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Identification of State Markers in Anorexia Nervosa: Replication and Extension of Inflammation-Associated Biomarkers Using Multiplex Profiling

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ABSTRACT

BACKGROUND: Proteomics offers potential for detecting and monitoring anorexia nervosa (AN) and its variant, atypical AN (atyp-AN). However, research has been limited by small protein panels, a focus on adult AN, and lack of replication.

METHODS: In this study, we performed Olink multiplex profiling of 92 inflammation-related proteins in females with AN/atyp-AN (n = 64), all of whom were ≤90% of expected body weight, and age-matched healthy control individuals (n = 44).

RESULTS: Five proteins differed significantly between the primary AN/atyp-AN group and the healthy control group (lower levels: HGF, IL-18R1, TRANCE; higher levels: CCL23, LIF-R). The expression levels of 3 proteins (lower IL-18R1, TRANCE; higher LIF-R) were uniquely disrupted in participants with AN in our primary model. No unique expression levels emerged for atyp-AN. In the total sample, 12 proteins (ADA, CD5, CD6, CXCL1, FGF-21, HGF, IL-12B, IL18, IL-18R1, SIRT2, TNFSF14, TRANCE) were positively correlated with body mass index and 5 proteins (CCL11, FGF-19, IL8, LIF-R, OPG) were negatively correlated with body mass index in our primary models.

CONCLUSIONS: Our results replicate the results of a previous study that demonstrated a dysregulated inflammatory status in AN and extend those results to atyp-AN. Of the 17 proteins correlated with body mass index, 11 were replicated from a previous study that used similar methods, highlighting the promise of inflammatory protein expression levels as biomarkers of AN disease monitoring. Our findings underscore the complexity of AN and atyp-AN by highlighting the inability of the identified proteins to differentiate between these 2 subtypes, thereby emphasizing the heterogeneous nature of these disorders.

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Anorexia nervosa (AN) and its related variant (i.e., atypical AN [atyp-AN]) are among the deadliest psychiatric disorders. AN is marked by significant low weight (body mass index [BMI] < 18.5), while in atyp-AN, weight remains within or above normal limits (1); however, psychiatric and medical severity are comparable (2,3). Recovery becomes less likely as the illnesses progress (4–6), which underscores the need for early intervention. However, AN and atyp-AN often go undetected (4), and individuals rarely initiate treatment on their own (5). Diagnosis is challenging because it relies on measured body weight (for which diagnostic weight thresholds are imperfectly defined) and self-reported symptoms (which individuals with AN/atyp-AN minimize or fail to recognize). Biomarkers could aid with illness detection and differential diagnosis, inform mechanistic understanding of these illnesses, and guide conceptualization of outcomes. To date, studies on protein biomarkers have focused only on adults with AN, usually years after the disorder emerged.

Inflammation is a regulatory process that occurs in response to injury, stress, or infection and is widely implicated across psychiatric disorders (6–9). Meta-analyses and narrative reviews have suggested that AN displays a unique profile of inflammatory molecule expression compared to primary malnutrition, with increases in circulating tumor necrosis factor α (TNF-α), interleukin (IL) 1β, IL-6, and TNF receptor-II and decreases in C-reactive protein and IL-6 receptor expression (10–12). A recent study that compared the largest battery of plasma inflammatory markers in 113 women with active AN, 113 women who had recovered from AN, and 114 normal-weight healthy control individuals (HCs) highlighted the promise of inflammation protein expression levels as state-specific markers of AN, associated only with low-weight status (13). The investigators observed a different proteomic plasma profile of inflammatory markers in women with AN than in women who had recovered from AN and HCs, whereas

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recovered women and HCs did not differ from each other. The proteins identified were also correlated with BMI, which suggests that an aberrant inflammatory profile is a state marker of AN. Because individuals with atyp-AN were not included, parsing whether those inflammation proteins were unique to AN and/or independent of low-weight status (i.e., present in both AN and atyp-AN but not in either group when recovered) was not possible.

