

Adipocytokines in Graves' orbitopathy and the effect of high-dose corticosteroids

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ABSTRACT

Graves' orbitopathy (GO) is a serious, progressive eye condition seen in patients with autoimmune thyroid disease. GO is characterized by inflammation and swelling of soft orbital tissues. Adipose tissue produces cytokine mediators called adipokines. The present study focuses on the relationship between serum levels of selected adipokines in patients with GO, comparing them with the control group, and uniquely describes the effect of high-dose systemic corticosteroids (HDSC) on their levels. For the purposes of this study, we collected blood samples before and after the treatment with HDSC from 60 GO patients and 34 control subjects and measured serum levels of adiponectin, AIF-1, A-FABP and FGF-21. Levels of adiponectin significantly differed among the three study groups (ANOVA $p = 0.03$). AIF-1 levels were also significantly different among the study groups (ANOVA $p < 0.0001$). AIF-1 was significantly associated with the presence of GO after adjusting for clinical factors (age, sex, smoking and BMI) and level of TSH (odds ratio 1.003, $p < 0.01$). This finding could enforce targeting macrophages in treatment strategies for GO since AIF-1 is considered as a marker of their activation.

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Introduction

Graves' Orbitopathy (GO) is a serious, progressive eye condition that is associated with autoimmune thyroid disease. GO is characterized by inflammation and swelling of orbital tissues, increasing the volume of connective, muscle and adipose tissue participating in the complex ocular disability. Early diagnosis and correct timing of immunosuppressive therapy are critical steps in the management of GO. Despite the severity of the disease, information on its pathogenesis is limited; however, novel pathophysiological mechanisms are appearing [1]. The orbital tissues are infiltrated by activated T-cells, plasmocytes, macrophages and mast cells, and correlation between T and B lymphocytes infiltrating orbital tissues and the activity of GO was reported [2,3]. Furthermore, it is thought that there is an occurrence of local adipo- and fibro-genesis [4]. The role of adipose tissue is under investigation in connection with many diseases. The ability of fibroblasts to trans-differentiate into adipocytes has been widely

described, especially it has been shown that GO fibroblasts undergo spontaneous and pressure-induced adipogenesis in 3D cultures [5,6]. This finding suggests that GO orbital fibroblasts have an intrinsic pro-adipogenic phenotype and that the pressure applied to normal orbital fibroblasts was sufficient to induce trans-differentiation into adipocytes [5]. Based on those findings, Li et al. postulated that the pathological intraorbital pressures encountered in GO could drive adipogenesis on its own, and potentially also explain the clinical results suggesting that orbital decompression can reduce orbital inflammation [5,7].

Adipocytes serve not only as a store of excess energy, but also as a functionally active cell, producing biologically active substances with different functions. Adipocytes can uniquely secrete adipocytokines, or adipokines, which are peptides signalling via endocrine, autocrine and paracrine mechanisms and can translate the functional status of adipose tissue towards intermediate and energy metabolism of the brain, liver and

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 Supplemental data for this article can be accessed [here](#)

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skeletal muscles. They are promising candidates for both novel pharmacological treatment strategies and diagnostic tools in a plethora of diseases. Only scarce data are available on the relationship between adipokines and GO, but several members of the adipokine family have previously been shown to be associated with thyroid disease, and their levels are reported to be altered by changes of thyroid function [8–10].

Adiponectin is a 244 amino acid protein highly expressed in human plasma. Multiple reports have shown that it provides anti-inflammatory effects, increasing insulin sensitivity and protecting against atherosclerotic development [11]. Serum level of adiponectin does not seem to be influenced by hypothyroidism; however, it can be normal or elevated in hyperthyroidism; the results of performed studies are discordant [11,12].

Allograft inflammatory factor 1 (AIF-1) is a novel member of the adipocytokine family, commonly studied in connection with obesity and insulin resistance [13]. However, it is thought to also play an important role in the development of several autoimmune diseases and processes [14]. AIF-1 was found to be produced by macrophages; its expression could be upregulated after T cell activation [15], by some being considered as a marker of macrophage activation [16]. To our knowledge, its role in connection to thyroid function or thyroid autoimmunity has not yet been elucidated.

