⁵N, ¹⁵N]-ureide excretion were found in either of the urine
17 fractions after administration of lactulose compared to baseline. In none of the urine samples
18 lactose-[¹⁵N, ¹⁵N]-ureide was detected.

19 **Conclusions:** In conclusion, the results obtained in the present study indicated that the percentage
20 dose glucose-[¹⁵N, ¹⁵N]-ureide recovered in urine is rather constant and not influenced by the
21 presence of lactulose.

22

23 **Key words:** lactose-[¹⁵N, ¹⁵N]-ureide, ammonia metabolism, stable isotopes, GC-MS

24

1 INTRODUCTION

2 Lactose ureide labelled with ^{13}C on the urea moiety has been proposed as a substrate for the
3 determination of oro-caecal transit time (OCTT) by Heine *et al.* (Heine *et al.*, 1995). Its application is
4 based on the observation that the bond between the urea and sugar moiety in lactose ureide is
5 completely resistant to human digestive processes, yet it is hydrolysed by the intestinal microbiota
6 upon arrival in the colon. The labelled urea generated in this way is rapidly hydrolysed to $^{13}\text{CO}_2$ and
7 NH_3 .

8 On the basis of the same principle, its ^{15}N -labelled analogue has been applied as a vehicle to
9 introduce a known amount of urea-nitrogen into the colon. The labelled NH_3 can either be used by
10 the colonic microbiota for their own metabolism which is followed by faecal ^{15}N -excretion or it can
11 be absorbed through the colonic wall and after conversion in the liver to labelled urea, be excreted in
12 the urine (Jackson *et al.*, 1999) (Figure 1). In this way, lactose- ^{15}N -ureide can be used to
13 evaluate the fate of the colonic urea-nitrogen metabolism. Using the same molecule, Jackson *et al.*
14 demonstrated that bacterially derived amino acids from ^{15}N -urea are available for colonic
15 absorption and thus for the host (Jackson *et al.*, 2004). We have proposed this molecule as a
16 biomarker to evaluate the influence of pre- and/or probiotic administration on the colonic ammonia
17 metabolism. It has been shown that bacterial activity and/or growth is stimulated upon
18 administration of pre- and/or probiotics which is reflected in a shift from urinary to faecal ^{15}N -
19 excretion (De Preter *et al.*, 2004; Geboes *et al.*, 2005).

20 Several methods have previously been applied to measure the ^{15}N -enrichment and total nitrogen
21 content in urine and faeces, however, they do not all allow **discrimination** between the different
22 chemical forms of the marker. Ruummele *et al.* showed that during the passage through the
23 gastrointestinal tract, lactose ureide is converted by β -galactosidase, located in the brush border of
24 the small bowel, to galactose and glucose ureide, and that glucose ureide is absorbed to a limited
25 extent from the small intestine and is excreted urinarily without being further metabolised
26 (Ruummele *et al.*, 1997). **The analytical methodology used by Jackson *et al.* (1999) did not allow to**

1 differentiate between ^{15}N originating from urea or glucose- $^{15}\text{N},^{15}\text{N}$ -ureide, leading to their
2 conclusion that a limited amount of labelled $^{15}\text{N},^{15}\text{N}$ -urea (5 %) can be absorbed unchanged from
3 the colon. Morrison *et al.* have qualitatively identified glucose- ^{13}C -ureide in urine after
4 administration of ^{13}C -labelled lactose ureide using thin layer chromatography (TLC). They estimated
5 the ^{13}C -recovery in urine at approximately 15% of administered dose (Morrison *et al.*, 2003). As a
6 consequence, it is likely that ^{15}N -labelled glucose ureide will be excreted in urine after administration
7 of ^{15}N -labelled lactose ureide and it might be necessary to correct urinary ^{15}N -data for the presence
8 of glucose- $^{15}\text{N},^{15}\text{N}$ -ureide.

9 Therefore, an analytical procedure using gas chromatography – mass spectrometry (GC-MS)
10 technology has been developed to quantify in a prospective study the urinary excretion of glucose-
11 $^{15}\text{N},^{15}\text{N}$ -ureide after oral administration of lactose- $^{15}\text{N},^{15}\text{N}$ -ureide. In addition, urine and faecal
12 samples obtained during a dietary intervention study with the prebiotic lactulose were
13 retrospectively analysed.