To determine whether inflammatory protein markers represent useful clinical biomarkers, follow-up steps are necessary: first, examination of inflammatory profiles in AN and atyp-AN compared with HCs could inform whether inflammatory markers reflect restrictive eating disorders more broadly (i.e., AN and atyp-AN), thus distinguishing them from HCs, or whether specific biomarkers could aid in differential diagnosis between individuals with AN and those with atyp-AN. Second, AN and atyp-AN occur most commonly in adolescents and young adults (14,15), but the previous study sample comprised adults with AN. Because inflammation proteins differ significantly across age (16), studying adolescents with AN and atyp-AN is a priority.

We quantified inflammatory proteins in a mixed sample of female adolescents with AN or atyp-AN and age-matched HCs to replicate and expand upon the findings of Nilsson *et al.* (13). We hypothesized that the combined AN/atyp-AN group would show a unique profile of inflammatory protein expression versus HCs. To understand the contribution of low-weight status, we also hypothesized that certain markers of immune dysregulation would be unique to AN (and therefore evident in comparisons of the AN group vs. the HC group and not in comparisons of the atyp-AN group vs. the HC group). In the total sample, we expected to see strong relationships between immune proteins and BMI.

METHODS AND MATERIALS

Sample

Data were derived from 2 parent studies (17,18). The sample included females with AN and atyp-AN who were ≤90% of median BMI (based on the 50th percentile of BMI [weight (kg)/height^2 (m)] for age and gender) and HCs. Participants were enrolled between April 1, 2014 and January 18, 2021 at Massachusetts General Hospital with institutional review board approval. Participants provided written informed consent or parental consent with assent from minors <18 years and were recruited from Massachusetts General Hospital and the greater Boston area. In this report, we included females with AN or atyp-AN and HCs with stored plasma samples (N = 108). Participant criteria and study procedures are included in Supplemental Methods in Supplement 1 and have been reported elsewhere (17).

Participants

We included females ages 10 to 22 years with DSM-5-defined AN or atyp-AN and HCs with no lifetime history of psychiatric diagnoses and with similar pubertal (Tanner) stages. The DSM-5 defines atyp-AN as meeting all criteria for AN except for the low-weight criterion (operationalized here as a BMI < 18.5 for adults or a BMI-for-age percentile of ≤ 10.99 for people <18 years) (Centers for Disease Control and Prevention). Screening for AN/atyp-AN included clinical evaluation to confirm the absence of any organic condition that could account for low body weight. Participants had no history of diabetes mellitus, gastrointestinal tract surgery, recent systemic hormone use, or pregnancy that could impact inflammatory protein levels. No participants had taken nonsteroidal anti-inflammatory drugs on the day of collection. We recorded nonsteroidal anti-inflammatory drug use over the preceding 2 months, which we used as a covariate in secondary models. See Supplemental Methods in Supplement 1 for details.

Blood Sampling and Processing

Trained nursing staff collected fasting blood at 8:45 AM using EDTA tubes. After centrifugation at 4° C, plasma samples were stored at −80° C. Samples were processed by Olink's Target inflammation 96 panel, which utilizes proximity extension assay technology followed by amplification using polymerase chain reaction (19). The panel quantifies 92 inflammation-related proteins simultaneously and reports the protein concentrations using a relative $\log_2$ scale (normalized protein expression). A difference of 1 normalized protein expression unit approximates a doubling of protein concentration. We assessed normality of protein concentrations by visually assessing the quantile-quantile plots. Because proteins are reported using a $\log_2$ scale and most (see Figure S1 in Supplement 1) were normally distributed, no additional normalization procedures were applied. Olink reports the limit of detection (LOD) for each assay estimated from negative controls (i.e., contain buffer only) plus 3 standard deviations; samples that did not pass quality control were flagged. For detailed information, see Assarsson *et al.* (19) and https://www.olink.com/resources-support/white-papers-from-olink/.