The adipocyte fatty-acid-binding protein (A-FABP, also known as FABP4 or aP2) was originally identified as an abundant cytoplasmic protein in adipocytes. It links lipid metabolism with inflammation and also increases thermogenesis by promoting the conversion of T4 to T3 in brown adipocytes [17]. The relationship between thyroid status and A-FABP levels has not been profoundly studied yet, and the only available data showed significantly higher A-FABP levels in hyperthyroid patients, followed by an A-FABP decrease after euthyroidism restitution [18].

Fibroblast growth factor-21 (FGF-21) is a member of the FGF family that is produced in the liver and adipose tissue. Presented data suggest that FGF-21 and thyroid hormones could interact to regulate metabolism. In mice, thyroid hormones regulate FGF-21 levels in the liver and in the adipose tissue, but in contrast, peripheral FGF-21 administration led to decreased circulating levels of thyroid hormones [19]. In humans, the results are contradictory; Xiao found an elevated FGF-21 level in hyperthyroid patients [20], but Lee reported that FGF-21 elevated in hypothyroid patients [21].

While data on adipokines exist, little is known about their relationship with the pathogenesis of GO. Here,

we attempt to elucidate the relationship between adipokines and Graves orbitopathy.

Patients and methods

Patients

Data of 60 patients with GO, seen at our tertiary endocrine centre, were included in this study; all patients were treated with high-dose systemic corticosteroids (HDSC). Our HDSC regimen consisted of intravenously applied methylprednisolone (ivMP) during three short hospitalizations, given over 3 months with two possible cumulative dosages: 7.5 g ivMP (twice 1 g for 3 days/month and once 0.5 g for 3 days/month) used until 2018 and then 6 g ivMP (once 1 g for 3 days/month and twice 0.5 g for 3 days/month). Between the first and second sessions, patients received 20 mg prednisolone orally, and between the second and third sessions they received 10 mg. Blood samples were obtained before initiation of HDSC (sample I./before HDSC), immediately after the third pulse of ivMP (sample II.) and at the outpatient visit 4–6 weeks after termination of the ivMP therapy (sample III). For technical reasons, we could not always obtain all three consecutive samples from all patients. The sample after HDSC therefore includes data from sample III. However, if this sample was unavailable then, the sample II. data was used, if both samples were available, than the mean of II. and III. was calculated. Numbers of samples per group are shown in Supplementary Table 1. As controls, data from healthy volunteers without known thyroid dysfunction were used (34 total). Clinical Activity Score (CAS) was assessed by an ophthalmologist experienced with GO patients according to the latest recommendations [22]. Informed consent was obtained from all participants. The ethics committee of the University Hospital in Olomouc approved this study (reference number 107/15).

Laboratory analyses

Venous blood samples were drawn in the morning after a 12-h-fasting period using a closed system Vacuette® tube with a clotting activator. After centrifugation, the serum was used for analyses. Routine biochemical parameters (fT4, TSH and TSI) were performed on the day of blood collection. Concentrations of adipokines were measured in the sample aliquot (stored at –80°C for no longer than 6 months, with no freeze–thaw cycles). fT4 and TSH were analysed using commercially available immunochemical (CMIA) kits using Architect i2000SR

Table 1. Baseline characteristics and adipocytokines levels in subjects of the control group and patients before and after HDSC.

	Controls	Before HDSC	After HDSC
Age (years)	40.53 ± 9.22	55.48 ± 13.44 †	NA
Sex (male/female)	10/24 [29/71]	15/45 [33/67]	NA
Smoker	4 [11.76]	5 [8.33]	NA
BMI (kg/m ²)	23.10 (20.62 - 25.98)	25.66 (22.83 - 29.51) *	NA
ft4 (pmol/l)	12.3 (12 - 13.5)	15.5(13.05 - 18.7) †	NA
TSH (mIU/l)	1.72 (1.44 -2.18)	0.2 (0.01 - 1.04) †¶	1.19 (0.21 -2.62)
TSI (IU/l)	NA	3.68 (0.75 - 11.5) ¶	0.86 (0.29 - 3.81)
CAS	NA	3.5 (2.5 - 4) ¶	1 (0.5 - 2)
Adiponectin (ug/ml)	11.1 (8.3 - 23.4)	14.1 (10 - 17.8) *	12.38 (9.85 - 56.35)
AIF-1 (pg/ml)	677.2 (558.8 - 824.3)	1135 (683 - 1688) †	1249 (901.8 - 2096) @
A-FABP (ng/ml)	25.5 (20.9 - 32.4)	31.9 (21.6 - 43.9)	27.35 (12.9 - 48)
FGF-21 (pg/ml)	267.2 (98.2 - 366.3)	133 (30 - 256.7)	132.4 (30 - 312.8)

