Accurate discrimination of Hartnup disorder from other aminoacidurias using a diagnostic ratio

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ABSTRACT

Introduction: Hartnup disorder is caused by a deficiency of the sodium dependent B⁰ AT¹ neutral amino acid transporter in the proximal kidney tubules and jejunum. Biochemically, Hartnup disorder is diagnosed via amino acid excretion patterns. However, these patterns can closely resemble amino acid excretion patterns of generalized aminoaciduria, which may induce a risk for misdiagnosis and preclusion from treatment. Here we explore whether calculating a diagnostic ratio could facilitate correct discrimination of Hartnup disorder from other aminoacidurias.

Methods: 27 amino acid excretion patterns from 11 patients with genetically confirmed Hartnup disorder were compared to 68 samples of 16 patients with other aminoacidurias. Amino acid fold changes were calculated by dividing the quantified excretion values over the upper limit of the age-adjusted reference value.

Results: Increased excretion of amino acids is not restricted to amino acids classically related to Hartnup disorder (“Hartnup amino acids”, HAA), but also includes many other amino acids, not classically related to Hartnup disorder (“other amino acids”, OAA). The fold change ratio of HAA over OAA was 6.1 (range: 2.4–9.6) in the Hartnup cohort, versus 0.2 (range: 0.0–1.6) in the aminoaciduria cohort (p < .0001), without any overlap observed between the cohorts.

Discussion: Excretion values of amino acids not classically related to Hartnup disorder are frequently elevated in patients with Hartnup disorder, which may cause misdiagnosis as generalized aminoaciduria and preclusion from vitamin B3 treatment. Calculation of the HAA/OAA ratio improves diagnostic differentiation of Hartnup disorder from other aminoacidurias.

1. Introduction

Aminoacidurias are caused by defective amino acid transport across the renal epithelium. Inborn errors of amino acid transporters include lysinuric protein intolerance (LPI) (MIM #222700), cystinuria (MIM #220100), iminoglycinuria (MIM #242600), dicarboxylic amino aciduria (MIM #222730) and Hartnup disorder (MIM #234500). Next to defective amino acid transporters, transport of amino acids can also be impaired by general dysfunction of the renal tubule, as occurs for example in Fanconi syndrome [5] and Lowe syndrome [8]. Aminoacidurias are biochemically classified according to their specific amino acid excretion pattern.

Hartnup disorder has an estimated frequency of 1:20.000 [9] and is caused by a deficiency of the sodium dependent B⁰ AT¹ neutral amino acid transporter, encoded by SLC6A19. This transporter is mainly expressed in the brush border membrane of the proximal kidney tubules and in the jejunum [7,12,13]. The disorder is biochemically characterized by increased excretion of neutral amino acids including alanine, serine, threonine, valine, leucine, isoleucine, phenylalanine, tyrosine, asparagine, glutamine, tryptophan, histidine and citrulline, whereas excretion of other amino acids is reported to be less affected (Nanto-Salonen et al. 2006, Vademecum Metabolicum).

The impaired renal and intestinal transport of neutral amino acids is a risk factor for developing amino acid deficiencies, tryptophan deficiency in particular. As tryptophan is the precursor for serotonin and nicotinamide, also known as vitamin B3, the clinical symptoms of
### Table 1
Amino acid excretion of patients with Hartnup disorder.

