The Journal of Clinical Endocrinology & Metabolism, 2023, **108**, 1290–1297
https://doi.org/10.1210/clinem/dgad004
Advance access publication 6 January 2023
Clinical Research Article

Systemic Activation of the Kynurenine Pathway in Graves Disease With and Without Ophthalmopathy

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Abstract

Context: Graves disease (GD) is one of the most common autoimmune disorders. Recent literature has shown an immune response involving several different inflammatory related proteins in these patients.

Objective: This work aimed to characterize the kynurenine pathway, activated during interferon-γ (IFN-γ)–mediated inflammation and cellular (T-helper type 1 [Th1] type) immunity, in GD patients with and without thyroid eye disease (TED).

Methods: We analyzed 34 biomarkers by mass spectrometry in serum samples from 100 patients with GD (36 with TED) and 100 matched healthy controls. The analytes included 10 metabolites and 3 indices from the kynurenine pathway, 6 microbiota-derived metabolites, 10 B-vitamers, and 5 serum proteins reflecting inflammation and kidney function.

Results: GD patients showed significantly elevated levels of 7 biomarkers compared with healthy controls (omega squared [ω^2] > 0.06; $P < .01$). Of these 7, the 6 biomarkers with the strongest effect size were all components of the kynurenine pathway. Factor analysis showed that biomarkers related to cellular immunity and the Th1 responses (3-hydroxykynurenine, kynurenine, and quinolinic acid with the highest loading) were most strongly associated with GD. Further, a factor mainly reflecting acute phase response (C-reactive protein and serum amyloid A) showed weaker association with GD by factor analysis. There were no differences in biomarker levels between GD patients with and without TED.

Conclusion: This study supports activation of IFN-γ inflammation and Th1 cellular immunity in GD, but also a contribution of acute-phase reactants. Our finding of no difference in systemic activation of the kynurenine pathway in GD patients with and without TED implies that the local Th1 immune response in the orbit is not reflected systemically.

Key Words: Graves disease, thyroid eye disease, kynurenine pathway, autoimmune, cellular immunity, Th1 response

Abbreviations: 3IS, 3-indoxyl sulfate; AA, anthranilic acid; BMI, body mass index; CAS, clinical activity score; CRP, C-reactive protein; EUGOGO, European Group on Graves' Orbitopathy; FMN, flavin mononucleotide; fT4, free thyroxine; GD, Graves disease; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; HKr, HK ratio; IAA, indole-3-acetate; IAld, indole-3-aldehyde; IAM, indole-3-acetamide; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-γ; ILA, indole-3-lactate; IPA, indole-3-propionate; KA, kynurenic acid; KTR, kynurenine-tryptophan ratio; Kyn, kynurenine; mNAM, N1-methylnicotinamide; NAM, nicotinamide; PA, 4-pyridoxic acid; PAr, PAR index; Pic, picolinic acid; PL, pyridoxal; PLP, pyridoxal 5-phosphate; QA, quinolinic acid; Qld, quinaldic acid; ROAS, Norwegian register of organ-specific autoimmune disorders; S100A, calprotectin; SAA, serum amyloid A; TED, thyroid eye disease; Th1, T-helper type 1; TMP, thiamine monophosphate; TPOAb, thyroid peroxidase antibody; TRAb, thyrotropin receptor antibody; Trp, tryptophan; TSH, thyrotropin; XA, xanthurenic acid.

Graves disease (GD) is one of the most common autoimmune disorders with an annual incidence of 20 to 30 per 100 000 individuals (1). Thyrotropin receptor antibodies (TRAbs) most often stimulate the thyroid gland to overproduce thyroid hormones, resulting in hyperthyroidism. A systemic activation of the immune system is seen, involving various cytokines, T and B lymphocytes (2, 3).

Approximately 40% of patients with GD develop orbital manifestations, known as thyroid eye disease (TED) (4). A cross-binding of TRAbs and autoantibodies against insulin-like growth factor 1 to CD34+ fibroblasts is essential in the initiation of the orbital inflammatory response (5). T-helper type 1 (Th1)-mediated immunity is consistently observed in TED (6).