Legend: Age is as mean ± standard deviation, all other values are as median (25th - 75th percentile), in [] percentage. Statistically significant differences are in bold. Normal ranges for ft4 are 9.1 - 19.1; for TSI 0 - 0.55; and TSH 0.35 - 4.94. Data outside normal ranges are in italics. HDSC - high dose systemic corticosteroids, BMI - body mass index, ft4 - free thyroxine, TSH - thyroid stimulating hormone, TSI - thyroid stimulating immunoglobulin, CAS - clinical activity score, NA - not available, *p < 0.05 and †p < 0.001 in patients before treatment vs. control group, §p < 0.05 and @p < 0.001 in patients after treatment vs. control group, ¶p < 0.05 and ¶¶p < 0.001 in patients before treatment vs. after treatment.

(Abbott, USA); TSI was determined immunologically (LEIA) on the Immulite 2000 analyser (Siemens Healthineers, Germany). Enzyme-linked immunosorbent assay (ELISA) kits (Bio-Vendor laboratory Medicine, Inc., Brno, the Czech Republic) were used to measure adipokines, according to the manufacturer's instructions. Adipokine measurements included FGF-21 (Cat. No. RD191108200R), A-FABP4 (Cat. No. RD191036200R), adiponectin (Cat. No. RD195023100) and AIF-1 (Cat. No. RD191204200R).

Statistical analysis

To test the data distribution, the Shapiro–Wilk normality test was utilized. Data are presented as mean ± standard deviation (t-test to detect differences) or as median and 25th–75th percentiles (Mann–Whitney to detect differences). To compare different group characteristics, a nonparametric ANOVA test (Kruskal–Wallis) was utilized with Dunn's Post-test (not paired). The association of adipokines and/or other variables with the presence of GO was analysed by multivariate logistic regression analysis. GraphPad Prism 8.4.3 for Windows (San Diego, California, USA) was used to perform analyses and create figures.

Results

The baseline characteristics and laboratory results of the study population are presented in Table 1. Our treated cohort was significantly older and had a higher BMI than the control group; however, the ratio of males and females was not statistically different nor was the ratio of smokers to non-smokers. Regarding the thyroid function tests, GO patients at

baseline had significantly higher levels of ft4 than the control group (15.5 vs. 12.3 pmol/l; p < 0.05); however, both values were within the normal reference range. The baseline TSH level of GO patients was significantly lower than in both the control group and the treated group after HDSC and was below the normal reference range. TSI levels were significantly higher before compared to after HDSC (3.68 vs. 0.86; p < 0.001).

Levels of adiponectin significantly differed among the three study groups (ANOVA p = 0.03), with the highest value in GO patients before initiation of HDSC, which was significantly higher than the control group (14.1 vs. 11.1ug/ml; p = 0.03). AIF-1 levels were also highly significantly different among the study groups (ANOVA p < 0.0001) with the highest value in GO patients after HDSC treatment, which was, however, not statistically different to the value in patients before HDSC treatment (p = 0.82). We did not observe any statistically significant difference in the levels of A-FABP or FGF-21 between controls and our patients, nor did HDSC treatment have an effect (ANOVA p = 0.19; p = 0.11). Levels of studied adipokines in controls, patients before HDSC and after HDSC levels are shown in Table 1 and Figure 1.

In our control group, serum levels of adiponectin negatively correlated with BMI, while A-FABP levels positively correlated with BMI both in the control population and in the treated group before HDSC. We did not observe any other statistically significant correlation between adiponectin, AIF-1, A-FABP or other measured parameters mentioned among the study groups. We found a statistically significant positive correlation between the levels of FGF-21 and TSH (r = 0.31, p = 0.04) and CAS (r = 0.35, p = 0.03) in patients before HDSC treatment. Correlation parameters are shown in Table 2.