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1 MATERIALS AND METHODS

2 GC-MS analysis

3 *Substrates*

4 Lactose-[¹⁵N, ¹⁵N]-ureide, glucose-[¹⁵N, ¹⁵N]-ureide and the corresponding unlabelled substrates were
5 synthesized according to the method of Schoorl (Schoorl MN 1903) as modified by Hofmann
6 (Hofmann E 1931) with [¹⁵N, ¹⁵N] urea obtained from Euriso-top (Saint-Aubain Cédex, France). Sugar
7 ureides are the condensation products of a reducing sugar and urea in mild aqueous acid. Their
8 synthesis is simple and avoids the use of toxic chemicals, making them safe for use in clinical practice.
9 Absence of [¹⁵N, ¹⁵N]-urea, lactose or glucose-[¹⁵N, ¹⁵N]-ureide in the synthesis of lactose-[¹⁵N, ¹⁵N]-
10 ureide, and absence of [¹⁵N, ¹⁵N]-urea or glucose in the synthesis of glucose-[¹⁵N, ¹⁵N]-ureide was
11 confirmed using TLC (Morrison *et al.*, 2001).

12

13 *Chemicals*

14 Meso-inositol and n-heptane were supplied by Vel (Leuven, Belgium), whereas acetone was obtained
15 from Chem-Lab NV (Zedelgem, Belgium). NH₂OH.HCl was purchased from Sigma-Aldrich (St. Louis,
16 USA), pyridine and BSTFA-TMCS (99:1) were obtained from Pierce (Rockford, USA).

17

18 *Sample preparation*

19 The glucose ureide and lactose ureide content was measured by gas chromatography–mass
20 spectrometry (type time-of-flight (TOF)) technology. Therefore, 40 µl internal standard (meso-inositol
21 (100mg/100ml)) was added to 960 µl of a standard solution (= final concentration range of glucose
22 ureide and lactose ureide between 0 and 30 mg/l). This solution was diluted with 3 ml aceton, mixed
23 for 1 min and centrifuged at 3000 rpm for 20 min to deproteinized the samples. One ml aliquots
24 were evaporated to dryness and 100 µl oxime reagent (NH₂OH.HCl in pyridine, 25 mg/l) was added to
25 the sample, mixed for 1 min and heated for 30 min at 80°C. The reaction was stopped at –20°C (10
26 min) and the samples were evaporated under a stream of nitrogen. Derivatisation was accomplished

1 by adding 50 μ l of BSTFA+TMCS (99:1) to the dried samples, thoroughly mixed and heated at 80°C.
2 After 35 min, samples were cooled down to room temperature. After adding 250 μ l pyrogeen-free
3 H₂O, samples were extracted with 300 μ l n-heptane and 0.5 μ l of this solution was analysed on a GC-
4 MS (Interscience, Louvain-la-Neuve, Belgium). The analytical column was a 30 m x 0.25 mm i.d., 0.25
5 μ m AT5-MS (Alltech Associates, Deerfield, USA). Helium GC grade was used as a carrier gas with a
6 constant flow of 1.3 ml/min. The oven temperature was programmed to increase from 200°C (1 min),
7 with 5°C/min to 270°C (isothermal for 15 min). Mass spectrometric detection was performed in full
8 scan mode from m/z 35 to m/z 600 at 10 scans/sec. Xcalibur-software was used for the
9 automatisation of the GC-TOF-MS and for data acquisition. The results for glucose-ureide and lactose
10 ureide were expressed as percentage of the administered dose recovered in the different urine
11 fractions.

12

13 **Study design**

14 *Subjects*

15 Nineteen healthy volunteers (10 men and 9 women; mean age 22) were included in the study. None
16 of the subjects had a history of gastrointestinal or metabolic disease or previous surgery (apart from
17 appendectomy). The subjects were free of antibiotics or any other medical treatment influencing gut
18 transit or intestinal flora for at least 3 months before the start of the study. The Ethics Committee of
19 the University of Leuven approved the study and all subjects gave informed consent.

20

21 *Prospective study to quantify the glucose-[¹⁵N, ¹⁵N]-ureide excretion*

22 Six healthy volunteers (3f / 3m) were included and performed one test. On the day of the test, they
23 provided a basal urine sample after which they received a standard pancake test meal (8.4 g
24 proteins, 11.2 g fat and 26.7 g carbohydrates (243.5 kcal)) containing 75 mg lactose-[¹⁵N, ¹⁵N]-ureide.
25 After intake of the test meal, a fractionated 24h urine collection was performed (0-3h, 3-6h 6-9h and
26 9-24h) to investigate the urinary excretion of glucose-[¹⁵N, ¹⁵N]-ureide in the different urine fractions.

