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CLINICAL RESEARCH ARTICLE

Hydrocortisone Affects Fatigue and Physical Functioning Through Metabolism of Tryptophan: A Randomized Controlled Trial

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Precis: Sorgdrager et al. showed that hydrocortisone treatment affected symptoms of fatigue and physical functioning by altering tryptophan metabolism in patients with secondary adrenal insufficiency.

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Abstract:

Context:

Hydrocortisone (HC) treatment influences health-related quality of life (HRQOL) in secondary adrenal insufficiency (AI). Glucocorticoids regulate tryptophan metabolism through the kynurenine pathway which modulates mood and energy homeostasis.

Objective:

This study investigated whether tryptophan metabolism mediated the effect of HC dose on HRQOL in patients with secondary AI.

Design, Setting and Patients:

Forty-seven patients with secondary AI participated in this double-blind randomized controlled cross-over trial in the University Medical Center Groningen.

Intervention:

Patients were treated for two 10-week periods with a daily HC dose of 0.2 - 0.3 mg and 0.4 - 0.6 mg/kg body weight, respectively.

Main outcome measures:

Diary data and questionnaires were used to assess HRQOL. Tryptophan, kynurenine and 3-hydroxykynurenine were measured in serum and dialyzed plasma and the kynurenine to tryptophan (kyn/trp) ratio was calculated.

Results:

A higher dose HC was associated with increased levels of tryptophan (95% CI for mean difference 0.37 to 12.5, $p= .038$), reduced levels of kynurenine (95% CI -0.49 to -0.10, $p= .004$) and 3-hydroxykynurenine (95% CI -10.6 to -2.35, $p= .003$) and a reduced kyn/trp ratio (95% CI -0.84 to -0.50, $p < .001$). The kyn/trp ratio mediated the effect of a higher dose HC on fatigue ($p = .041$) and physical functioning ($p = .005$).

Conclusion:

Metabolism of tryptophan through the kynurenine pathway is reduced after a 10-week treatment with a higher dose HC and plays a role in the effect of HC on fatigue and physical functioning in patients with secondary AI.

Keywords: Tryptophan; kynurenine; glucocorticoids; adrenal insufficiency; fatigue; physical functioning

1. Introduction

Individuals who suffer from adrenal insufficiency (AI) report reduced quality of life due to a variety of mental and physical symptoms such as fatigue, depression and physical disabilities (1,2). Because of inadequate production of glucocorticoids, these persons require lifelong glucocorticoid replacement with hydrocortisone (HC). Health-related quality of life (HRQOL), a concept that encompasses physical, mental and social functioning in relation to disease, was shown to be affected by changes in the HC dose (3), the dose scheme (4) and the mode of HC administration (5). The mechanisms behind this are poorly understood.

Metabolism of tryptophan is regulated by glucocorticoids and is tightly linked to mood and energy homeostasis. Tryptophan is an essential amino acid that drives *de novo* synthesis of serotonin and nicotinamide adenine dinucleotide (NAD). Serotonin modulates behavioral and neuropsychological processes whereas NAD is a co-factor with several cellular functions crucial for energy homeostasis (6,7). Around 95% of the available tryptophan is processed through the kynurenine pathway to produce NAD. Kynurenine plays a role in immune functioning and several downstream metabolites, including 3-hydroxykynurenine, kynurenic acid and quinolinic acid, play a role in glutamate functioning (8). Changes in the functioning of serotonin, tryptophan, kynurenine and NAD have been described in a wide variety of inflammatory, metabolic, neurodegenerative and psychiatric diseases (9–11). These findings suggest that tryptophan metabolism modulates mental and physical functioning by affecting distinct biological processes.

Systemic levels of tryptophan reflect the rate at which tryptophan is processed through the kynurenine pathway in various organs (12). The first and rate-limiting step of the kynurenine pathway is catalyzed by two enzymes: tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO). TDO is highly expressed in hepatocytes and regulates systemic tryptophan levels. Extrahepatic activity of TDO and IDO - e.g. in blood cells, kidneys, lungs, spleen, muscles, lymph nodes and adipose tissue – is thought to regulate systemic levels of kynurenine and its downstream metabolites (13,14). IDO activity, which is low under normal circumstances, is induced by pro-inflammatory cytokines (15). TDO is induced by glucocorticoids (12). In healthy individuals, tryptophan levels were reduced shortly after administration of potent glucocorticoids (16). On the contrary, we recently showed that in recurrently depressed individuals, increased levels of basal endogenous glucocorticoids were associated with a decreased kynurenine to tryptophan (kyn/trp) ratio (17). These contrasting findings support the idea that acute and

more sustained exposure to glucocorticoids could have a differential effect on tryptophan metabolism along the kynurenine pathway (10,18).