| Age group | Upper limits age groups | Patient 1 | Patient 2 | Patient 3 | Patient 4 |
|-----------|-------------------------|-----------|-----------|-----------|-----------|
|           | 1.1 1.2 1.3 1.4 1.5     | 2.1 2.2 2.3 2.4 | 3.1 3.2 3.3 3.4 3.5 3.6 | 4.1 4.2 4.3 4.4 4.5 |
| Age group | 05 06 07                | 07 07      | 07 07     | 07 07     | 07 07     |
| Alanine   | 1099 1443 1102 539 969 | 407 264 472 479 | 1026 621 1020 968 1041 1108 | 474 590 585 460 1429 |
| Serine    | 1471 2015 1220 724 1145 | 754 536 700 655 | 1550 1002 1108 1154 997 988 | 736 745 892 672 1631 |
| Threonine | 348 325 455 423         | 891 724 506 550 564 567 | 513 376 485 374 854          |
| Valine    | 344 1049 329 381 550   | 500 560 635 663 | 605 388 363 338 416 389 | 441 349 377 243 803 |
| Leucine   | 98 776 141 281          | 193 239 287 332 | 199 220 81 117 151 138 | 181 67 87 33 189 |
| Isoleucine| 155 510 143 262         | 131 175 197 229 | 189 168 88 88 115 131 | 192 105 135 50 199 |
| Phenylalanine | 119 353 95 122 156 | 151 134 161 175 | 152 128 84 76 96 101 | 109 95 110 37 123 |
| Tyrosine  | 623 949 536 432 616   | 299 233 254 249 | 717 488 377 431 521 272 | 375 327 436 231 656 |
| Asparagine| 760 972 520 569        | 317 316 437 357 | 504 382 417 430 389 | 388 355 441 312 492 |
| Glutamine | 2658 4684 3176 1190 1873 | 1571 1859 1257 1793 | 3009 1422 1858 1560 2030 1443 | 1052 1197 1272 997 879 |
| Histidine | 836 1259 732 804       | 436 424 396 443 | 58 710 602 656 640 515 | 489 557 587 477 713 |
| Citruline | 38 134 34 90           | 54 51 44 53     | 50 50 23 31 18 14 | 8 2 1 8 26 |
| Arginine  | 237 751 154 202 359   | 67 59 80 103    | 118 208 57 70 59 57 | 87 37 40 32 84 |
| Lysine    | 38 134 34 90           | 54 51 44 53     | 50 50 23 31 18 14 | 8 2 1 8 26 |
| Aspartic acid | 237 751 154 202 359 | 67 59 80 103    | 118 208 57 70 59 57 | 87 37 40 32 84 |
| Glutamic acid | 237 751 154 202 359 | 67 59 80 103    | 118 208 57 70 59 57 | 87 37 40 32 84 |
| Glycine   | 237 751 154 202 359   | 67 59 80 103    | 118 208 57 70 59 57 | 87 37 40 32 84 |
| Cysteine  | 24 71 22 26 34        | 65 31 45 46     | 66 60 28 24 21 20 | 32 20 26 25 40 |
| Ornithine | 52 100 13 25           | 13 21 12 32     | 75 22 475 40 19 22 | 71 193 78 17 46 |
| α-aminobutyric acid | 28 56 43 50 | 25 38 32 35 | 54 48 22 35 24 25 | 44 42 42 23 53 |

Amino acid excretion patterns of patients with Hartnup disorder in mmol/mmol creatinine. Age group: age adjusted reference values were obtained from literature, taking into account seven age groups: first week (1), first week till first month (2), first month till four months (3), four months till two years (4), two years till ten years (5), ten years till eighteen years (6) and above eighteen years (7) [1]. Hartnup amino acids: alanine to glutamine. Other amino acids: arginine to α-aminobutyric acid.
Hartnup disorder are those of a nicotinamide deficiency. Reported symptoms include dermatological symptoms, particularly a pellagra-like rash and light-sensitive dermatitis, intermittent cerebellar ataxia and psychiatric symptoms as emotional instability, delirium and hallucinations [3]. All symptoms respond well to treatment with vitamin B3 [15].

To date, many individuals remain asymptomatic [14], likely because of a sufficiently high intake of protein, tryptophan, vitamin B3 or a combination thereof [3]. However, even in asymptomatic patients, accurate diagnosis of Hartnup disorder is essential [4,14], to ensure correct differentiation of Hartnup disorder from other aminoacidurias, which would demand alternative diagnostic trajectories. Biochemically, Hartnup disorder is diagnosed based on the amino acid excretion profile. Here, we demonstrate that patients with Hartnup disorder may present with an amino acid excretion pattern that closely resembles generalized aminoaciduria [3,3,6]. We show that this potential misdiagnosis can be overcome by quantification, visualization and computation of urinary amino acids, enabling us to correctly discriminate Hartnup disorder from other causes of aminoaciduria.

2. Methods

2.1. Patient inclusion

Twenty-seven urine samples of 11 patients with Hartnup disorder were analyzed. Four of these patients were included at the University Medical Centre Utrecht. Hartnup disorder was confirmed through PCR amplification followed by Sanger sequencing of SLC6A19. Patient 1 is compound heterozygous for the pathogenic SLC6A19 c.517G > A (p.Asp173Asn) and c.1173+2T > G (p.?) mutations [13]. Patient 2, 3 and 4 are homozygous for the common SLC6A19 c.517G > A (p.Asp173Asn) mutation. Patient 3 and 4 are siblings. To extend the Hartnup disorder cohort, amino acid excretion patterns of seven patients with Hartnup disorder previously published by Potter et al. were included [11]. Hartnup disorder was genetically confirmed in these patients by Seow et al. [13]. One patient (patient 2/II) was excluded, because of heterozygosity for cystinuria type II. In the patients from Potter et al., amino acid excretion patterns were quantified using a Beckman 6300 amino acid analyzer [11].

To differentiate Hartnup disorder from other aminoacidurias, 10 samples of 7 patients with generalized aminoaciduria, 16 samples of 2 patients with LPI and 42 samples of 7 patients with cystinuria were included, coming to a combined aminoaciduria cohort of 68 samples of 16 patients, all from the University Medical Centre Utrecht. All diagnoses were genetically confirmed.