1 *Influence of lactulose on the glucose-[¹⁵N, ¹⁵N]-ureide excretion*

2 In a retrospective study, urine and faecal samples obtained from 13 healthy volunteers (6f / 7m) who
3 participated in a study to investigate the effects of the prebiotic lactulose, were analysed. These
4 volunteers had consumed three times a standard pancake test meal labelled with 75 mg lactose-[¹⁵N,
5 ¹⁵N]-ureide; the first time in baseline conditions, the second time together with 10g lactulose and the
6 third time after a 4-week dietary intervention period with lactulose (2x 10g/d). Each time, the
7 volunteers provided a basal urine sample before consumption of the test meal and collected all urine
8 for 48h (0-24h and 24-48h fractions) and stools for 72h.

9

10 *Samples*

11 All urine was collected in recipients to which neomycin was added for prevention of bacterial growth.
12 After measurement of the volume, samples were taken and stored at –20 °C until analysis. Upon
13 delivery of the faecal samples, all stools collected on the same day were combined, diluted fivefold
14 with sterile pyrogen-free water and homogenized. The homogenate was ultracentrifuged at 25000g
15 during 120 min (MR22i, Jouan, St-Herblain, France) and the supernatans was subsequently filtered
16 through a 0.2-µm syringe filter (Supor Acrodisc 32, Gelman Sciences, Ann Arbor, USA) in order to
17 discard the ultimate faecal rests and the bacteria. The final filtrate was used for determination.
18 Aliquots were frozen at –20°C.

19 For analysis, 960 µl of urine or faecal extract were prepared according to the standard solution to
20 determine the excretion of glucose-[¹⁵N, ¹⁵N]-ureide and lactose-[¹⁵N, ¹⁵N]-ureide.

21

22 **Statistical analysis**

23 Results were expressed as median and interquartile range (IQR). The statistical analysis was
24 performed with SPSS software (SPSS 16.0 for Windows; SPSS Inc., Chicago, IL, USA). Given the low
25 number of subjects in the treatment groups, non-parametric statistical analysis was used. Statistical

1 evaluation of the data was performed by applying the Wilcoxon and Mann-Whitney test. The level
2 for statistical significance was set at $p < 0.05$.

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1 RESULTS

2 *GC-MS analysis*

3 GC-MS analysis of a derivatisation reaction of glucose ureide showed a peak eluting at 11.02 min
4 which was identified as the internal standard and a peak at 15.37 min, identified as glucose ureide.
5 Similarly, lactose ureide was found to elute from the column at 24.85 min. A calibration curve was
6 constructed using dilutions ranging from 0 – 30 mg/l ($r^2=0.9923$). The limit of detection was
7 established at 1.25 mg/l. A good interday and intraday reproducibility was found in the analysis of
8 the urine samples (<10% RSD)

9

10 *Prospective study to quantify the glucose-[¹⁵N, ¹⁵N]-ureide excretion*

11 Figure 2 shows the glucose-[¹⁵N, ¹⁵N]-ureide excretion in the different urine fractions. Within the first
12 3h of ingestion of the marker, a small fraction of the labelled glucose-ureide (i.e. 0.64 % dose
13 glucose-[¹⁵N, ¹⁵N]-ureide (IQR 0.21 – 0.86)) was already excreted in the urine indicating an early
14 absorption from the upper small intestine. Urinary excretion reached its maximum level in the 3-6h
15 urine collection (5.45 % dose glucose-[¹⁵N, ¹⁵N]-ureide (IQR 4.89 – 6.25) and decreased in the 6-9h
16 urine collection to 4.03 % dose glucose-[¹⁵N, ¹⁵N]-ureide (IQR 3.74 – 5.46). The label was still
17 detectable in the 9-24h urine collection (3.27 % dose glucose-[¹⁵N, ¹⁵N]-ureide (IQR 1.53 – 3.90). The
18 cumulative excretion of ¹⁵N-labelled glucose ureide after 24h amounted 12.91 % dose glucose-[¹⁵N,
19 ¹⁵N]-ureide (IQR 11.40 – 15.30) and is shown in Figure 3. In none of the samples lactose-[¹⁵N, ¹⁵N]-
20 ureide was detected.

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22 *Influence of lactulose on the glucose-[¹⁵N, ¹⁵N]-ureide excretion*

23 The urinary and faecal excretion of glucose-[¹⁵N, ¹⁵N]-ureide in the three different test situations is
24 represented in Table 1. The individual recovery of glucose-[¹⁵N, ¹⁵N]-ureide over 24h is shown in
25 Figure 4. No statistically significant differences were found in either of the urine fractions. No
26 glucose-[¹⁵N, ¹⁵N]-ureide was found in the 0-24h and 24-48h urine collections in 1 of the volunteers

1 after short-term administration of lactulose and was probably due to the fact that these urine
2 fractions were significantly diluted. In none of the urine samples lactose-[¹⁵N, ¹⁵N]-ureide was
3 detected. Neither glucose-[¹⁵N, ¹⁵N]-ureide nor lactose-[¹⁵N, ¹⁵N]-ureide was found in the analysis of
4 the faecal samples.