In a randomized controlled cross-over study, our group showed that a 10-week treatment with a higher dose HC improved HRQOL in persons with secondary AI. On a higher dose, patients reported fewer symptoms of depression, pain, general fatigue and mental fatigue in addition to increased motivation and better physical functioning, vitality and perceived health (19). We hypothesized that the effect of HC on mental and physical health in these patients could be mediated by metabolism of tryptophan through the kynurenine pathway. Using data from this cohort, the aim of the current study was twofold: i) to investigate the effect of lower and higher physiological levels of glucocorticoids on plasma concentrations of bound and unbound tryptophan, kynurenine and 3-hydroxykynurenine and ii) to determine whether the kynurenine pathway mediated the effects of HC on HRQOL that were previously described in these patients.

2. Subjects and Methods

Subjects

For the current study, 63 individuals with secondary adrenal insufficiency were recruited from a population of 624 pituitary patients from the endocrine outpatient clinic of the University Medical Center Groningen (UMCG), a tertiary referral center for pituitary surgery in the Netherlands. Inclusion criteria were (i) age between 18 and 75, (ii) weight between 50 and 100 kg, (iii) a minimal time interval of a year between study entry and tumor treatment (surgery and/or radiotherapy), (iv) a minimal duration of glucocorticoid substitution therapy of six months prior to the study entry and (v) adequate treatment of other pituitary hormone deficiencies for at least six months prior to entry of the study. Secondary AI was diagnosed according to internationally accepted biochemical criteria and principally included early morning (0800 – 0900 h) serum cortisol measurements and, if necessary, an insulin tolerance test. In our center, the applied early morning cut-off cortisol level (< 230 nmol/l) has been previously validated against an insulin tolerance test with an internationally accepted cut-off level (< 500 nmol/l) providing a 100% specificity for adrenal insufficiency (20). Out of all patients that were reviewed for study eligibility, the medical evaluation included unstimulated cortisol measurements in approx. 560 cases (± 90%). A

four-week run-in phase was included during which patients using cortison acetate were converted to a bioequivalent dose of HC. A total of 60 patients completed the run-in phase. Additional methods (exclusion criteria, study design, safety and sample size calculation), in accordance with CONSORT guidelines, are described in detail elsewhere (20).

The study protocol was approved by the local ethics committee and the study is registered with ClinicalTrials.gov, number NCT01546922. All patients provided written informed consent.

Intervention

In this double-blind cross-over study, patients were randomized using a computer-generated treatment allocation list with a block size of four to receive tablets containing either a lower or higher dose HC in the first treatment period by Tiofarma Inc.. The randomization code was known by the local pharmacy of the UMCG in case premature unblinding was necessary. Both the investigator and the participant were blinded for the HC dose and group. 'Group 1' received a lower dose of HC for 10 weeks, followed by a higher dose for an additional 10 weeks. 'Group 2' first received a higher dose of HC, followed by a lower dose. Dosing schemes were adjusted for weight. On the lower dose, patients received a cumulative daily dose of 15 - 20 mg HC (respectively 7.5, 5.0 and 2.5 mg for patients weighing 50 - 74 kg; 10.0, 5.0 and 2.5 mg for patients weighing 75 - 84 kg; and 10.0, 7.5 and 2.5 mg for patients weighing 85 - 100 kg). On the higher dose, patients received the double amount (respectively 15.0, 10.0 and 5.0 mg for patients weighing 50 - 74 kg; 20.0, 10.0 and 5.0 mg for patients weighing 75 - 84 kg; and 20.0, 15.0 and 5.0 mg for patients weighing 85 - 100 kg). The total daily amount was divided over three oral dosages and had to be taken before breakfast, before lunch and before dinner. Upon intercurrent illness or fever, patients were allowed to double or triple their HC dose according to predefined criteria. This was allowed for a maximum of seven days (i.e. 10% of the study time and of the cumulative HC dose) excluding the week preceding the visits. Compliance with the study medication was assessed by (i) checking patient's daily medication diaries, (ii) counting the tablets returned at the end of each study period and (iii) comparing cortisol concentration in plasma between the two study periods. Out of 60 patients, 47 individuals (22 of Group 1 and 25 of Group 2) completed the study period and were used for analyses.