Received: 7 November 2022. **Editorial Decision:** 29 December 2022. **Corrected and Typeset:** 18 January 2023

Interferon γ (IFN-γ) is an activator of the Th1 immune response and stimulates the release of neopterin from macrophages. In addition, IFN-γ stimulates the oxidative cleavage of tryptophan (Trp) to kynurenine (Kyn) by enhancing the activity of indoleamine 2,3-dioxygenase (IDO) (7). This is the first (enzymatic) step of the kynurenine pathway where Kyn degrades further to other metabolites collectively termed *kynurenines* (Supplementary Fig. S1 (8)). Several of the kynurenines have immunomodulatory properties, and the serum kynurenine-tryptophan ratio (KTR) and neopterin are sensitive markers of IFN-γ–mediated inflammation (9).

The kynurenine pathway plays a role in a broad spectrum of inflammatory diseases. Elevated Kyns have been associated with cardiovascular disease (10), inflammatory bowel disease (11), and rheumatoid arthritis (12). Increased levels of kynurenines and metabolites have also been reported in patients with chronic kidney disease (13). There is evidence of alterations in the kynurenine pathway in different neurodegenerative and psychiatric disorders (14, 15). Interleukin-6 has been proposed as an inducer of the kynurenine pathway in patients with chronic schizophrenia (16).

We recently showed that IFN-γ was significantly raised in patients with GD in general, with particularly high levels of interleukin-6 in the subgroup with TED (17). Here, we further explore the IFN-γ–mediated inflammation in this cohort of GD patients with and without TED by mapping the intermediates of the kynurenine pathway, relevant vitamin cofactors, and microbial-derived breakdown products of tryptophan (indoles).

Materials and Methods

Participants

In the time period 2013 to 2021, 100 patients with GD were randomly included into the Norwegian register of organ-specific autoimmune disorders (ROAS). At inclusion they all underwent a general clinical examination and blood samples were collected. From a pool of healthy control samples stored in our biobank, we selected samples from 100 age- and sex-matched controls (Table 1). All individuals signed a letter of consent for participation in future research projects. Before inclusion, they received written information about the present study and were offered the possibility to withdraw if they so desired. The Regional Committee for Medical and Health Research Ethics, Western Norway approved the ROAS biobank (institutional review board No. 00001872, ref. 2013/1504) and the present study (ref. 2021/7624).

Clinical Data

Clinical data were obtained from ROAS and from hospital records. GD patients were categorized as having TED if they had characteristic symptoms or signs when included in ROAS. Development of TED after inclusion were recorded at follow-up. Severity of TED was classified according to the European Group on Graves' Orbitopathy (EUGOGO) classification (18) and inflammatory activity by clinical activity score (CAS) (19). We defined active TED as a CAS of 3 out of 7 or higher.

Thyroid Status

Serum levels of free thyroxine (fT4) (Roche catalog No. 12017709, RRID:AB_2756378), thyrotropin (TSH) (Roche catalog No. 11731459, RRID:AB_2756377), 3,5,3′-triiodothyronine (T3) (Roche catalog No. 11731360122, RRID:AB_2827369), thyroid peroxidase antibody (TPOAb) (Roche catalog No. 11820818, RRID:AB_2631044), and TRAb (Roche catalog No. 04 388 780 190, RRID:AB_2801453) were analyzed at the Laboratory of Clinical Biochemistry, Haukeland University Hospital, using electrochemiluminescence immunoassay (ECLIA) (Roche Cobas).

Table 1. Basic characteristics of patients and healthy controls at inclusion

	Patients with GD	HCs
Demographic characteristics		
n	100	100
Age, y	42 (15-70)	39 (15-70)
Female sex	77 (77%)	77 (77%)
Body mass index	25 (17-49)	24 (16-36)
Systolic blood pressure, mm Hg	120 (90-192)	124 (90-154)
Smoking history		
Current smoker	22 (22%)	4 (4%)
Ex-smoker	18 (18%)	0 (0%)
Never smoker	60 (60%)	96 (96%)
Status of thyroid disease		
Hyperthyroidism	40 (40%)	
Euthyroid	59 (59%)	
Hypothyroidism	1 (1%)	
Biochemical tests		
s-Free-thyroxine (pmol/L)	20 (7-81)	
s-Triiodothyronine (pmol/L)	7.1 (1.3-31)	
TSH, mU/L	0.01 (0.01-9.0)	
TRAb, U/L	5.4 (1-40)	
TPOAb positive	37 (37%)	
Current thyroid treatment		
Antithyroid drugs	87 (87%)	
Levaxin substitution	14 (14%)	
None	11 (11%)	
Unknown	2 (2%)	
Autoimmune comorbidities[a]	30 (30%)	0 (0%)
Addison disease	17 (17%)	
Diabetes mellitus type 1	7 (7%)	
Vitiligo	6 (6%)	
Celiac disease	5 (5%)	
Vitamin B_{12} deficiency	2 (2%)	
Hypoparathyroidism	2 (2%)	
Sjögren syndrome	1 (1%)	
Guillain-Barré syndrome	1 (1%)	
Rheumatoid arthritis	1 (1%)	