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1 DISCUSSION

2 The aim of the present study was to develop an analytical procedure using GC-MS analysis to
3 quantify in a prospective study the urinary excretion of glucose-[¹⁵N, ¹⁵N]-ureide after oral
4 administration of lactose-[¹⁵N,¹⁵N]-ureide. The appearance of glucose-[¹⁵N, ¹⁵N]-ureide in urine is the
5 result of hydrolysis, absorption and urinary filtration of the disaccharide bond in lactose-[¹⁵N, ¹⁵N]-
6 ureide by the brush border enzyme β -galactosidase to release galactose and glucose-[¹⁵N, ¹⁵N]-
7 ureide.

8 Quantitative evaluation of the urinary glucose-[¹⁵N, ¹⁵N]-ureide excretion in the prospective study
9 has indicated that the urinary excretion of ¹⁵N-labelled substrate reaches its maximum excretion
10 level in the 3-6h urine collection, which is assumed to represent the *in vivo* absorption from the small
11 intestine. We determined previously the oro-caecal transit time, i.e. the time to reach the colon, of
12 the standard test meal used in the present study and found it to approximate 360 min (Verbeke *et*
13 *al.*, 2005). Thus, the time for appearance of the label in urine within the first 6 hours, after
14 administration is due to hydrolysis of lactose-[¹⁵N, ¹⁵N]-ureide to glucose-[¹⁵N, ¹⁵N]-ureide and
15 galactose by the enzyme β -galactosidase which is located in the brush border of the small intestine,
16 subsequent absorption and distribution in the body, followed by renal excretion. However, also in
17 the 6-9h and 9-24h urine collection, a small amount of ¹⁵N-labelled glucose ureide was recovered,
18 suggesting that a certain percentage of glucose-[¹⁵N, ¹⁵N]-ureide might remain in circulation in the
19 body before being excreted. As a consequence, the urinary ¹⁵N content recovered in those urine
20 collections does not all originate from the ¹⁵N-labelled urea formed after bacterial degradation of
21 the test substrate, but a minor fraction of the ¹⁵N can be attributed to ¹⁵N-labelled glucose ureide
22 excretion.

23 Since lactose-[¹⁵N, ¹⁵N]-ureide has been proposed as a biomarker to evaluate the influence of pre-
24 and/or probiotics on the colonic fate of urea-nitrogen, it was investigated whether the metabolism of
25 lactose-[¹⁵N, ¹⁵N]-ureide was influenced by administration of the pre- and/or probiotics. For this

1 purpose, urine and faecal samples which were already available from a dietary intervention study
2 with lactulose have been re-analysed in the second part of the study (De Preter *et al.*, 2006).

3 It is unlikely that probiotics would influence the lactose-[¹⁵N, ¹⁵N]-ureide metabolism, but it is well
4 known that certain non-digestible carbohydrates may influence transit and digestion in the
5 gastrointestinal tract. Lactulose has previously been shown to significantly delay gastric emptying by
6 increasing viscosity of the gut contents and to accelerate small intestinal transit (Diggory *et al.*, 1985;
7 Holgate *et al.*, 1983; Wutzke *et al.*, 1997). As a consequence, the absorption of glucose-[¹⁵N, ¹⁵N]-
8 ureide could be decreased due to either the shorter time available for absorption or to a decreased
9 hydrolysis of lactose-[¹⁵N, ¹⁵N]-ureide to glucose-[¹⁵N, ¹⁵N]-ureide. However, in the present study the
10 mean glucose-[¹⁵N, ¹⁵N]-ureide absorption and urinary excretion was not influenced by the presence
11 of lactulose in the test meal, although the individual results of the urinary glucose-[¹⁵N, ¹⁵N]-ureide
12 excretion are more variable compared to baseline and long-term lactulose administration. In five
13 individuals, inclusion of lactulose in the test meal resulted in less glucose-[¹⁵N, ¹⁵N]-ureide excretion
14 suggesting that the absorption of glucose-[¹⁵N, ¹⁵N]-ureide was decreased due to the shorter time
15 available for absorption or to a decreased hydrolysis of lactose-[¹⁵N, ¹⁵N]-ureide to glucose-[¹⁵N, ¹⁵N]-
16 ureide, whereas in four individuals more glucose-[¹⁵N, ¹⁵N]-ureide was recovered.