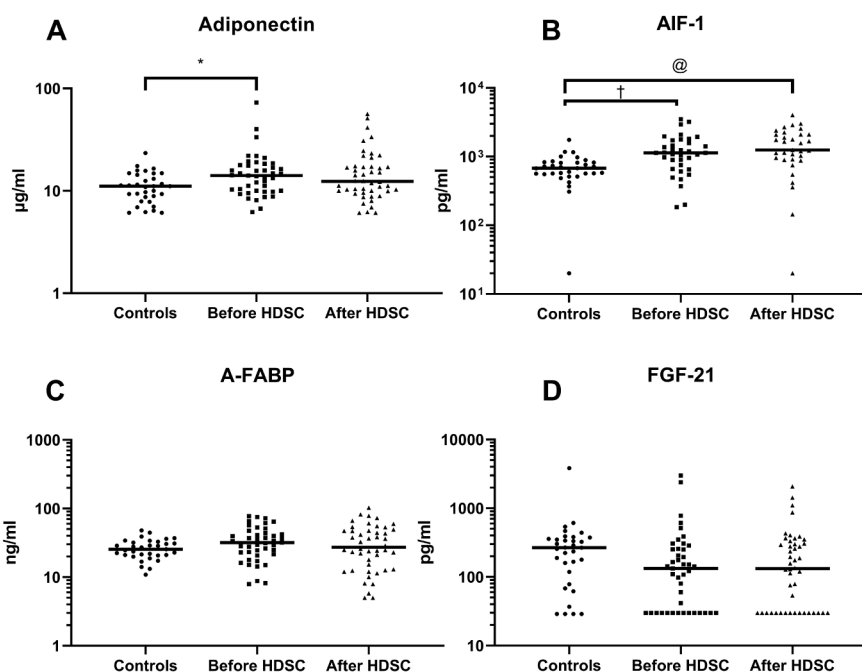


Figure 1 Levels of studied adipokines in controls, patients before HDSC and after HDSC. Panel A: adiponectin ($\mu\text{g/ml}$); Panel B: AIF-1 (pg/ml); Panel C: A-FABP (ng/ml); Panel D: FGF-21 (pg/ml). Y axis concentrations are in log 10. * $p < 0.05$ and † $p < 0.001$ in patients before treatment vs. control group, @ $p < 0.001$ in patients after treatment vs. control group.

Table 2. Correlation of serum adiponectin, AIF-1, A-FABP, FGF-21 with clinical variables in controls, patients before HDSC (1) and after HDSC (2).

	Adiponectin			AIF-1			A-FABP			FGF-21		
	Control	Before HDSC	After HDSC	Control	Before HDSC	After HDSC	Control	Before HDSC	After HDSC	Control	Before HDSC	After HDSC
n	34	43	46	34	43	41	34	43	46	34	43	46
Age	0.095 (0.598)	0.26 (0.092)	NA	-0.145 (0.422)	-0.137 (0.426)	NA	0.061 (0.737)	-0.017 (0.912)	NA	0.285 (0.108)	0.027 (0.866)	NA
BMI	-0.394 (0.028)	0.27 (0.08)	NA	0.262 (0.154)	0.202 (0.237)	NA	0.682 (<0.001)	0.337 (0.027)	NA	0.267 (0.147)	0.029 (0.854)	NA
ft4	0.134 (0.446)	-0.225 (0.158)	NA	0.024 (0.898)	-0.145 (0.412)	NA	0.108 (0.555)	-0.105 (0.515)	NA	-0.1 (0.585)	-0.222 (0.164)	NA
TSH	-0.082 (1)	0.068 (0.666)	NA	-0.013 (0.943)	0.167 (0.33)	NA	0.099 (0.583)	0.149 (0.34)	NA	0.026 (0.885)	0.31 (0.043)	NA
TSH	NA	NA	0.071 (0.645)	NA	NA	0.098 (0.593)	NA	NA	-0.121 (0.427)	NA	NA	0.062 (0.688)
TSI	NA	0.092 (0.569)	NA	NA	0.189 (0.277)	NA	NA	-0.165 (0.304)	NA	NA	0.17 (0.288)	NA
TSI	NA	NA	0.002 (0.992)	NA	NA	0.233 (0.215)	NA	NA	-0.212 (0.178)	NA	NA	-0.134 (0.397)
CAS	NA	-0.12 (0.455)	NA	NA	-0.104 (0.553)	NA	NA	-0.144 (0.368)	NA	NA	0.348 (0.026)	NA
CAS	NA	NA	-0.279 (0.07)	NA	NA	0.1 (0.587)	NA	NA	-0.2 (0.199)	NA	NA	-0.1 (0.523)

Legend: Spearman correlation index r (p-value) was used to determine significance; statistically significant correlations are highlighted in bold. Correlation indexes for age, BMI, ft4 and TSH were calculated for controls and for patients before therapy. Correlation indexes for TSH, TSI and CAS were also further calculated before (1) and after (2) HDSC. HDSC - high dose systemic corticosteroids, BMI - body mass index, ft4 - free thyroxine, TSH - thyroid stimulating hormone, TSI - thyroid stimulating immunoglobulin, CAS - clinical activity score, NA - not available

AIF-1 was in a multivariate analysis significantly associated with the presence/absence of GO after adjusting for clinical factors (age, sex, smoking and

BMI) and level of TSH (Odds ratio 1.003, $p < 0.01$). No other adipokine showed this association (Table 3).