Categorical data are given as n (%); continuous data are given as median (range).
Abbreviations: GD, Graves disease; HCs, healthy controls; s-, serum-; TED, thyroid eye disease; TPOAb, thyroid peroxidase antibody; TRAb, thyrotropin receptor antibody; TSH, thyrotropin.
[a]Patients with Addison disease were treated with cortisone, diabetes mellitus type 1 with insulin, vitamin B_{12} deficiency with supplementation, celiac disease had dietary restriction, and hypoparathyroidism used calcium and vitamin D supplementation. None of the patients were on anti-inflammatory treatment.

Analysis of Inflammatory Markers and Metabolites

Serum samples from all participants were obtained nonfasting between 0900 hours and 1400 hours, and stored at −80 °C.

Analyses for biomarkers were performed at the laboratory of Bevital, Bergen, Norway (Bevital.no). Trp, kynurenines, and downstream metabolites, indoles, neopterin, cotinine, and B-vitamers were analyzed by liquid chromatography–tandem mass spectrometry (LC/MS-MS). C-reactive protein (CRP), serum amyloid A (SAA), calprotectin (S100A), and cystatin C were assayed by matrix-assisted laser desorption-ionization time of flight mass spectrometry. Details regarding the limit of detection and coefficient of variation for the essential biomarkers are published on Bevital's home pages at Bevital.no. Cotinine and trans-3′-hydroxycotinine were applied as indicator of recent nicotine exposure (20).

As a part of the tryptophan-kynurenine pathway, we analyzed serum samples for concentrations of Trp, Kyn, kynurenic acid (KA), quinaldic acid (Qld), 3-hydroxykynurenine (HK), anthranilic acid (AA), xanthurenic acid (XA), 3-hydroxyanthranilic acid (HAA), picolinic acid (Pic), and quinolinic acid (QA). We measured neopterin as a marker of IFN-γ activity.

We also analyzed 6 different microbiota-derived metabolites of Trp collectively called indoles: indole-3-aldehyde (IAld), indole-3-acetate (IAA), indole-3-propionate (IPA), indole-3-lactate (ILA), 3-indoxyl sulphate (3IS) and indole-3-acetamide (IAM).

The following markers related to B-vitamin status were analyzed: pyridoxal 5-phosphate (PLP), pyridoxal (PL), 4-pyridoxic acid (PA), riboflavin, flavin mononucleotide (FMN), nicotinamide (NAM) and N1-methylnicotinamide (mNAM). Thiamine monophosphate (TMP) concentration was used to adjust for variation in serum samples handling before freezing, as TMP is converted to thiamine at room temperature (21).

Ratios and Indices

We calculated the ratio between Kyn and Trp (KTR) as a measure of IDO activity, which catalyzes the first, rate limiting, and IFN-γ–responsive step of the kynurenine pathway (22). Many of the metabolites downstream of Kyn are considered immunomodulatory and their formation are catalyzed by vitamin B_6- and B_2-dependent enzymes. We calculated the HK ratio (HKr), composed of HK and the 4 kynurenines that are products of the PLP-dependent enzymes, kynurenine transaminase and kynureninase (HK: (KA + AA + XA + HAA)). HKr is a functional marker of B_6 status (23). The PAR index (PAr) was calculated as the ratio of PA divided by the sum of PLP plus PL (PA: (PLP + PL)), and reflects altered vitamin B_6 metabolism during inflammation (24).