2.2. Quantification of amino acid excretion

Amino acid excretion was quantified at the University Medical Centre Utrecht using a Biochrom amino acid analyzer (Isogen Life Sciences, de Meern, the Netherlands) according to diagnostic standards. Amino acid excretion was expressed in mmol/mol creatinine. Age-adjusted reference values were obtained from literature, taking into account seven age groups: first week (1), first week till first month (2), first month till four months (3), four months till two years (4), two years till ten years (5), ten years till eighteen years (6) and above.
2.3. Statistical analysis

Amino acid fold changes were calculated by dividing the quantified excretion values over the upper limit of the age-adjusted reference value. Amino acids were grouped into Hartnup amino acids (HAA) versus other amino acids (OAA). HAA included alanine, serine, threonine, valine, leucine, isoleucine, phenylalanine, tyrosine, asparagine, glutamine, tryptophan, histidine and citrulline (Nanto-Salonen et al. 2006, Vademecum Metabolicum), and OAA included arginine, lysine, aspartic acid, glutamic acid, glycine, cysteine, methionine, proline, ornithine, taurine and alpha-aminobutyric acid. Statistical analyses were performed using R programming language. Results were visualized in both heatmaps and scatter plots. Data files and R code are available upon request.

3. Results

Amino acid excretion patterns of the patients with Hartnup disorder in the Utrecht cohort are presented in Table 1. This table displays that increased excretion of amino acids is not restricted to HAA, but also includes many OAA, including cystine (in all 20 samples), alpha-aminobutyric acid (in 19/20), glycine and lysine (both in 17/20), citrulline and glutamic acid (both in 15/20), aspartic acid (in 14/20) and arginine (in 12/20). This precludes discrimination of Hartnup disorder from other aminoacidurias and induces the risk of misclassification as generalized aminoaciduria (Fig. 1A).

We assessed whether the degree of increase could aid differentiation of Hartnup disorder from other aminoacidurias. Indeed, quantification of amino acid excretion values and computation of amino acid fold changes enabled visual discrimination between Hartnup disorder and generalized aminoaciduria (Fig. 1B). The heatmap demonstrating the fold changes shows that, unlike in patients with generalized aminoaciduria, in both Hartnup disorder cohorts the fold changes of HAA were strikingly higher than the fold changes of OAA. LPI and cystinuria could also be recognized easily: LPI based on clear increases of arginine, lysine and ornithine and cystinuria based on the additional increase of cystine (Fig. 1B).

In patients with Hartnup disorder, the mean fold change of HAA ranged from 8.1 for phenylalanine to even 49.7 for valine. Histidine, an amino acid classically related to Hartnup disorder, was unexpectedly only slightly elevated, with a mean fold change of 3.1. Surprisingly, this was even lower than in generalized aminoaciduria, for which a mean fold change of 7.8 was calculated. Histidine excretion, while increased over the mean fold change of glycine and histidine clearly discriminated Hartnup disorder from generalized aminoaciduria, with a mean Ala/(Gly + His) ratio in the Hartnup cohort of 4.3, contrasting with a mean Ala/(Gly + His) ratio of only 0.7 in the generalized aminoaciduria cohort. Even for this limited ratio, there was no overlap between the two cohorts, as the minimum value in the Hartnup cohort was 2.7, while the maximum value for the ratio in generalized aminoaciduria was 1.3 (Table 2, Fig. 2B).

4. Discussion

In this study we demonstrated that quantitative assessment of the degree of the increases, rather than qualitative assessment of increases of amino acid excretion, enhances correct discrimination of Hartnup disorder from other aminoacidurias. We introduce the HAA/OAA ratio as a new and easily applicable diagnostic tool to discriminate Hartnup disorder from other aminoacidurias. Moreover, we demonstrate that even the limited Ala/(Gly + His) ratio, requiring quantification of only three amino acids, can distinguish Hartnup disorder from generalized aminoaciduria.