Table 3. Multivariate logistic regression analysis with presence of Graves' Orbitopathy as the dependent variable.

Independent variables	Odds ratio (95% CI)	p-value
Age	1.156 (1.067 - 1.291)	0.0024
Sex (male)	0.196 (0.01288 - 1.571)	0.1781
Smoking	0.5502 (0.01983 - 9.570)	0.7008
BMI	0.8368 (0.6385 - 1.013)	0.1343
TSH	0.544 (0.2898 - 0.9327)	0.0328
Adiponectin	1.089 (0.9321 - 1.441)	0.4985
AIF-1	1.003 (1.001 - 1.005)	0.0087
A-FABP	1.013 (0.9404 - 1.101)	0.7503
FGF-21	0.9995 (0.9964 - 1.001)	0.7421

Legend: Statistically significant parameters/odds ratios are in bold. CI – confidence interval, BMI - body mass index, TSH - thyroid stimulating hormone

We did not find any statistically significant difference between the levels of any adipokine among multiple blood samplings (samples I, II and III) nor between the two different HDSC regimens. Data for AIF-1 are only for 7.5 g ivMP dosage regimen. This profound analysis is shown in Supplementary Table 1.

Discussion

In the present study, of a relatively large cohort of 60 patients with GO, we could present data on serum levels of selected adipocytokines and their changes associated with HDSC treatment. To our knowledge, the effect of HDSC on adipocytokines has not been reported previously. We present a novel role for AIF-1 as a potentially important parameter associated with GO. Furthermore, we confirmed increased levels of adiponectin in GO patients. For the first time, we describe a positive correlation of FGF-21 levels with CAS in GO patients.

Patients in our treated cohort had both median and mean fT4 within the normal reference range (15.5 vs. 16.52); however, their median TSH level (not the mean) was below the norm (TSH 0.02 resp. 1.01 mIU/l), suggesting a higher prevalence of hyperthyroidism in our study group. Multivariate analysis found that higher age and lower TSH were significantly associated with the presence of GO; however, the former could be attributed to the age distribution of our control group and the latter to the increased incidence of hyperthyroidism in patients with GO. Smoking is a well-known risk factor, which we could not confirm in this study due to a low number of smoking patients/controls.

In our control group, serum levels of adiponectin negatively correlated with BMI, as shown in previous studies [11,23]. Contrarily, this correlation was not present in our cohort ($r = 0.27$, $p = 0.08$), where the patients had statistically significantly higher levels of

adiponectin than the control group (14.1 vs. 11.1 ug/ml; $p = 0.03$), but in the multivariate regression analysis, we did not find adiponectin as an independent marker of GO (Odds 1.089, $p = 0.5$). Our data are therefore in concordance with the study of Kim et al., who also reported significantly higher levels of adiponectin among patients with GO, but did not confirm it as an independent marker of GO. However, in this study, adiponectin positively correlated with CAS, which we did not confirm ($r = -0.12$, $p = 0.46$) [24]. Levels of adiponectin were not affected by HDSC (14.1 vs. 12.4 ug/ml; $p > 0.99$).