Statistical Analyses

Descriptive statistics were used to describe cases and controls. All measured biomarkers were checked for missing data, and log-transformed before conducting analyses. Missing data for body mass index (BMI) (13.5%) and CRP (4.5%) were imputed once (single imputation) using the predictive mean matching algorithm as implemented in the *mice* package in R (25). The Spearman correlation coefficient (*r*) was used to map the correlations between biomarkers. Information on active smoking was obtained using both data on self-reported smoking and measured serum cotinine levels. All individuals with a serum cotinine level above 85 nmol/L were considered active smokers (20).

To estimate the difference in log-transformed biomarker data between GD and healthy controls, we used linear regression models with robust SE estimation to account for potential correlation that arose by matching of cases and controls. The estimated differences were further adjusted for age, BMI, and smoking, as well as for sample storage (TMP), and reported together with 99% CIs or adjusted *P* values. As healthy controls were sex-matched one-to-one with GD cases, adjustment for sex were not found necessary. Age matching allowed for a variation between ±5 years, and adjustments for age were therefore performed. To account for nonlinear relationship with the biomarkers, the variables age and BMI were included as polynomial quadratic regression terms (ie, age + age^2 and BMI + BMI^2).

To quantify the effect size of the estimated difference in biomarkers between the disease groups, we calculated the partial omega squared (ω^2) statistics (26, 27), which measure how much of the variation in the biomarker is explained by the difference between GD and healthy controls. The calculation of the partial ω^2 statistics was based on type 3 sum of squares from the adjusted linear regression models, excluding robust SE estimation, as this option is currently not available in the *effectsize* package in R (28). The following thresholds of the ω^2 statistics have been suggested for the strength of associations: ω^2 less than 0.01 (very small); 0.01 less than or equal to ω^2 and less than 0.06 (small); 0.06 less than or equal to ω^2 and less than 0.14 (medium); ω^2 greater than or equal to 0.14 (large) (29). The ω^2 statistics can in some cases be negative. Finally, to identify the most important biomarkers for GD, the paired combination of –log 10 (adjusted *P* value) and partial ω^2 statistics for each biomarker is depicted in a graphical format with the aforementioned ω^2 thresholds. The level of statistical significance was set to .01, corresponding to a –log 10 (adjusted *P* value) of 2.

Factor analysis was performed on a subset of the biomarkers: the kynurenines, PLP, the protein biomarkers, CRP, SAA, S100A, and cystatin C, and the covariates smoking and BMI. We chose to present a 2-factor solution where the 2 factors explained 40.3% of the total variation (a third factor increased the total explained variation to only 47.0%). The individual scores of the 2 factors, labeled as GD patients or healthy controls, were used to generate a score plot (see Supplementary material for further details (8)). Statistical analyses were conducted using R (version 4.2.0).

Results

Participants

A total of 100 GD patients (77 females) were included with a median age of 41.5 (15-70) years. Fifty-nine of these patients were included within 2 months after presentation of hyperthyroidism. When included, 87 patients had started treatment for their hyperthyroidism, while 13 were treatment naive. Basic characteristics of the study patients and their treatments at inclusion are given in Table 1.

Sixty-four percent of the patients were included during their first episode of hyperthyroidism, and the median number of relapses of hyperthyroidism was 1 (range, 0-4). After a median follow-up time of 36 months (range, 6-240 months) from the first episode of hyperthyroidism, 34 had received definitive treatment by either radioiodine (n = 18), total thyroidectomy (n = 10), or a combination of both modalities (n = 6). In 9 patients, the indication for definitive treatment was TED. During radioiodine treatment, 7 patients received prednisolone according to EUGOGO guidelines (18). After radioiodine treatment, 3 patients developed TED; 1 of these patients had received prednisolone.

Twenty-nine patients had clinical characteristics of TED at inclusion, of whom 7 patients had a CAS score of 3 or greater. The median time after diagnosis of GD to development of TED was 0 months (range, 0-240 months). Seven patients

developed TED within 72 months after inclusion, 2 of them within the first 6 months. The severity of TED according to the EUGOGO classification was mild in 27, moderate to severe in 8, and sight-threatening in 1 patient.

There were no significant differences in age, sex, and BMI between the GD patients with and without TED. There were more smokers among patients with TED (39%) compared with patients without TED (22%) ($P < .05$). No significant differences in serum levels of TRAb or fT4 were observed between the 2 groups.