Tryptophan, kynurenine, 3-hydroxykynurenine and the kynurenine to tryptophan ratio

At the end of each treatment period, fasting blood samples were drawn. Plasma and serum samples were stored at -80°C. For the determination of unbound plasma tryptophan, kynurenine and 3-hydroxykynurenine, plasma equilibrium dialysis was performed at the department of Laboratory Medicine of the University Medical Center Groningen using 10-kD cellulose membranes (Harvard Apparatus) as discussed previously (21). Next, tryptophan, kynurenine and 3-hydroxykynurenine concentrations were measured (total levels in serum and unbound levels in dialyzed plasma) at the department of Laboratory Medicine of the University Medical Center Groningen using a validated automated online solid-phase extraction-liquid chromatographic-tandem mass spectrometric (XLC-MS/MS) method with deuterated internal standards (22). The kyn/trp ratio was calculated for all included participants by dividing the level of kynurenine by the level of tryptophan and multiplying this value by 100. Samples of both treatment periods were available for 43 out of 47 patients (90 out of 94 samples).

Health-related quality of life

Several self-administered tools were used to measure domain-specific and generic HRQOL. First, patients were instructed to keep a daily mood and symptom diary throughout the whole study period consisting of items of the Patient Health Questionnaire-9 (PHQ-9) and the Patient Health Questionnaire-15 (PHQ-15) (23,24). The PHQ-9 consists of nine items that correspond to the DSM-IV diagnostic criteria for major depressive disorder whereas the PHQ-15 includes 15 items closely related to the most prevalent DSM-IV symptoms of somatization disorder. The depression score included all nine items of the PHQ-9.To produce a composite pain score, we combined all five PHQ-15 items that consider pain ("stomach pain", "back pain", "joint pain", "headache" and "chest pain"). Both questionnaires asked the patient to rate symptom severity over the preceding 24 hours on a scale from 1 to 7. Daily scores on depression and pain therefore ranged from 9 to 63 and 5 to 35 respectively. A daily score was not computed in the case of one or more missing items. A weekly score was calculated for both scales by taking the average of the available daily scores for each study week. For the analyses, diary data from

the final four weeks of each treatment period were pooled and averaged to give a stable measure of symptom severity.

Patients were also instructed to fill out questionnaires at home on the day before the end of each study period regarding their mental and physical health. The Hospital Anxiety and Depression Scale (HADS) was used to evaluate symptoms of depression and anxiety. It consists of 14 items and originally asks the patient to rate symptom severity over the past week on a scale from 0 to 3 (25). An adapted version asking to rate severity of symptoms over the past four weeks was used. The Multidimensional Fatigue Inventory 20 (MFI-20) was used to rate symptoms of fatigue over the past days on five subdimensions (general fatigue, physical fatigue, reduced activity, reduced motivation and mental fatigue) with scores ranging from 4 to 20 on each subscale (26). Finally, the RAND-36 was used as generic tool to assess general health perception on eight domains (physical functioning, role limitation due to physical health problems, bodily pain, general health perception, vitality, social functioning, role limitations due to emotional problems and mental health) in the past four weeks (with the exception to general health perception), which are each scored on a range from 0 to 100 (27). Results from the HADS, MFI-20 and RAND-36 were standardized using Dutch normative data (z-scores) (matched on age and sex) (5,28–30).

Higher scores on PHQ-9, PHQ-15, HADS and MFI-20 suggest worse quality of life or increased symptom severity, whereas a higher score on RAND-36 indicates better perceived health. Diary data (PHQ-9 and PHQ-15) were available for both study periods for 45 out of 47 patients due to missing data. The questionnaire data (HADS, MFI-20 and RAND-36) were available for all patients for both study periods.

Statistical analyses

Analyses were performed using IBM SPSS statistics 23 (IBM Corp, 2014) and Stata13 (StataCorp, 2013). Number (n), mean and standard deviation of the mean (SD) or percentages are reported for the baseline characteristics. To compare baseline characteristics we used Chi-square tests for dichotomous variables and independent sample t-tests for continuous variables.

To investigate the treatment effect of the two HC doses on measurements of tryptophan metabolism, we used paired sample t-tests. Values were transformed in case of non-normality and are

reported after back-transformation. List-wise exclusion was used in case of missing values. A 95 % confidence interval (CI) for the mean difference and a *p*-value are reported.

Next, we used a mediation analysis to determine whether the effect of a lower or a higher dose HC on mental and physical health was mediated by the kyn/trp ratio (31). In order to reduce the number of analyses (thus reducing the chance of type 1 errors), only the outcome measures that were previously shown to be affected by HC dose within this cohort were included in this analysis (symptoms of depression and pain obtained from diary data (PHQ-9 and PHQ-15), depressive symptoms (HADS, z-score), general fatigue, mental fatigue, reduced motivation (MFI, z-score) and physical functioning, vitality and general health (RAND, z-score)) (19). Using linear mixed modeling, we first fitted models investigating the association between lower and higher dose HC and the kyn/trp ratio as dependent variable (path a). Then, we constructed two models for each outcome measure (mental and physical health scores) investigating the association between (i) the kyn/trp ratio and the outcome (path b) controlled for HC dose (path c') and (ii) HC dose and the outcome (path c) (Figure 1). The mediation effect (indirect effect) (path a * path b), the direct effect (path c') and the total effect (path c) are reported. A *p*-value (based on the partial posterior method) for the indirect effect was calculated (32). In case of a statistical significant indirect effect, the ratio between the indirect effect and the total effect was constructed as a measure of the effect size for the mediation effect (33).