AIF-1 levels significantly differed among the study groups, with the highest value in patients after HDSC treatment. No correlation of AIF-1 with any of the screened laboratory or clinical factors was found. Values of AIF-1 were significantly higher in patients being treated for GO before and also after the treatment with HDSC. We can only speculate, why the level of AIF-1 did not decrease with the ivMP treatment. Only limited data on this topic are available, but in the study focused on cardiac rejection after heart transplant, Autieri et al. found decrease in AIF-1 tissue expression after immunosuppressive therapy (including methylprednisolone) [25]. This discrepancy could be caused by a short time of follow-up in our study or different effects of immunosuppression on AIF-1 as a possible pro-fibrotic factor in GO [26]. The adipokines multivariate analysis revealed that the serum level of AIF-1 was significantly associated with the presence of GO after adjusting for clinical factors (age, sex, smoking and BMI). Previously, a similar association with the presence of GO showed other adipokines, which were not included in this study (Leptin and Resistin) [24]. Also, previously AIF-1 concentrations were reported to inversely correlate with high-density lipoprotein cholesterol level [16], and also its level could affect the response to ivMP therapy [27]. The role of metabolic factors in the development of GO and its treatment strategies was recently reviewed by Lanzolla et al. [28]. Since AIF-1 could be considered as a marker of macrophage activation [16], we postulate that our results confirm the role of macrophages in GO. With respect to attempts to find novel, nonsteroidal therapeutic options for GO patients, targeting macrophages could be a promising option. For example, Adalimumab and Etanercept block tumour necrosis factor alpha (TNF- α), a crucial macrophage chemoattractant, and therefore may have a role in the treatment of active GO with prominent inflammatory symptoms [2,29].

Levels of A-FABP were found at similar levels in controls and patients, both before and after treatment with HDSC. Its level positively correlated with BMI in

controls, as previously shown by our group and others [30,31] and also in the patient group. We did not find any other significant correlation of A-FABP with any clinical/laboratory marker of GO, nor did multivariate analysis confirm its role in GO. Therefore, we suggest that the levels of A-FABP are not affected in patients with GO.

We did not observe any significant differences in FGF-21 levels between the control group and our patients before or after the treatment (ANOVA $p = 0.12$). The median serum level of FGF-21 in the control group was almost double the median level in the patient group before treatment (267.2 vs. 133 pg/ml), but this result is likely due to FGF-21 plasma variation, as the difference was not statistically significant ($p = 0.14$) (Figure 1, panel D). Additionally, the levels of FGF-21 did not change with the treatment ($p > 0.99$); therefore, we can postulate that the level of FGF-21 is not affected by HDSC. We found a positive correlation between TSH and the level of FGF-21 in the patient group before treatment; therefore, we can report a decreasing level of FGF-21 with increasing hyperthyroidism, which is contradictory to some of the previous reports [20,32]. It is, however, of note that the effect of thyroid hormones on FGF-21 is not entirely elucidated yet, for example, Lee et al. showed increased FGF-21 plasma levels in hypothyroid patients [21].

In a more profound substudy, including several time points (I, II and III) and dosage regimens (7.5 g and 6 g), we did not observe any statistically significant differences; therefore, those data are only complimentary (Supplementary Table 1). Data for AIF-1 are only for 7.5 g ivMP dosage regimen because the biochemical analysis was available only in the first years of the study.

As a study limitation, we consider the inability to perform repeated measurements for analysis, due to the unavailability of consecutive patients' samples limits the power of the study. Furthermore, the minor change in methylprednisolone cumulative dose in 2018 according to the latest European Group On Graves' Orbitopathy (EUGOGO) recommendation could potentially affect our observed results in the treated population. The possible effect of those two cumulative dosages on levels of adipokines was not the primary goal of this study; however to monitor and to exclude its potential effect, we performed a subanalysis and did not record any difference after altering HDSC dose (Supplementary Table 1), and therefore we omitted this difference for analyses in Table 1 and 2. To quickly stabilize thyroid function, we advise our patients to undergo a total thyroidectomy after the second ivMP pulse, and most of the patients included in this study underwent this procedure (approx. 90%). Unfortunately, we could not get an age-matched control group without known thyroid disorder, but the multivariate regression analysis was

adjusted for differing parameters of age, BMI, and smoking.

In this tertiary centre-based clinical study, we report the levels of several adipokines in patients with GO before and after treatment with HDSC. Adiponectin levels were repeatedly higher in GO patients; however, they were not an independent marker of GO. A multivariate analysis of adipokines revealed a significant association between the presence of GO and serum level of AIF-1 after adjusting for clinical factors and TSH levels. This finding could enforce role of macrophages as a potential target for GO treatment strategies, as AIF-1 is considered as a marker of macrophage activation. We found low levels of FGF-21 in patients with GO; however, a large intersample variation reduces the interpretability of the results. We conclude that the adipose tissue in the orbit affected by GO seems to be actively participating in the course of the disease and targeting its activation may lay the groundwork for the development of new therapeutic options.

Data availability statement

The data that support the findings of this study are available from the corresponding author [JS], upon reasonable request. The data are not publicly available due to an ethics committee statement.

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