Kynurenines and Markers of Inflammation

Four out of 34 biomarkers (cotinine, trans-3′-hydroxycotinine, IAM, and TMP) were excluded from further analyzes because of low detection rate (< 60%) both in patients and healthy controls. The concentrations of the remaining 30 biomarkers are given in Table 2. Adjusted for age, smoking, and BMI, by linear regression, 7 biomarkers were significantly elevated with a medium effect size ($P < .01$; $0.06 < \omega^2 < 0.14$) (Fig. 1) in patients with GD compared to healthy controls. The 6 most elevated biomarkers included neopterin and metabolites that were part of or functionally linked to the kynurenine pathway, namely HKr, PAr, Kyn, KTR, and QA. In addition, cystatin C was significantly elevated with medium effect size (Fig. 2).

For the other biomarkers including CRP, S100A, SAA, indoles, and B-vitamers, the effect sizes were small ($0.01 \leq \omega^2 < 0.06$) or very small ($\omega^2 < 0.01$) (Fig. 1).

Table 2. Levels of biomarkers in patients with Graves disease and healthy controls

Biomarker	All patients with GD	HCs	*P*
Tryptophan and kynurenines			
Tryptophan, µmol/L	66.7 (38.6-111.0)	70.8 (38.0-120.0)	.71
Kynurenine, µmol/L	1.9 (0.94.4)	1.6 (0.8-4.3)	< .001
Kynurenic acid, nmol/L	46.2 (14.7-162.0)	52.3 (20.7-128.0)	.14
Quinaldic acid, nmol/L^{L}	7.6 (2.1-28.5)	8.2 (3.2-26.1)	.23
3-Hydroxykynurenine, nmol/L	54.2 (1.2-232.0)	46.2 (4.4-101)	< .001
Anthranilic acid, nmol/L	17.3 (5.0-202.0-)	17.2 (5.6-85.7)	.45
Xanthurenic acid, nmol/L	17.5 (4.9-55.1)	20.9 (5.1-51.4)	< .001
3-Hydroxyanthranilic acid, nmol/L	43.4 (1.2—132.0)	42.6 (2.6-105.0)	.83
Picolinic acid, nmol/L	45.1 (11.5-140.0)	56.8 (19.4-118.0)	.003
Quinolinic acid, nmol/L	419.0 (201.0-1260.0)	331.0 (169.0-3740.0)	< .001
Indoles			
Indole-3-aldehyde, µmol/L	11.1 (3.4-148)	17.3 (7.0-191.0)	.30
Indole-3-acetate, µmol/L	1.9 (0.6-7.8)	2.1 (0.7-5.4)	.99
Indole-3-propionate, µmol/L	1.6 (0.1-36.2)	1.7 (0.3-7.9)	.04
Indole-3-lactate, nmol L^{-1}	0.56 (0.43-0.68)	0.64 (0.54-0.78)	.002
3-Indoxyl sulphate, µmol/L	5.0 (0.1-18.9)	4.7 (0.7-13.5)	.49
Ratios and indexes			
Kynurenine-tryptophan ratio	30 (15-68)	20 (12-113)	< .001
3-Hydroxykynurenine ratio	42 (1-221)	34 (3-53)	< .001
PAr index	0.4 (0.1-1.6)	0.3 (0.1-0.6)	< .001
B-vitamers			
Pyridoxal 5-phosphate, nmol/L	33.7 (3.9-247.0)	39.7 (11.5-163.0)	.03
Pyridoxal, nmol/L	18.8 (4.7-2630.0)	20.3 (6.4-80.5)	.18
4-Pyridoxic acid, nmol/L	20.6 (5.7-1088.3)	19.0 (7.2-49.7)	.01
Riboflavin, nmol/L	16.2 (4.6-202.0)	14.1 (4.8-53.1)	.02
Flavin mononucleotide, nmol/L	5.2 (1.7-56.9)	4.4 (1.7-15.5)	.23
Nicotinamide, nmol/^{L}	171.0 (57.9-1220.0)	205.9 (69.1-478.0)	.15
N1-methylnicotinamide, nmol/L	110.5 (25.5-990.0)	107.0 (40.4-302.0)	.97
Markers of inflammation			
C-reactive protein, µg/mL	1.5 (0.1-24.6)	0.9 (0.1-7.3)	< .003
Serum amyloid A, µg/mL	2.4 (0.7-53.8)	1.8 (0.5-13.2)	.02
Calprotectin, S100A, µg/mL	0.6 (0.1-6.7)	1.1 (0.2-2.5)	< .001
Neopterin, nmol/L	17.7 (5.1-58.4)	8.9 (4.5-64.3)	< .001
Others			
Cystatin C, µg/mL	0.8 (0.4-1.9)	0.7 (0.3-2.0)	< .001

Data are given as median (range). *P* value estimated by Mann-Whitney *U* test.
Abbreviations: GD, Graves disease; HCs, healthy controls.