Statistical Analyses

Statistical analyses were performed using R version 4.2.2. All 108 samples passed the general Olink quality control. Protein values lower than the LOD were replaced with the corresponding LOD value. Proteins with over 20% of data lower than the LOD were excluded from the analyses, which left a total of 73 proteins for analysis. Moreover, we visually inspected the summary plot from the principal component analysis to identify outliers.

We conducted multiple linear regression analyses to examine group differences for each protein between the AN/atyp-AN group and the HC group (aim 1) and the AN versus atyp-AN versus HC groups (aim 2) followed by associations between BMI z scores and each protein in the total sample (aim 3). We tested 2 primary models for each aim (Table 1). We adjusted multiple comparisons using the Benjamini-Hochberg correction (20). A false discovery rate (FDR) < .05 was considered statistically significant, and an FDR of .06 to .1 was considered marginally significant. In Supplement 2, we have also listed the median estimates for all proteins individually and for each group.

Following our primary analyses (models 1 and 2), we conducted secondary multiple linear regression models to test the robustness of the primary analyses, including factors shown to influence inflammation levels [i.e., smoking (21,22),

Table 1. Model Details

Models	Covariate(s)	Participants Removed
Main Models		
Model 1	Age	–
Model 2	Age, smoking status, body temperature, any antihistamine use, any psychiatric medication use, and any psychiatric comorbidity	–
Secondary Models		
S1	Age	Smoking, $n = 4$
S2	Age	Body temperature >37.2° C, $n = 8$
S3	Age, psychiatric comorbidity	–
S4	Age, anxiety/depressive symptoms	–
S5	Age, antidepressant use	–
S6	Age, antihistamine use	–

antihistamine use (23), antidepressant use (24), and depression/anxiety comorbidity (9)]. Model details are shown in Table 1. All R code can be downloaded from GitHub https://github.com/laurenbreithaupt-mgh/Targeted-Inflammation-Proteomics-AN-atypAN-Cross_Sectional.

RESULTS

We included 108 females (38 with AN, 26 with atyp-AN, 44 HCs), with similar percentages of premenarchal participants in each group. As expected, BMI *z* score differed by group (BMI *z* score mean [SD]: AN = −2.26 [0.741], atyp-AN = −0.864 [0.264], HC = 0.118 [0.556]; $F_{2,105} = 171.1$, $p < .001$). Individuals with AN were older than HCs (Tukey's honestly significant difference test, $p = .01$); however, there were no age differences between the AN and atyp-AN groups or the atyp-AN and HC groups. AN and atyp-AN groups did not differ on duration of illness, frequency of binge/purge symptoms, number of psychiatric comorbidities, levels of depression, anxiety, or psychiatric medication use. Clinical characteristics are summarized in Table 2 and Table S1 in Supplement 1. We excluded 19 proteins (MCP-3, IL-17C, IL-2RB, IL-1 alpha, IL2, TSLP, SLAMF1, FGF-5, IL-22 RA1, Beta-NGF, IL-24, IL13, ARTN, IL-20, IL33, IL4, LIF, NRTN, and IL5) from further analysis due to over 20% of data being invalid (values lower than the LOD). We identified and removed 2 outliers from the HC group through visual inspection of the principal component analysis summary plot (see Figure S2 in Supplement 1) based on data from the 73 proteins. All proteins included in the analysis are in Table S2 in Supplement 1.

Inflammatory Markers in the Combined AN/atyp-AN Group Versus the HC Group

After adjusting for age in model 1, 5 proteins differentiated the combined AN/atyp-AN group from the HC group (Figure 1A). Compared with HCs, participants in the AN/atyp-AN group had lower plasma concentrations of TRANCE, IL-18R1, and HGF and higher concentrations of LIF-R and CCL23. Figure 2A illustrates the $\log_2$ fold change of protein concentrations in the 2 groups. After adjusting for covariates in model 2, TRANCE, IL-18R1, and LIF-R remained different between the AN/atyp-AN group and the HC group; additionally, MMP-1 was increased in the AN/atyp-AN group.