17 Long-term dietary intake of lactulose on the other hand, is known to cause changes in the metabolic
18 activity and the relative composition of the colonic microbiota. Acidification of colonic luminal
19 environment has probably no effect on the absorption of glucose-[¹⁵N, ¹⁵N]-ureide in the small
20 intestine, but could hypothetically influence the bacterial hydrolytic processes in the colon, resulting
21 in an incomplete hydrolysis of ¹⁵N-labelled glucose ureide. As a consequence, unhydrolysed glucose-
22 [¹⁵N, ¹⁵N]-ureide might be detected in the faecal samples. However, analysis of faecal samples has
23 indicated that nor glucose-[¹⁵N, ¹⁵N]-ureide neither lactose-[¹⁵N, ¹⁵N]-ureide appeared in the faeces.

24 The results of both studies have indicated that meanly 13.16 ± 2.19 % of glucose-[¹⁵N, ¹⁵N]-ureide is
25 recovered in urine after administration of ¹⁵N-labelled lactose ureide and remains quite constant
26 within the different individuals and even between the different test circumstances. This percentage

1 dose recovered in urine was in line with the estimated ^{13}C -recovery after oral administration of
2 lactose- ^{13}C -ureide, as determined by Morrison *et al.* (Morrison *et al.*, 2003).

3 In Table 2, the original (total) ^{15}N data and corrected ^{15}N data are shown. For each volunteer, the
4 original ^{15}N data was corrected with the actual individual percentage glucose- ^{15}N , ^{15}N -ureide
5 recovered. As was expected, the correction for the presence of glucose- ^{15}N , ^{15}N -ureide did not have
6 a significant influence on the previously observed effects. Since it would be very time-consuming and
7 labour intensive to measure in each urine sample the amount of glucose- ^{15}N , ^{15}N -ureide besides the
8 ^{15}N -enrichment and because of the small variability in amounts of retrieved glucose- ^{15}N , ^{15}N -ureide,
9 we suggest not to correct the data for total ^{15}N excretion for the presence of glucose- ^{15}N , ^{15}N -
10 ureide.

11 In conclusion, the results obtained in the present study have indicated that the percentage dose
12 glucose- ^{15}N , ^{15}N -ureide recovered in urine is rather constant and not influenced by the presence of
13 lactulose.

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25 Belgium).

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1 **Figure Legends**

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4 **Figure 1:** Schematic representation of the metabolism of lactose-[¹⁵N, ¹⁵N]-ureide

5

6 **Figure 2:** Appearance of glucose-[¹⁵N, ¹⁵N]-ureide in the different urine collections after oral
7 administration of lactose-[¹⁵N, ¹⁵N]-ureide (horizontal bar = median)

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9 **Figure 3:** Cumulative % dose glucose-[¹⁵N, ¹⁵N]-ureide recovered in 24h (horizontal bar = median)

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11 **Figure 4:** Individual urinary excretion pattern of glucose-[¹⁵N, ¹⁵N]-ureide over 24h before, during and
12 after lactulose intake

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Table 1: Influence of short-term (ST) and long-term (LT) lactulose administration on the urinary and faecal excretion of glucose- ^{15}N , ^{15}N]-ureide (GUR) (n=13)

		Collection	Baseline	Lactulose (ST)	Lactulose (LT)	p-value
% dose GUR rec	Urine	0-24h	12.91 (IQR 11.26-14.15)	12.36 (IQR 10.35-13.99)	13.17 (IQR 11.62-14.80)	NS
		24-48h	4.70*	ND	4.06*	NS
	Faeces	72h	ND	ND	ND	NS

* In 1 volunteer, GUR was still present in the 24-48h urine fraction.

Table 2: Correction of the urinary ^{15}N -data (% dose/48h) for the presence of glucose- ^{15}N , ^{15}N]-ureide before and after short-term (ST) and long-term (LT) lactulose intervention

		Baseline	ST Lactulose	p-value	LT Lactulose	p-value
Cum ^{15}N rec (%dose/48h)	Before	52.62	29.27		43.18	
	correction	(IQR 39.48-56.06)	(IQR 20.96-34.60)	p=0.002	(IQR 32.83-49.23)	p=0.013
	After	37.95	18.40		29.26	
	correction	(IQR 25.25-44.29)	(IQR 5.39-21.42)	p<0.001	(IQR 19.77-34.60)	p=0.013







