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Increased plasma levels of soluble programmed death ligand 1 (sPD-L1) and fibroblast growth factor 23 (FGF-23) in patients with Graves' ophthalmopathy in comparison to hyperthyroid patients without Graves' ophthalmopathy

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ABSTRACT

Background: Management of Graves' ophthalmopathy (GO) is still a challenge in Graves' disease (GD). Moreover, 40% of GD patients show radiological muscle enlargement without clinically apparent GO. Delayed treatment of GO may lead to deterioration in prognosis.
Methods: Thirty GD patients with overt hyperthyroidism were included in this study, 17 of whom either had GO at diagnosis or developed GO during the study period. Samples were collected at the beginning of the study, at 6 months, and at 24 months. Plasma samples were analyzed for 92 cytokines using the Olink Target 96 inflammation panel.
Results: After adjustment for multiplicity testing using the false discovery rate approach, soluble programmed death ligand 1 (sPD-L1) and fibroblast growth factor 23 (FGF-23) were significantly elevated in GO patients.
Conclusion: Using a broad cytokine panel we show that patients with Graves' ophthalmopathy have elevated PD-L1 and FGF-23 levels. The findings support previous suggestions that PD-L1 may serve as a treatment target.

1. Introduction

The incidence of hyperthyroidism in Sweden is 27.6 cases per 100,000 inhabitants, 75% of which are due to the autoimmune condition known as Graves' disease (GD). Graves' orbitopathy (GO) is considered to be an inflammatory autoimmune disease affecting the orbita [1,2]. Approximately 50% of GD patients develop GO, but with heterogeneous clinical manifestations. Consequences of GO include dryness/tearing/redness of the eye, eyelid retraction, chemosis, periorbital swelling, proptosis, diplopia, and impaired vision [3]. Current treatment has focused on the use of glucocorticoids aiming to reduce the inflammation in the orbit [4]. Additional and alternative treatments have long been sought, but with little progress. In 2020, the FDA approved the monoclonal antibody teprotumumab, an inhibitor for insulin-like growth factor-1 receptor [5,6] which has shown promising results, in particular with respect to reducing proptosis.

Treatment of GO focuses on the reduction of the inflammatory process, which involves a large number of cytokines that may both enhance and suppress the production of other cytokines [7]. Due to this complexity, it may not be sufficient to study a single cytokine; rather, the goal should be to gain an overview of the cytokine networks in order to reveal the complicated inflammatory processes that are present in patients with GD. Traditional cytokine ELISA methods often need sample volumes around 100–200 uL, and so if a large number of cytokines are to be measured the total sample volume will be quite large. To allow the simultaneous quantification of a large number of substances with a reasonable sample volume, we used the Proseek Multiplex Inflammation panel from Olink (Uppsala, Sweden), which allows the determination of 92 cytokines related to inflammatory processes [8,9].

The aim of the present study was to investigate a large group of inflammatory cytokines in patients with hyperthyroid disease with and without GO. We wanted to find markers in patients who had GO or developed GO during the study period, that would allow early discrimination from hyperthyroid patients without GO. We also investigated the association between individual cytokine levels and TSH, fT3, fT4, and TSH receptor antibodies (TRAb), and the effects of anti-thyroid

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drug treatment (ATD) on cytokine levels.

2. Materials and methods

2.1. Study population

Thirty patients with *de novo* Graves' disease were recruited at Uppsala University Hospital during February–November 2017, all with low TSH and positive TRAb. One patient had TRAb below the reference range (1.7 IE/L, reference < 1.75). This patient had symptoms and laboratory findings typical of GD, which was in line with a homogeneous uptake at scintigraphy.

During the first visit, all patients underwent an examination including recording of demographic characteristics, medical history, family history, and concomitant medication. Blood samples were taken at baseline and at six months and 24 months after treatment start to measure TSH, fT4, fT3, TRAb, thyroglobulin, and cytokines. In all 30 participants, ATD comprising methimazole or propylthiouracil was initiated in conjunction with the first visit and given in a block-replace regimen beginning with 10–20 mg methimazole or 150–300 mg propylthiouracil. Thyroxine was added and was adjusted with the aim of keeping TSH levels in the low normal range during the follow-up. Care was taken throughout to avoid treatment-induced episodes of hypothyroidism.