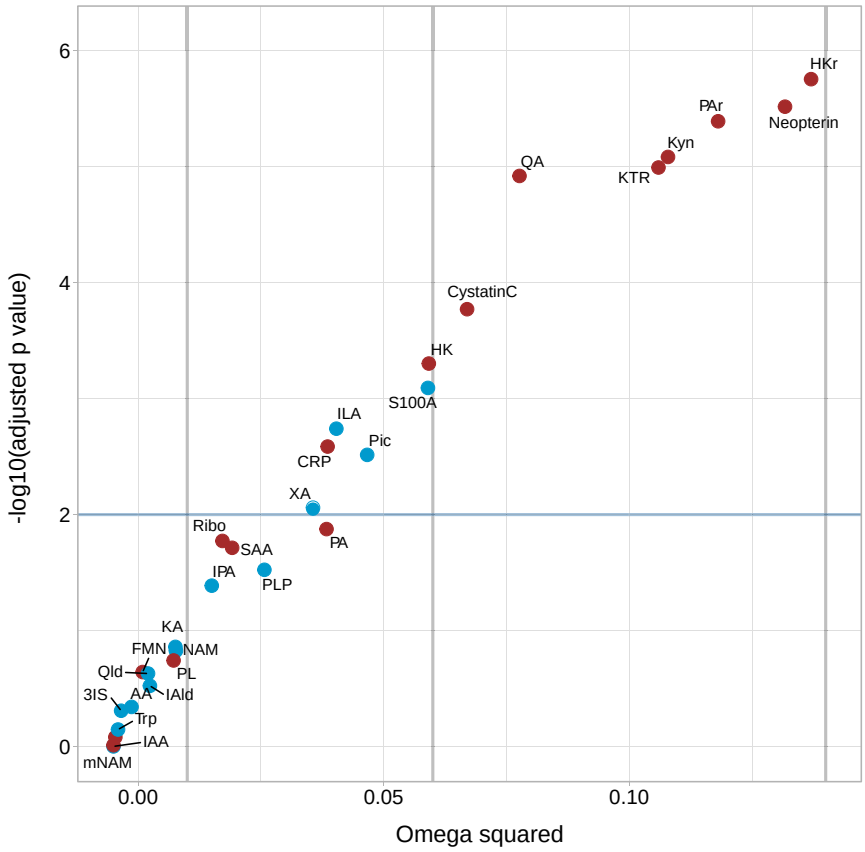

Figure 1. Combination plot of effect size against significance level in biomarkers between GD and healthy controls. X-axis: effect size estimated by omega squared (ω^2). Y-axis: significance level as −log 10 (adjusted *P* value). Blue line: represents significance level at $P = .01$. Blue dots: biomarker suppressed in GD. Red dots: biomarker elevated in GD. ω^2 and *P* values for difference in biomarkers were obtained from a linear regression model, adjusted for age, BMI, and smoking. AA, anthranilic acid; BMI, body mass index; CRP, C-reactive protein; FMN, flavin mononucleotide; GD, Graves disease; HK, 3-hydroxykynurenine; IAA, indole-3-acetate; IAld, indole-3-aldehyde; ILA, indole-3-lactate; IPA, indole-3-propionate; KA, kynurenic acid; KTR, kynurenine-tryptophan-ratio; Kyn, kynurenine; mNAM, N1-methylnicotinamide; NAM, nicotinamide; PA, 4-pyridoxic acid; Pic, picolinic acid; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; QA, quinolinic acid; Qld, quinaldic acid; Ribo, riboflavin; SAA, serum amyloid A; S100A, calprotectin; Trp, tryptophan; XA, xanthurenic acid; 3IS, 3-indoxyl sulfate.