Robust standard errors were estimated to adjust for non-normality of the residuals. Since mixed models can deal appropriately with missing values, these were not imputed. All models were adjusted for age, gender and included a random intercept. In cross-over studies, a treatment effect can be accompanied by a period effect and a carry-over effect. In two-period, two-treatment cross-over studies, the carry-over effect can be expressed as a period-by-treatment interaction effect. All models were adjusted for a period effect (included as a dichotomous variable). Routine testing for carry-over effects is not recommended due to low statistical power (34). However, due to the fact that we could not include a washout period between the two treatments, we explored the possibility of carry-over effects by adding a period-by-treatment interaction effect to all mixed models and testing its significance. In case of statistical significance, carry-over effects were included in the models. Both the inclusion of a random slope and the determination of the applied covariance structure were based on goodness-of-fit principles by comparing the Bayesian information criterion (BIC) of the models. Regression coefficients (B) and their

respective robust standard error (SE) are reported as a measure of association. A p-value below the 0.05 level was considered statistically significant.

Figure 1 Graphical display of mediation model
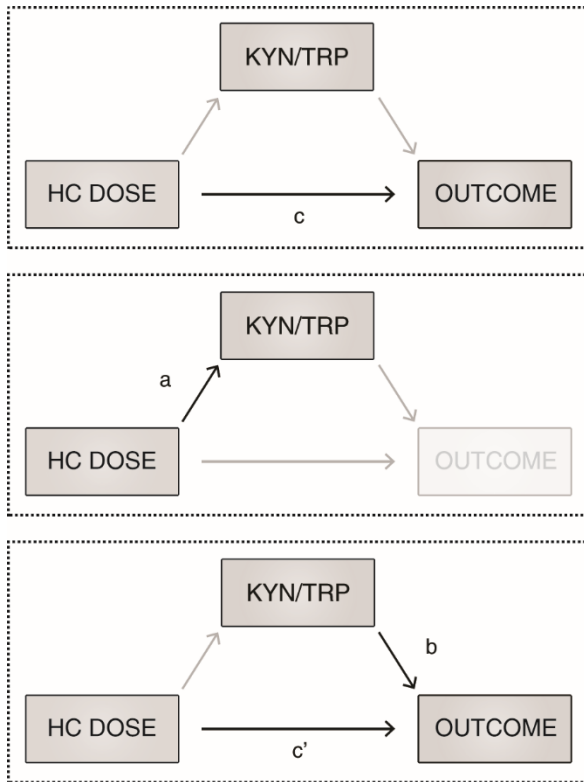
Graphical display of the mediation analysis. The different letters (a, b, c, c') denote the different paths in the analyses. Path c (the total effect) is the effect of lower and higher dose HC on the outcome variable. Path a is the effect of lower and higher dose HC on the kyn/trp ratio. Path b is the association between the kyn/trp ratio and the outcome variable controlling for HC dose. Path c' (direct effect) is the coefficient of HC dose in this same model. The mediation effect (indirect effect) is the effect of HC dose on the outcome variable through the kyn/trp ratio (path a * path b).

Abbreviations: HC, hydrocortisone; kyn/trp ratio, kynurenine to tryptophan ratio.

3. Results

Baseline characteristics

Table 1 shows the characteristics of the study sample for both patients who first received a lower dose HC followed by a higher dose HC (group 1) (n= 22) and patients who first received a higher dose HC followed by a lower dose (group 2) (n=25). These results indicated no significant differences between both groups.

Table 1 Baseline characteristics

	Group 1 (n= 22)	Group 2 (n= 25)	*p*-value
Demographics			
Gender, % female	40.9	36.0	.771
Age, years	52.5 (12.5)	50.6 (17.0)	.661
Physical parameters			
Body weight, kg	82.1 (11.8)	83.1 (15.7)	.808
BMI, kg/m^2	27.2 (2.6)	26.7 (5.0)	.683
AI parameters			
Age at diagnosis, years	30.7 (17.8)	36.4 (15.2)	.247
Surgery, %	59.1	76.0	.347
Radiotherapy, %	27.3	52.0	.136
HC treatment prior to randomization			
Duration of HC treatment, years	17.2 (12.0)	12.8 (11.0)	.193
Total daily dose HC, mg/day	24.7 (5.4)	26.0 (5.4)	.398

Table showing mean and SD or, when indicated, percentage (%) on demographics, physical parameters and disease parameters for patients included in group 1 (lower dose followed by higher dose) and group 2 (higher dose followed by lower dose).