We tested the robustness of models using complementary sensitivity analyses (models S1–S6). In each secondary model, we adjusted for different covariates while also accounting for age to ensure comparability with model 1. TRANCE, IL-18R1, and LIF-R were consistently significant in all models (Figure 3A). HGF and CCL23 were significant in models S1, S2, S3, and S6. CXCL1 and TNFSF14 showed significance only in model S2, and Flt3L showed significance only in model S4.

Across primary and secondary models, 9 proteins differentiated the AN/atyp-AN group from the HC group (summarized in Figure 3A): CCL23, CXCL1, Flt3L, HGF, IL-18R1, LIF-R, MMP-1, TNFSF14, and TRANCE.

Inflammatory Expression Levels Unique to Low-Weight AN

When we compared the AN group with the HC group in model 1, we observed a lower TRANCE and IL-18R1 concentration and higher LIF-R concentration in the AN group (see Figure 1B). Figure 2B shows the $\log_2$ fold change for protein concentrations comparing AN versus HC. After adjusting for covariates in model 2, TRANCE and LIF-R remained different between the AN group and the HC group. Across primary and secondary models, 3 proteins differentiated participants with low-weight AN from HCs (summarized in Figure 3B): TRANCE, IL18-R1, and LIF-R.

We did not observe differences in protein expression when we compared AN with atyp-AN or atyp-AN with HCs in our primary models. In secondary models, levels of 2 proteins (LIF-R and Flt3L) were higher in participants with atyp-AN than in HCs in model S4; no proteins emerged in the other secondary models.

Inflammatory Markers and BMI in the Total Sample

In the total sample, we analyzed associations between BMI *z* scores and each protein. In model 1, controlling for age, 17 proteins were associated with BMI after correcting for multiple testing. Among them, 12 proteins (TRANCE, IL-12B, IL-18R1, CXCL1, IL18, HGF, ADA, TNFSF14, CD6, FGF-21, SIRT2, and CD5) were positively associated and 5 proteins (LIF-R, CCL11, FGF-19, IL8, OPG) were negatively associated with BMI (Figure 1C and 2C).

In model 2, TRANCE, IL-12B, and IL-18R1 remained positively associated with BMI, while LIF-R, CCL11, FGF-19, and IL8 remained negatively associated with BMI.

We tested the robustness of models through complementary sensitivity analyses (models S1–S6). When we tested for associations with BMI, 7 proteins (TRANCE, IL-12B, LIF-R, CCL11, IL8, IL-18R1, and FGF-19) were significant in all models (model 1 and models S1–S6). OPG was significant in all models except models S4 and S5 (FDR > .1). CXCL1 was significant in models 1, S1, S2, S3, and S6 and at the trend-level (FDR < .1) in model S4 and nonsignificant in model S5 (FDR > .1). ADA was significant in all models except models