Quantification of all urinary amino acid concentrations revealed that, in addition to the amino acids reported to be excreted excessively in Hartnup disorder (Nanto-Salonen et al. 2006, Vademecum Metabolicum), cystine, alpha-aminobutyric acid, glycine, lysine, citrulline, glutamic acid, aspartic acid and arginine can be increased as well in the urine of patients with Hartnup disorder [2,3,6]. Unexpectedly, the excretion of histidine, an amino acid of which the intestinal uptake and tubular reabsorption is expected to be affected (Nanto-Salonen et al. 2006, Vademecum Metabolicum), was increased only modestly in 18/20 samples, contrasting with the extent of the excretion of HAA. Whether the complete range of amino acids excreted by patients with Hartnup disorder can be explained by a broader substrate specificity of the B₀ AT1 transporter than currently described, or whether the aberrant transport of amino acids in the proximal tubule of patients with Hartnup disorder affects (saturation of) other amino acid

| Table 2 |
|------------------------|------------------------|------------------------|------------------------|
| HAA/OAA ratio and Ala/(Gly + His) ratio in Hartnup disorder versus other aminoacidurias. | | | |
| | Patients | Samples | FULL RATIO: HAA/OAA | LIMITED RATIO: Ala/(Gly + His) |
| | Mean FC HAA | Mean FC OAA | HAA/OAA ratio (mean (range)) | FC Alanine and His | Mean FC Gly and His | Ala/(Gly + His) ratio (mean (range)) |
| Hartnup disorder – Utrecht | 4 | 20 | 19.7 3.7 | 6.1 (3.1–9.2) | 10.5 | 2.5 | 4.2 (2.7–9.8) |
| Hartnup disorder – Potter et al. | 7 | 7 | 12.0 1.9 | 6.2 (2.4–9.6) | 11.9 | 2.5 | 4.7 (4.0–6.2) |
| Hartnup disorder – Combined | 11 | 27 | 17.7 3.2 | 6.1 (2.4–9.6) | 10.9 | 2.5 | 4.3 (2.7–9.8) |
| Generalized aminoaciduria | 7 | 10 | 7.2 6.2 | 1.0 (0.5–1.6) | 4.7 | 7.2 | 0.7 (0.4–1.3) |
| Lysinuric protein intolerance | 2 | 16 | 1.6 10.5 | 0.2 (0.1–0.5) | 2.5 | 1.7 | 1.8 (1.0–3.5) |
| Cystinuria | 7 | 42 | 1.2 37.0 | 0.0 (0.0–0.1) | 0.7 | 1.6 | 0.6 (0.2–1.7) |
| Aminoaciduria – Combined | 16 | 68 | 2.3 25.7 | 0.2 (0.0–1.6) | 1.8 | 2.6 | 0.9 (0.2–3.5) |

Abbreviations: Ala: alanine; FC: fold change; Gly: glycine; HAA: Hartnup amino acids; His: histidine; OAA: other amino acids.

Bold signifies P < 0.0001
transporters remains to be elucidated.

It is of interest that the Hartnup disorder cohort derived from Potter et al. described a less generalized aminoaciduria in their patients, even though the same reference values were used. We speculate that differences in the patient age at time of sampling (all adults in Potter et al.) may affect the amino acid excretion pattern. Moreover, differences in nutrition, particularly a higher protein intake, could have contributed to the here observed more pronounced generalized aminoaciduria [3].

Still, despite these differences, the distribution of the calculated ratios is comparable, corroborating the accuracy of these ratios in discriminating Hartnup disorder from other aminoacidurias. However, given the relatively small sample sizes of the two cohorts, it would be of interest to assess the generalizability of the calculated ratio in another, independent cohort of patients with Hartnup disorder.

As nutritional intake, including protein intake, has been increasing in many countries over the past decades [3], the degree to which individuals with Hartnup disorder demonstrate amino acid excretion patterns mimicking generalized aminoaciduria might increase as well, explaining why quantification of urinary amino acid concentrations was not required in the past, but is expedient now.

In conclusion, we here report that excretion values of amino acids not classically related to Hartnup disorder, are frequently elevated in patients with Hartnup disorder. This may induce a risk of misdiagnosis as generalized aminoaciduria and preclusion from vitamin B3 treatment. By changing the focus from absolute to relative increase of amino acid excretion and by calculating the HAA/OAA ratio, we introduce a diagnostic tool that enhances correct discrimination of Hartnup disorder from other aminoacidurias.

Take home message

The fold change ratio of Hartnup amino acids over other amino acids ensures correct diagnostic differentiation of Hartnup disorder from other aminoacidurias.

Guarantor for the article

J.J.M. Jans declares that she will accept full responsibility for the work and the conduct of the study. She had access to all the data and controlled the decision to publish.

Compliance with ethics guidelines

All procedures followed were in accordance with the ethical standards of the University Medical Center Utrecht and with the Helsinki Declaration of 1975, as revised in 2000. No patient informed consent was required for this study, since all patient data was anonymized.
Details of contributions of individual authors

J.J.M.J. and P.M.v.H. conceptualized and designed the study and supervised data collection and analysis. J.J.M.J., H.C.M.T.P. and M.G.M.S.v.d.V. collected the data. H.A.H. performed the data analysis and drafted the initial manuscript. All authors critically reviewed the initial manuscript and approved the final version as submitted.

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