Abbreviations: BMI, body mass index; HC, hydrocortisone; AI, adrenal insufficiency; SD, standard deviation.

Effect of hydrocortisone on tryptophan metabolism

Figure 2 displays tryptophan, kynurenine and 3-hydroxykynurenine levels and the kyn/trp ratio in patients from both groups on lower and higher dose HC. The analyses showed increased levels of tryptophan (95% CI for mean difference 0.37 to 12.5, *p*= .038) for patients on a higher dose HC, reduced levels of kynurenine (95% CI -0.49 to -0.10, *p*= .004) and 3-hydroxykynurenine (95% CI -10.6 to -2.35, *p*= 0.003), and a lowered kyn/trp ratio (95% CI -0.84 to -0.50, *p*< .001) in patients on the higher dose HC.

Figure 2 Total serum levels of tryptophan, kynurenine and 3-hydroxykynurenine and the kyn/trp ratio in secondary AI patients on lower and higher dose HC
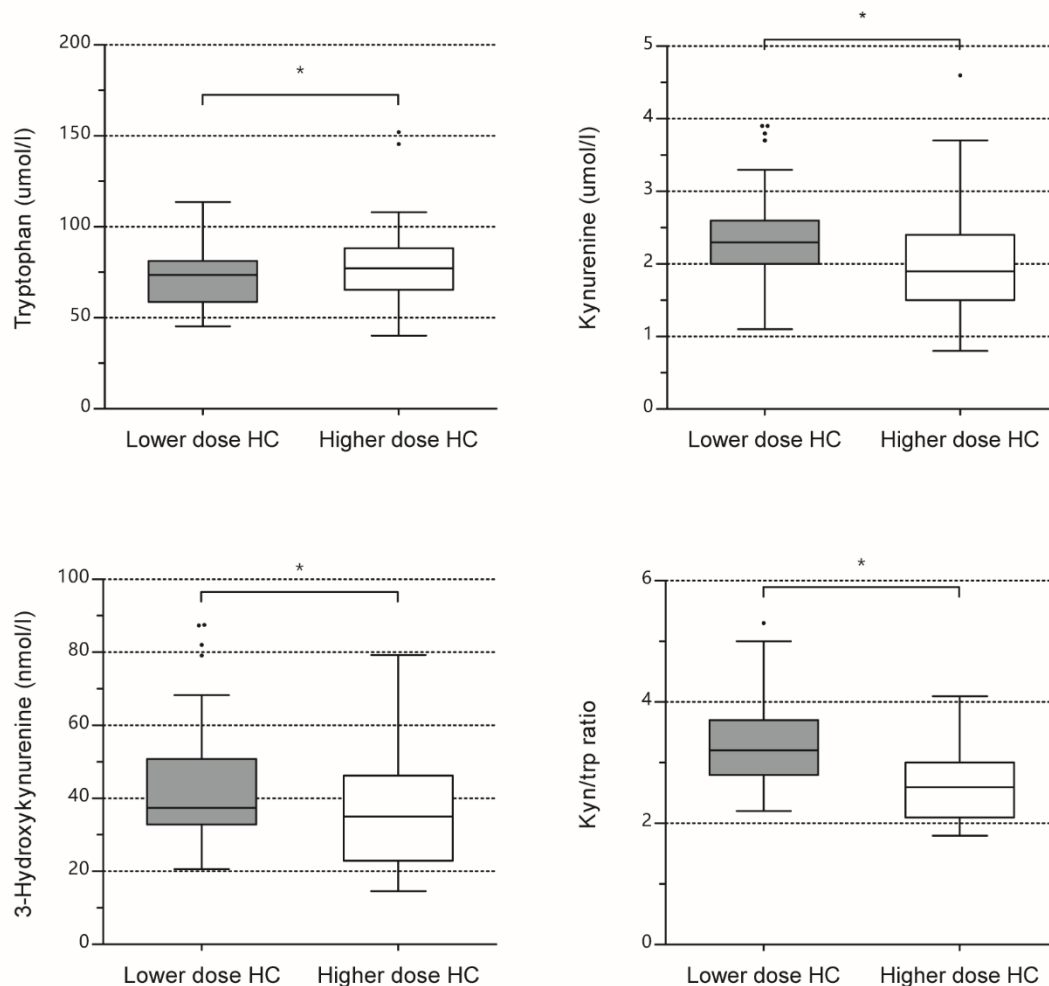

Figure showing median and interquartile range of serum tryptophan, kynurenine, 3-hydroxykynurenine and the kyn/trp ratio after lower and higher dose HC. Dots represent outliers. Asterisk (*) denotes statistical significance (p < 0.05).