Five patients were referred to radioiodine treatment (RAI) during the follow-up, four of them due to persistently elevated TRAb after 10–15 months of ATD treatment. One patient received RAI after 5 months at the request of the patient. Post-RAI, one patient experienced mild GO not requiring steroid medication. Three patients underwent total thyroidectomy, two of them due to neutropenia, which developed directly after start of methimazole in one case and after 10 months on methimazole in the other. In these cases, neutropenia persisted, and a fear of agranulocytosis led to the recommendation of surgery. One female patient was operated after five months because of pregnancy desire. Of the remaining 22 patients, 21 received ATD for 18–24 months until negative TRAb. One non-GO patient had a spontaneous recovery before treatment initiation.

All patients received clear information about GD, and were examined for the presence of eye symptoms and signs at each visit by a doctor and a nurse with experience in managing GD. They were also repeatedly asked to contact the clinic if they began to experience eye symptoms. GO was defined as eye symptoms and signs related to GD according to guidelines from the European Group on Graves' Orbitopathy (EUGOGO) [10]. The severity was classified as mild when the GO manifested with symptoms such as gritty sensation and tearing due to dry eyes, caruncle swelling and/or redness, or upper eyelid retraction; or as moderate to severe in the instance of redness and/or swelling of the eyelids, chemosis, pressure or pain in the eyes, exophthalmos, diplopia, or signs of optic nerve compression. Eye signs and symptoms were documented in the medical records by both the endocrinologist and the research nurse at every visit.

2.2. Proximity extension assay (PEA)

The PEAs were analyzed using the Proseek Multiplex Inflammation I panel (Olink Bioscience, Uppsala, Sweden). Briefly, 1 μL plasma was mixed with 3 μL incubation mixture containing two probes (antibodies labeled with unique corresponding DNA oligonucleotides). The mixture was first incubated at 8 °C overnight. Next, 96 μL extension mix containing PEA enzyme and PCR reagents was added, and the samples were incubated for 5 min at room temperature before the plate was transferred to the thermal cycler for 17 cycles of DNA amplification. A 96.96 Dynamic Array IFC (Fluidigm, South San Francisco, CA, USA) was prepared and primed according to the manufacturer's instructions. In a separate plate, 2.8 μL of sample mixture was mixed with 7.2 μL detection mix from which 5 μL was loaded into the right side of the primed 96.96 Dynamic Array IFC. The unique primer pairs for each cytokine were loaded into the left side of the 96.96 Dynamic Array IFC, and the protein expression program was run in a Fluidigm BioMark reader according to the instructions for Proseek. The Proseek kits measured 92 biomarkers.

2.3. Routine thyroid tests

TSH, fT4, fT3, TRAb, and thyroglobulin (Tg) were analyzed on a Cobas immunoassay instrument (Roche Diagnostics, Rotkreuz, Switzerland).

2.4. Statistical analysis

Cytokine values above or below the highest and lowest standard points were assigned the values of these points. Protein levels were measured with the Proseek kit on a log 2 scale and further transformed to an SD scale in order to be easily comparable. Analyzing a large number of correlations increases the risk of false discoveries, and so the p-values were adjusted for multiplicity using the false discovery rate approach. Associations between thyroid markers and with cytokine levels were analyzed using Spearman rank correlations. STATA 16 (Stata Inc, College Station, TX, USA) was used for the calculations.

3. Results

3.1. Patient characteristics

Thirty GD patients with overt hyperthyroidism were included. Median age was 55 years (range: 35–72 years), 29 were women, and 2 were smokers. Of the 17 patients with GO, 14 had mild GO and the remaining 3 had moderate to severe GO; these 3 were referred to an ophthalmologist, who also examined and advised on 6 mild cases. Eleven patients had GO at diagnosis, while 6 patients developed GO during the follow-up. Of the 22 patients who were not referred for RAI or thyroidectomy during the study period, 12 were classified as GO (at diagnosis or during treatment) and ten as non-GO. Thyroid hormonal status, TRAb, and Tg at baseline and end of study are presented in Tables 1 and 2.

3.2. Cytokine levels in GO patients

Patients with GO had generally higher cytokine levels. Even if not significantly associated on the individual level, 81 cytokines showed higher values in GO patients, while 2 were equal and 9 cytokines had lower values (Table 3).

After adjusting for multiplicity testing, soluble programmed death ligand 1 (sPD-L1) and fibroblast growth factor 23 (FGF-23) were significantly elevated in GO patients compared to the group of patients with GD without GO.

An adjusted p-value of < 0.05 was considered statistically significant. P-values are presented before and after multivariate adjustment.

Table 1
Thyroid hormone and TRAb (median and range) at diagnosis of 30 patients with *de novo* GD, divided into an all-GO group (including 6 patients who developed GO after the first visit) and a non-GO group.