In GD patients, we observed moderate correlation of fT4 with KTR ($r = 0.31$; $P = .002$) and cystatin C ($r = 0.37$; $P < .001$). None of the biomarkers correlated with the level of TRAb. The correlation between Kyn and QA was strong ($r = 0.7$; $P < .05$).

When comparing the treatment-naive subgroup (n = 13) with patients on antithyroid drugs (n = 87), we did not find significant differences in any biomarker levels.

We did not observe significant differences with medium or higher effect size in any biomarkers between GD patients with and without TED using the same analyses as previously mentioned. When separated further into subgroups of patients with active TED (CAS score ≥ 3), moderate to severe TED, TED at inclusion, or development of TED after inclusion, none of these groups had significantly different biomarker concentrations.

Factor Analysis

A factor analysis was performed to simplify the interpretation of our data. Fig. 2 shows the factor analysis of selected biomarkers labeled by outcome status (GD or healthy control). The 2-factor solution indicates that variations in kynurenine levels are organized roughly along 2 axes. Factor 1 represents acute-phase inflammation with negative loadings of CRP and SAA. In contrast, factor 2 was dominated by strong loadings of Kyn, QA, and HK, and thereby Th1-mediated immunity. Factor 2 was more strongly associated with GD than Factor 1. A more thorough description of the factor analysis including loading pattern of the different biomarkers on factors 1 and 2 are given in the Supplementary material (8).

Discussion

We observed elevated levels of 7 different inflammatory-related biomarkers in GD patients compared with healthy controls using modern mass spectrometry methods. Elevated levels of neopterin, Kyn, KTR, and QA demonstrate enhanced metabolism along the kynurenine pathway and the cell-mediated Th1 response, while increased HKr and PAr indicate alterations in vitamin B_6 status and metabolism in GD patients, irrespective of TED status.

The autoimmune T-helper type 2 response in GD is well known, but the importance of Th1-mediated immunity is debated (2, 3). By factor analysis (see Fig. 2), we demonstrated biomarkers related to cellular immunity and Th1 response to be associated with GD.

The observed alteration in serum levels of neopterin, KTR, and kynurenines (QA and Pic) indicates increased activity of the kynurenine pathway. We confirmed elevated serum

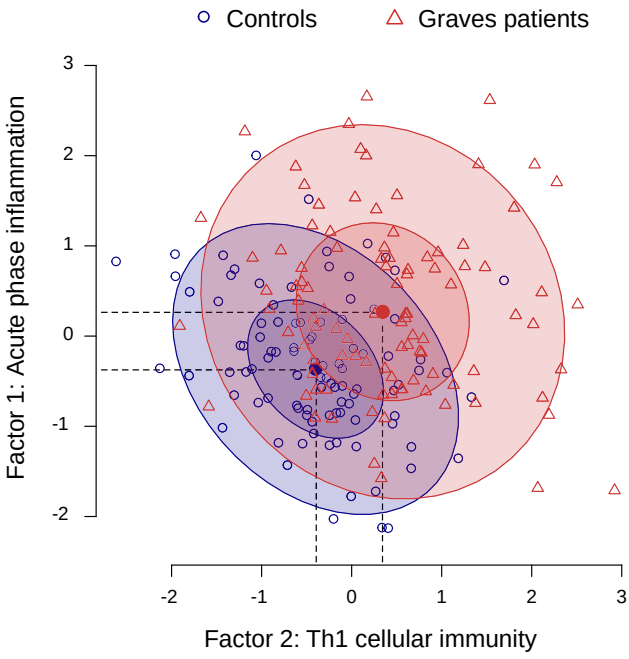

Figure 2. Score plot from factor analysis showing scores from factor 1 (acute-phase inflammation) and 2 (Th1 cellular immunity) labeled by outcome status (GD or healthy control). The filled symbols indicate the central estimates, and the dark-shaded zones and the light-shaded zones the 50 percentile and the 95 percentile, respectively. The scale on each axis is SD of the factor scores. The difference between Graves disease (GD) and healthy control was 0.69 SD on factor 1 and 0.83 SD on factor 2 as indicated by dashed lines.