The effects of HC dose on the plasma protein binding showed reduced levels of unbound kynurenine and 3-hydroxykynurenine in patients on higher dose HC, but not on tryptophan. Further analyses indicated no effect of the treatment on the percentage of unbound tryptophan (95% CI -0.91 to 2.18, $p=$ .409), kynurenine (95% CI -0.76 to 2.07, $p=$ .355) and 3 -hydroxykynurenine (95% CI -2.37 to 3.14, $p=$ .781) (Supplemental Table 1).

Mediation by the kyn/trp ratio of the effect of hydrocortisone on mental and physical health

Results on the effect of HC dose on HRQOL have been previously described in detail elsewhere (19). In short, on a higher HC dose, with regard to the diary data (PHQ-9 and PHQ-15) patients reported reduced symptoms of depression (median [interquartile range] of 9.13 [10.00 – 12.87] versus 10.63 [9.07 – 14.52] on a lower dose, Cohen's d effect size (d)= 0.2, $p=$ .041) and reduced symptoms of pain (12.32 [11.28 – 14.76] versus 12.89 [11.13 – 16.04], d= 0.2, $p=$ 0.023). With regard to the questionnaire data, patients reported reduced symptoms of depression (2.0 [0.0 – 5.0] versus 3.5 [1.0 – 6.0], d= 0.3, $p=$ 0.016) (HADS), general fatigue (10.0 [6.0 – 15.0] versus 11.0 [8.0 – 16.0], d= 0.3, $p=$ 0.004), mental fatigue (8.0 [5.0 – 13.0] versus 10.5 [5.8 – 16.0], d= 0.3, $p=$ 0.003), better motivation (8.0 [5.0 – 12.0] versus 9.5 [6.0 – 12.3], d= 0.3, $p=$ 0.021) (MFI-20) and improved physical functioning (95 [85 – 100] versus 90 [80 – 95], d= 0.1, $p=$ 0.041), vitality (70 [50 – 80] versus 65 [45 – 71], d= 0.3, $p=$ 0.025) and general health perception (65 [55 – 80] versus 60 [40 – 75], d= 0.3, $p=$ 0.013) (RAND-36). Independently of the HC dose, patients reported increased mental fatigue, reduced vitality and lower general health compared to the Dutch reference populations. On a higher dose patients scored similar to the Dutch reference population regarding symptoms of depression (HADS), general fatigue and motivation while reporting improved physical functioning. On a lower dose patients scored similar to the reference population regarding physical functioning whereas they reported increased symptoms of depression (HADS) and general fatigue and reduced motivation.

Table 2 shows the results of mediation by the kyn/trp ratio of the effect of HC on perceived mental and physical health. Linear mixed models showed that the higher dose of HC was associated

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with a reduced kyn/trp ratio in all models (path a). They further depicted an association between

increased dose of HC and decreased symptoms of depression (HADS and PHQ-9) and pain (PHQ-15)

and better physical functioning, increased vitality and improved general health perception (all RAND-36)

(path c). The effect of HC dose on general fatigue and physical functioning was found to be mediated by

the kyn/trp ratio (mediation effect; p= 0.041 and p= 0.005 respectively). For general fatigue, the ratio

between the mediated effect and the total effect was 0.89 suggesting that almost 90% of the effect of HC

dose on general fatigue was mediated by the kyn/trp ratio. For physical functioning, the mediation effect

was larger than the total effect (path c) and the ratio was 1.86. This suggests the presence of an

(unmodeled) opposing mediational effect resulting in a relatively small overall total effect. None of the

models indicated a significant carry-over effect.