	TSH	F-T4	F-T3	TRAb
All GD (*n* = 30)	0.005 (0.005–0.040)	41.0 (17.6–100.0)	15.6 (5.6–34.0)	5 (1.7–78)
GO (*n* = 17)	0.005 (0.005)	48 (25–100)	18.3 (9–34)	6.3 (2.7–78)
Non-GO (*n* = 13)	0.005 (0.005–0.040)	35 (17.6–60)	12.4 (5.6–28)	4.9 (1.7–29)
p	0.157	0.007	0.025	0.053

Table 2
Longitudinal thyroid hormone and TRAb (median and range) determined at baseline and after 24 months, divided by GO (n = 10) and non-GO (n = 12) groups. RAI and thyroidectomy cases are excluded.

Visit	GO	TSH	F-T4	F-T3	TRAb
0	GO	0.005 (0.005)	47.5 (25–85)	17.7 (9–34)	5.7 (2.7–20)
	non-GO	0.005 (0.005–0.04)	34 (17.6–60)	12.3 (5.6–28)	4.55 (1.7–29)
24 m	GO	2 (0.07–4.47)	16 (10–19.7)	4.6 (3.7–5.1)	0.3 (0.3–1.8)
	non-GO	1.79 (0.2–2.6)	15.6 (12.8–25)	4.6 (3.4–6.9)	0.3 (0.3–3)

3.3. Effects of treatment on cytokine levels

The treatment the patients received resulted in generally decreased cytokine levels. Table 4 presents the cytokines according to p-value for the difference between first and last sample. Overall, 39 cytokines showed a significant difference between the two sampling times. All but two were reduced over time when comparing the first and last sample times. PD-L1 and FGF-23 were significantly reduced over the treatment period.

3.4. Associations between cytokine levels and thyroid markers

There were strong Spearman rank correlations between the thyroid markers fT3, fT4, and TRAb (Table 5). Of the studied cytokines, PD-L1 showed the strongest correlation with TRAb (Spearman r = 0.55). Both FGF-23 and PD-L1 showed significant positive Spearman rank correlations with several of the thyroid markers (PD-L1: fT4 0.26, fT3 0.32, TRAb 0.55, and Tg 0.35; FGF-23: fT4 0.15, fT3 0.26, TRAb 0.24, and Tg 0.38).

4. Discussion

The present results show that in patients with GD, individuals who had or developed GO exhibited significantly higher levels of sPD-L1 and FGF-23. GO is characterized by orbital inflammation, T cell infiltration, and fibroblast activation. The finding that 81 out of 92 studied cytokines were elevated in the GO group supports previous reports on GO being associated with inflammation [11–13].

The exact cause of GO is not fully known, but it is believed to involve an autoimmune response targeting the tissues around the eyes. This immune response involves the activation of immune cells and the release of various chemical mediators and pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha), which further contribute to the inflammatory process. Increased levels of these cytokines have been observed in the orbital tissues of individuals with GO [14].

Inflammation in GO affects various components of the eye, including the extraocular muscles, the connective tissues, and the lacrimal gland [15,16]. The swelling and inflammation can cause enlargement of the extraocular muscles, resulting in protrusion of the eyes (exophthalmos), and can also lead to eyelid retraction, double vision (diplopia), and other visual disturbances. It is important to note that the severity and progression of inflammation in GO can vary between individuals [12]. The treatment of GO typically involves a multidisciplinary approach with the aim of controlling inflammation, managing symptoms, and preventing long-term complications associated with the condition [17].

Considering that GO has a highly heterogeneous clinical phenotype, it would be valuable to have additional biomarkers to evaluate the severity of the disease and to better monitor the treatment. Programmed death-ligand 1 (PD-L1), which is also known as CD274 [18], plays an important role in suppressing the adaptive immune systems in autoimmune disease [19]. Binding of PD-L1 to PD-1 produces an inhibitory

Table 3
Comparison of patients who had or developed GO during the study period (n = 17) with hyperthyroid patients without GO (n = 13).