neopterin levels in patients with GD (30). Neopterin is a direct marker of IFN-γ activity and activation of the cellular immune system (31). Elevated IFN-γ levels have previously been observed in patients with GD both compared to healthy controls and compared to patients with Hashimoto thyroiditis (32, 33). KTR reflects IDO activity (34) and is highly correlated with neopterin. Conflicting observations of KTR in GD have been published (35, 36). Genetic factors, disease stage, and presence of leukocyte infiltration in the thyroid gland could explain the discrepancy. Our observations support increased KTR and IDO activity in GD patients. Further, we found increased QA and suppressed Pic in GD patients. High QA has earlier been found in relation to multiple neurologic diseases (37), but QA and the QA/Pic ratio has not been studied in GD. A high QA/Pic ratio may reflect insufficient amino-β-carboxymuconate-epsilon-semialdehyde-carboxylase (ACMSD) activity when the kynurenine pathway is upregulated due to inflammation, thereby directing the pathway toward NAD+ synthesis to support mitochondrial electron transport and energy production (38).

We observed elevated levels of cystatin C, a sensitive biomarker for changes in glomerular filtration rate often used to diagnose and evaluate patients with kidney disease (39). Inconsistent reports exist on cystatin C and thyroid disease (40-43). Our findings support an increased cystatin C level in patients with GD with and without TED. Instead of a decrease in renal function, stimulatory effects of 3,5,3′-triiodothyronine and transforming growth factor β1 on cystatin C production may be the underlying mechanism in GD (44).

HKr is established as a functional marker of B_6 status (23), and PAr reflects increased catabolism of vitamin B_6 during inflammation (24). We observed high values of HKr and PAr in GD patients indicating decreased vitamin B_6 status possibly caused by increased demand (HKr), and alterations in vitamin B_6 handling and metabolism (PAr). These changes were associated with only a moderate reduction in PLP in GD patients. To our knowledge, altered vitamin B_6 status has not been reported in GD before. Supplementation with B_6 should be investigated for this group of patients.

We did not observe any differences in components of the kynurenine pathway in GD patients with and without TED. This indicates that changes specific to orbital Th-1–mediated immune response (6) is not sufficiently reflected in the circulation, possibly due to the relatively small volume of the orbital compartment compared to the total body volume.

Factor analysis demonstrated that biomarkers related to Th1-cellular immunity were strongly associated with GD. Interestingly, cystatin C was closely linked to the Th1-related factor. In addition, we found a moderate association between the acute-phase–related factor and GD. Chronic low-grade, acute-phase–like inflammation has earlier been observed in other autoimmune diseases, including systemic lupus erythematosus, primary biliary cirrhosis, and rheumatoid arthritis (45, 46). Altogether, our observations support the presence of a chronic low-grade, acute-phase–like inflammation in addition to the cellular Th1-immune reaction in GD.

Smoking is a known modulator of the immune system, and we found differences in smoking habits between patients with GD and healthy controls. Therefore, smoking was adjusted for in the linear regression analysis and accounted for in the factor analysis.

Our study has some weaknesses. Coexistence of additional autoimmune disorders in 30% of the patients may have influenced our findings. In addition, only 7 out of 36 TED patients had active disease at inclusion. A higher proportion of active TED might have increased our chances to detect a specific biomarker pattern for TED. Furthermore, serum samples were not drawn in the fasted state, which potentially could yield different concentrations both of Trp and the kynurenines.

In conclusion, our study promotes the role of Th1-mediated immunity in GD, as we demonstrate an increased activity of the kynurenine pathway and IFN-γ–mediated immune response. We also found evidence for decreased functional vitamin B_6 status and altered metabolism of vitamin B_6 vitamers in GD patients as indicated by the biomarkers HKr and PAr. Further, association of acute-phase reactants and GD speaks in favor of a chronic low-grade inflammation. Lack of difference in IFN-γ–mediated inflammation between GD patients with and without ophthalmopathy indicates that the immune response in the orbit is not reflected systemically. This supports local immunological features as being important in the development of TED.

Acknowledgments

The professional assistance of Prof Emeritus Per Magne Ueland and technician Elisabeth Tombra Halvorsen is greatly appreciated.

Financial Support

This work was supported by grants from the Novo Nordisk Foundation and Regional Health Authorities of Western Norway.

Disclosures

The authors have nothing to disclose.

Data Availability

All data sets generated during and/or analyzed during the present study are not publicly available but are available from the corresponding author on reasonable request.

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