Table 2. Clinical Characteristics of the Study Sample

	AN, *n* = 38	atyp-AN, *n* = 26	HC, *n* = 44	Overall, *N* = 108
Age, Years	20.0 [18.5–21.1]	19.0 [15.8–21.1]	18.8 [15.6–20.3]	19.2 [17.3–20.9]
Race				
Asian	7 (18.4%)	3 (11.5%)	7 (15.9%)	17 (15.7%)
White	30 (78.9%)	23 (88.5%)	36 (81.8%)	89 (82.4%)
Ethnicity				
Hispanic	3 (7.9%)	0 (0%)	2 (4.5%)	5 (4.6%)
Not Hispanic	35 (92.1%)	26 (100%)	42 (95.5%)	103 (95.4%)
Body Mass Index	17.00 [15.98–17.58]	18.80 [18.00–19.46]	21.50 [20.20–22.58]	18.71 [17.21–20.79]
Body Mass Index *z* Score	−2.02 [−2.75 to −1.70]	−0.88 [−1.03 to −0.79]	0.07 [−0.31–0.51]	−0.83 [−1.75 to 0.00]
Premenarchal				
Yes	2 (5.3%)	3 (11.5%)	6 (13.6%)	11 (10.2%)
No	36 (94.7%)	23 (88.5%)	38 (86.4%)	97 (89.8%)
ED Duration, Years				
Mean (SD)	4.91 (3.28)	3.67 (3.60)	NA	NA
Missing	1 (2.6%)	0 (0%)	NA	NA
Binge/Purge				
Yes	11 (28.9%)	7 (26.9%)	0 (0%)	18 (16.7%)
No	27 (71.1%)	19 (73.1%)	44 (100%)	90 (83.3%)
Smoking				
Yes	3 (7.9%)	1 (3.8%)	0 (0%)	4 (3.7%)
No	35 (92.1%)	25 (96.2%)	44 (100%)	104 (96.3%)
Psychiatric Medication Use				
Yes	22 (57.9%)	16 (61.5%)	0 (0%)	38 (35.2%)
No	16 (42.1%)	10 (38.5%)	44 (100%)	70 (64.8%)
Antihistamine Use				
Yes	3 (7.9%)	5 (19.2%)	2 (4.5%)	10 (9.3%)
No	35 (92.1%)	21 (80.8%)	42 (95.5%)	98 (90.7%)
Antihistamine and NSAID Use				
Yes	6 (15.8%)	8 (30.8%)	9 (20.5%)	23 (21.3%)
No	32 (84.2%)	18 (69.2%)	35 (79.5%)	85 (78.7%)
Psychiatric Comorbidity				
Yes	23 (60.5%)	15 (57.7%)	0 (0%)	38 (35.2%)
No	15 (39.5%)	11 (42.3%)	44 (100%)	70 (64.8%)

Values are presented as median [IQR], *n* (%), or mean (SD).

AN, anorexia nervosa; atyp-AN, atypical anorexia nervosa; ED, eating disorder; HC, healthy control individual; NA, not applicable; NSAID, nonsteroidal anti-inflammatory drug.

S1 (FDR < .1) and S4 (FDR < .1), and FGF-21 was significant in all models except models S1 and S5 (FDR < .1). IL18 was significant in all models except models S3 (FDR < .1) and S5 (FDR < .1). In model S2, 9 other proteins (AXIN1, CCL23, CD244, CD40, IL6, LAP TGF-beta-1, STAMBP, TNFRSF9, and TRAIL) were significant. Model S4 identified IL-10RB as significant.

Across primary and secondary models, 27 proteins were significantly correlated with BMI (summarized in Figure 3C).

DISCUSSION

For the first time in a sample that included adolescents, we analyzed 73 inflammation proteins in a cohort of females with AN and atyp-AN and age-matched HCs. Consistent with our hypothesis, inflammation protein profiles distinguished adolescents with AN/atyp-AN from HCs. Differences between the AN/atyp-AN and HC groups were driven by 5 proteins: TRANCE, IL-18R1, HGF, LIF-R, and CCL23. Furthermore, 3 proteins uniquely distinguished participants with low-weight AN from HCs: TRANCE, LIF-R, and IL-18R1. Seventeen proteins, including TRANCE, LIF-R, IL-18R1, HGF, and CCL23, were correlated with BMI in the total sample. Robust sensitivity analyses supported the validity of our findings, including disrupted levels of TRANCE, IL-18R1, and LIF-R being unique to low-weight AN and associations of these proteins with BMI. While the results of this study do not provide evidence for an immunological contribution in AN/atyp-AN, they highlight the state-specific inflammation present in both AN and atyp-AN. Our findings also underscore the complexity of AN and atyp-AN by highlighting the inability of the identified proteins to differentiate between these 2 subtypes, which emphasizes the heterogeneous nature of these disorders.