Biomarker	p-value	Difference	Adjusted p-value
PD-L1	0.00	0.40	0.031
FGF-23	0.00	0.86	0.048
NT-3	0.00	0.37	0.087
IL8	0.01	0.38	0.122
uPA	0.01	0.35	0.122
LIF-R	0.01	0.30	0.122
ARTN	0.01	0.21	0.122
ADA	0.01	0.37	0.122
TNFB	0.01	0.43	0.122
TGF-alpha	0.02	0.21	0.181
LAP.TGF-beta-1	0.03	0.32	0.201
HGF	0.03	0.29	0.201
CD5	0.03	0.30	0.201
EN-RAGE	0.03	0.22	0.201
MCP-2	0.02	0.55	0.201
TNFRSF9	0.03	0.46	0.201
CDCP1	0.05	0.34	0.233
IL-12B	0.04	0.41	0.233
CXCL6	0.05	0.47	0.233
CD6	0.05	0.34	0.245
OPG	0.07	0.22	0.313
SIRT2	0.08	0.40	0.329
TNFSF14	0.09	0.35	0.331
IL10	0.09	−0.09	0.331
CCL20	0.09	0.63	0.348
IL-15RA	0.10	0.26	0.365
CXCL11	0.11	0.49	0.370
CXCL10	0.11	0.42	0.370
TNF	0.12	0.20	0.376
CD40	0.12	0.25	0.376
IL-10RB	0.14	0.22	0.416
CCL3	0.14	0.26	0.416
TSLP	0.16	0.13	0.425
IL4	0.16	0.12	0.425
IL-24	0.16	NA	0.426
MCP-1	0.17	0.18	0.434
IL5	0.18	0.36	0.456
FGF-21	0.23	−0.25	0.513
IL-20	0.23	0.32	0.513
CASP-8	0.21	0.20	0.513
CSF-1	0.23	0.12	0.513
CXCL9	0.25	0.30	0.537
SCF	0.26	−0.11	0.537
IL18	0.26	0.28	0.537
SLAMF1	0.26	0.15	0.537
VEGFA	0.28	0.17	0.539
MCP-4	0.28	0.28	0.539
CCL19	0.28	0.30	0.539
IL-20RA	0.30	0.32	0.553
IFN-gamma	0.30	0.32	0.553
CD244	0.39	0.18	0.554
IL-17C	0.34	−0.24	0.554
AXIN1	0.39	0.21	0.554
IL2	0.32	0.15	0.554
IL-10RA	0.34	0.16	0.554
IL-22.RA1	0.39	0.10	0.554
IL-18R1	0.39	0.19	0.554
CCL28	0.34	0.15	0.554
DNER	0.32	0.17	0.554
FGF-19	0.36	0.44	0.554
LIF	0.34	0.10	0.554
NRTN	0.39	0.15	0.554
CCL25	0.36	−0.20	0.554
STAMBP	0.39	0.19	0.554
IL-17A	0.41	0.10	0.578
IL33	0.43	0.09	0.603
CCL4	0.48	0.16	0.643
4E-BP1	0.48	0.14	0.643
TWEAK	0.48	0.13	0.643
CST5	0.51	0.21	0.669
CX3CL1	0.54	0.13	0.694
IL7	0.62	0.12	0.751
TRAIL	0.59	0.13	0.751

(continued on next page)

Table 3 (*continued*)

Biomarker	p-value	Difference	Adjusted p-value
IL-1.alpha	0.62	0.14	0.751
CXCL1	0.62	0.02	0.751
IL13	0.62	0.00	0.751
MCP-3	0.65	0.15	0.767
TRANCE	0.65	0.03	0.767
FGF-5	0.68	−0.03	0.782
ST1A1	0.68	0.11	0.782
MMP-10	0.71	0.18	0.797
CCL23	0.71	−0.01	0.797
CCL11	0.74	−0.05	0.822
IL-2RB	0.77	0.04	0.847
IL6	0.80	0.04	0.871
OSM	0.84	0.09	0.895
GDNF	0.87	0.00	0.919
CD8A	0.93	0.01	0.934
MMP-1	0.93	−0.10	0.934
Beta-NGF	0.93	0.06	0.934
CXCL5	0.93	0.01	0.934
Flt3L	0.90	0.01	0.934

Table 4
Differences in cytokine levels between inclusion and end of the study. Only cytokines with an adjusted p-value of < 0.05 between inclusion and end of study are included in the table. The analysis included all 30 patients.