Table 2 Analysis of mediation by the kyn/trp ratio of the effect of HC on mental and physical health of patients with secondary AI

Dependent	Path a	Path b	Path c	Path c'	Mediation effect
	HC dose → kyn/trp ratio	kyn/trp ratio → outcome	HC dose → outcome	HC dose → outcome	path a * path b
PHQ[a]					
Depression	-0.66 (0.09)***	-0.09 (0.48)	-0.90 (0.35)*	-0.96 (0.50)	0.06 (0.04)
Pain	"	0.25 (0.18)	-0.71 (0.21)*	-0.54 (0.26)*	-0.17 (0.02)
HADS					
Depression	-0.70 (0.09)***	<0.01 (0.15)	-0.46 (0.14)**	-0.45 (0.21)*	<0.01 (0.01)
MFI-20					
General fatigue	-0.70 (0.09)***	0.34 (0.14)*	-0.27 (0.17)	-0.03 (0.19)	-0.24 (0.01)*
Mental fatigue	"	0.18 (0.21)	0.04 (0.22)	0.18 (0.27)	-0.13 (0.02)
Reduced motivation	"	0.47 (0.70)	-1.13 (0.98)	-0.81 (0.99)	-0.33 (0.06)
RAND-36					
Physical functioning	-0.70 (0.09)***	-0.37 (0.11)***	0.17 (0.07)*	-0.12 (0.08)	0.26 (0.01)**
Vitality	"	-0.15 (0.16)	0.33 (0.14)*	0.23 (0.16)	0.10 (0.01)
General health	"	-0.04 (0.12)	0.25 (0.10)**	0.23 (0.13)	0.03 (0.01)

Table showing coefficients (B) and robust standard errors (SE) for the association between HC dose and

kyn/trp ratio (path a), the kyn/trp ratio and different outcome variables adjusted for HC dose (path b), HC

dose and the outcome variable (path c), HC dose and the outcome variable adjusted for the kyn/trp ratio (path c') and the mediation effect (calculated as the product of path a and path b (indirect effect). All models are additionally adjusted for age, gender and a period effect. * p <.05, ** p<.01, *** p <.001.

[a] Available for at least one study period for 46 out of 47 patients.

Abbreviations: HADS, Hospital Anxiety and Depression Scale; PHQ, Patient Health Questionnaire; MFI-20, Multidimensional Fatigue Inventory; RAND-36, Research And Development 36-Item Health Survey. Other abbreviations as in Figure 1.

4. Discussion

We investigated the effect of a lower and a higher dose HC on tryptophan metabolism through the kynurenine pathway in relation to mental and physical health in patients suffering from secondary AI. Using data from a randomized controlled cross-over study, our analyses showed that 10 weeks of treatment with the higher dose of HC resulted in increased levels of tryptophan, reduced levels of kynurenine and 3-hydroxykynurenine and a lowered kyn/trp ratio without affecting their binding to plasma proteins. Mediation analyses showed that the kyn/trp ratio mediated the effect of a higher HC dose on general fatigue and physical functioning.

Systemic levels of tryptophan are regulated in the liver by intrahepatic TDO activity (12). Our results thus suggest that a higher dose HC inhibits the activity of TDO. These findings seem to contradict several studies showing that administration of glucocorticoids induces expression and activity of TDO in the liver (16,35–37). Several factors could explain these contrasting findings. First, the current study was conducted in humans whereas most mentioned studies were conducted in mice. It is not known to what extent the regulation of tryptophan-degrading enzymes by glucocorticoids differs across species. Secondly, previous studies administered a single shot of highly dosed and very potent glucocorticoids whereas patients in the current study were treated for a more prolonged period (twice 10 weeks) with a physiological dose of hydrocortisone. We hypothesize that similar to the process in which immune cells get resistant to glucocorticoid activation, more prolonged exposure to elevated levels of glucocorticoids could reduce intrahepatic activity of TDO (10,17). Additionally, in germ-free mice, tryptophan levels were found to be increased while kynurenine levels were reduced (38). As such, glucocorticoid-induced changes to the microbiome could be involved in our findings (39). Finally, glucocorticoids could inhibit IDO activity as part of their immunomodulatory role. Even though IDO is not an important regulator of

systemic tryptophan levels, its inhibition could cause reduced levels of kynurenine. Besides having important metabolic effects (10), tryptophan, kynurenine and 3-hydroxykynurenine play a role in neuropsychiatric and neurodegenerative disorders as they can cross the blood-brain barrier depending on their free fraction in relation to other branched chain amino acids (40,41). Although we found no effect on plasma protein binding, the finding that HC significantly alters the equilibrium between tryptophan and kynurenine warrants further research on the long-term effects this might have on the onset of metabolic and neurological diseases in patients with secondary AI and other patients who are chronically treated with glucocorticoids.

In accordance with several studies, our results showed that the HC dose plays a role in HRQOL reported by patients with secondary AI (42). More specifically, HC dose seems to affect both the physical and the psychological domain of HRQOL, which includes energy, vitality, pain and depressive symptoms. Our analyses showed that the kyn/trp ratio mediated almost 90% of the effect of HC on symptoms of general fatigue. Fatigue has been described in relation to induced tryptophan metabolism along the kynurenine pathway in a range of other medical conditions, including cancer (43), obesity (44), stroke (45) and schizophrenia (46). Our results are in agreement with these results and highlight the importance of the tryptophan metabolism in fatigue in patients with secondary AI. Although there is little substantial evidence on the precise mechanism in which tryptophan metabolism could play a role in fatigue, hypotheses include effects of serotonin (47), kynurenine metabolites (48) and NAD (49). Secondly, our results showed that the kyn/trp ratio mediated the effect of HC on physical functioning. During physical exercise, skeletal muscles use tryptophan for energy production resulting in reduced serum levels of tryptophan and increased levels of kynurenine (10). Increased levels of tryptophan were also associated with increased aerobic fitness in athletes (50). In light of these findings, a higher dose HC could increase physical functioning by increasing kynurenine pathway activity in skeletal muscles. Despite improvements in HC replacement strategies, symptoms of fatigue and reduced physical functioning remain very prevalent in persons with AI (51,52). Our findings suggest that nutritional or pharmaceutical interventions aiming to restore the tryptophan equilibrium could be beneficial for patients with secondary AI suffering from fatigue or significantly reduced physical functioning when increasing the dose of HC is not desirable.

Strengths of this study include its study design, the use of robust statistical models and the analysis of both free and total metabolite levels. Our results are limited by the associative nature of

mediational analyses. Additionally, results from cross-over studies can be affected by period and carry-over effects. As it was not feasible to add a washout period to the study, we tested for carry-over effects (which were non-significant) and adjusted our analyses for period effects. Moreover, we did not use the AddiQOL, a disease-specific questionnaire that assesses HRQOL in adrenal patients (53), because a translated version was not yet available at the time the current trial underwent evaluation by the ethical committee. However, we believe that HRQOL was adequately evaluated in our study as we (i) used both generic and domain-specific, internationally accepted, questionnaires, (ii) monitored treatment response using longitudinal (diaries) and cross-sectional (questionnaires) measures and (iii) compared scores to Dutch reference populations. Another limitation is that some of the questionnaires considered different timeframes. To deal with this, we adapted the HADS (which originally asks about the past week) to consider the past four weeks in order to improve comparability to the RAND36, which is the most commonly used tool to measure HRQOL in adrenal patients and also considers the past four weeks (42). Similarly, we averaged the weekly scores from the final four weeks of each study period for the diary data (depression and pain, PHQ). Results from the MFI-20 may be less comparable to the other outcome measures as it considered the past few days. Finally, our analyses showed low to moderate effect sizes for the effect of HC on HRQOL. However, these effect sizes should be interpreted taking into account the chronic nature of HC therapy in AI and the fact that patients reported an effect of HC dose on several distinct aspects of HRQOL. In addition, for the RAND36 a three-point difference is regarded clinically meaningful. Patients reported a five-point difference on six out of eight domains of the RAND36. We therefore believe that at least a number of the described changes are clinically relevant.

In conclusion, this randomized controlled cross-over trial showed that a 10-week treatment with a higher dose HC lowered metabolism of tryptophan through the kynurenine pathway, which mediated the effect of HC on fatigue and physical functioning in patients with secondary AI. These results prompt further investigation of the relevance of tryptophan metabolism along the kynurenine pathway as a mechanism of glucocorticoid-induced mental and physical health impairments.

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dialysis. FJHS and EHB performed the statistical analyses. FJHS managed the literature searches and analysis and wrote the first draft of the manuscript. EHB, APvB and IPK critically revised the manuscript.

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Supplemental Table 1 Unbound plasma levels of tryptophan, kynurenine and 3-hydroxykynurenine in patients on lower and higher dose HC

	Lower dose	Higher dose	95% CI for mean difference	*p*-value
Unbound levels				
Tryptophan, umol/l	17.4 (3.70)	17.9 (3.65)	[-0.08, 0.03]	.308
Kynurenine, umol/l	0.55 (0.14)	0.46 (0.14)	[0.14 , 0.30]	< .001
3-Hydroxykynurenine, nmol/l	11.9 (4.37)	9.79 (3.79)	[0.14 , 0.33]	< .001
Unbound percentage				
Tryptophan	24.0 (3.61)	23.4 (4.29)	[-0.99 , 2.25]	.435
Kynurenine	23.1 (3.48)	22.6 (3.97)	[-1.05 , 1.99]	.538
3-Hydroxykynurenine	28.2 (6.22)	27.8 (6.26)	[-2.37 , 3.14]	.781

Table showing cumulative mean and SD of the unbound levels or percentage unbound tryptophan, kynurenine and 3-hydroxykynurenine for patients on lower dose HC and higher dose HC.

Abbreviations: As in Table 1.