Biomarker	Difference between inclusion and last visit	Adjusted p-value
VEGFA	−0.223	0.0031
CDCP1	−0.366	0.0031
IL7	−0.312	0.0031
LAP.TGF-beta-1	−0.581	0.0031
uPA	−0.425	0.0031
MCP-1	−0.380	0.0031
TRAIL	−0.238	0.0031
IL18	−0.554	0.0031
TNFSF14	−0.432	0.0031
CCL19	−0.314	0.0031
IL-15RA	−0.413	0.0031
PD-L1	−0.392	0.0031
TRANCE	−0.477	0.0031
HGF	−0.372	0.0031
IL-12B	−0.647	0.0031
TNF	−0.252	0.0031
CD5	−0.363	0.0031
CCL3	−0.446	0.0031
Flt3L	−0.270	0.0031
CXCL6	−0.384	0.0031
CXCL10	−0.640	0.0031
4E-BP1	−0.552	0.0031
CASP-8	−0.867	0.0031
CX3CL1	−0.580	0.0031
TNFRSF9	−0.992	0.0031
NT-3	−0.145	0.0031
ADA	−0.293	0.0031
MCP-3	−0.230	0.0082
CST5	−0.226	0.0082
CCL28	0.165	0.0082
ST1A1	−0.348	0.0082
CD6	−0.171	0.0107
IL-17A	−0.201	0.0129
FGF-23	−0.259	0.0129
CSF-1	−0.119	0.0244
IL-17C	0.302	0.0307
AXIN1	−0.370	0.0315
CCL23	−0.147	0.0421
CCL11	0.132	0.0451

signal, reducing the proliferation of antigen-specific T-cells [20]. The discovery of the PD-L1/PD-1 pathway led to the development of immune checkpoint inhibitors, a type of immunotherapy that targets and blocks the interaction between PD-L1 and PD-1. PD-L1 expression levels in tumors have been used to identify patients who are more likely to respond to immune checkpoint inhibitor treatment. Tumors with higher

Table 5
Spearman rank correlations between thyroid markers. The correlations are based on all measurements.

	fT4	fT3	TRAb	Tg
fT4	1.00	0.77	0.56	0.04
fT3	0.77	1.00	0.53	0.21
TRAb	0.56	0.53	1.00	0.36
Tg	0.04	0.21	0.36	1.00

PD-L1 expression may indicate a greater likelihood of response to therapy.

PD-L1 is also involved in regulating immune responses in autoimmune diseases, infections, and transplantations. The increased PD-L1 levels found in the present study indicate that GO patients may be influenced by immune checkpoint inhibitor treatments.

Recent research has shown an association between the PD-1/PD-L1 pathway and GD [21,22]. A recent study reported that the PD-1/PD-L1 pathway was involved in fibroblast activation and suggested that lack of PD-L1 on orbital fibroblasts could be the cause of orbital inflammation in GO patients [23]. The authors also reported the involvement of the CD40-CD40L pathway and up-regulation of CD40. This contrasts with the present study, where there was no significant effect on CD40 levels.

The use of animal models would allow further exploration of which cytokines are the main players in GO. Treatment with PD-L1 antibodies has been shown to inhibit T cell activity, reduce fibroblast activation, and reduce cytokine production. In vivo treatment with immune checkpoint inhibitors and anti-PD-L1 antibodies have been shown to be associated with autoimmune diseases and thyroid dysfunction [24,25]. Recently, apparent cure of GD was described following treatment with a monoclonal directed against PD-1 in a case with metastatic lung cancer [26].

The reduced cytokine levels at the end of the observation period in comparison with the initial values show that the ATD treatment in the present study had a broad anti-inflammatory effect. The treatment resulted in significantly reduced levels of both PD-L1 and FGF-23. The study was not designed to differentiate between direct effects of ATD on inflammation and mediation through the remission of hyperthyroidism, and so we can only say that the treatment resulted in a broad anti-inflammatory effect.

Hyperphosphatasemia occurs in patients with hyperthyroidism, including patients with GD. FGF-23 regulates phosphate concentration in the circulation via a PTH-independent mechanism [27]. A previous study showed that GD patients had higher serum phosphate and calcium levels than healthy controls, and that they also had elevated FGF-23 levels [28]. Moreover, it has been shown that ATD treatment resulted in a decrease in FGF-23 levels [29]. This is in agreement with the present findings. Our finding of increased levels of FGF-23 in GO patients in relation to GD patients without GO has not been reported previously and raises questions regarding the effects on mineral metabolism in GO patients. Further studies are warranted on the levels of calcium, phosphate, and PTH in GO patients.

5. Strengths and limitations

The limitations of our study are the small sample size and the fact that all patients were white and in the age range of 35–72 years. It is not clear if the results can be extrapolated to other ethnicities and age groups.

6. Conclusion

We have shown that Graves' ophthalmopathy was associated with elevated levels of circulating PD-L1 and FGF-23 levels. The findings support previous suggestions that the PD-1/PD-L1 pathway may serve as

a treatment target in hyperthyroid patients to prevent Graves' ophthalmopathy.

CRediT authorship contribution statement

Selwan Khamisi: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Visualization. **F Anders Karlsson:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Visualization. **Östen Ljunggren:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Visualization. **Mans Thulin:** Data curation, Formal analysis, Methodology, Resources, Visualization. **Anders Larsson:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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