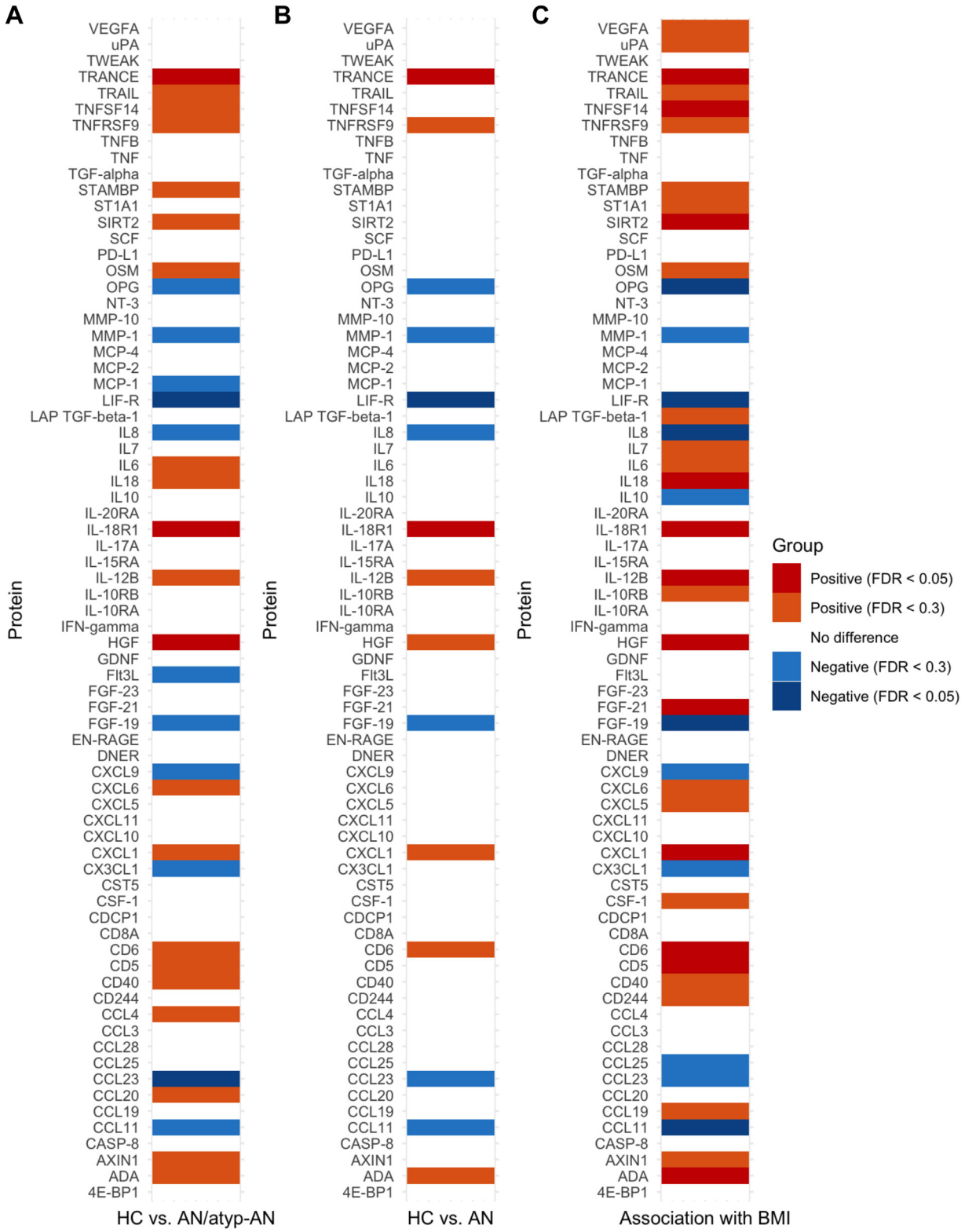

Figure 1. Plasma protein concentrations that differ between HC vs. AN/atyp-AN groups **(A)**, differ between HC vs. AN groups **(B)**, and are associated with BMI **(C)** after adjusting for age (model 1). A positive result indicates either the protein has a higher plasma concentration in the HC group or is positively associated with BMI. No significant results were observed between HC vs. atyp-AN or between AN vs. atyp-AN. AN, anorexia nervosa; atyp-AN, atypical anorexia nervosa; BMI, body mass index; FDR, false discovery rate; HC, healthy control individual.

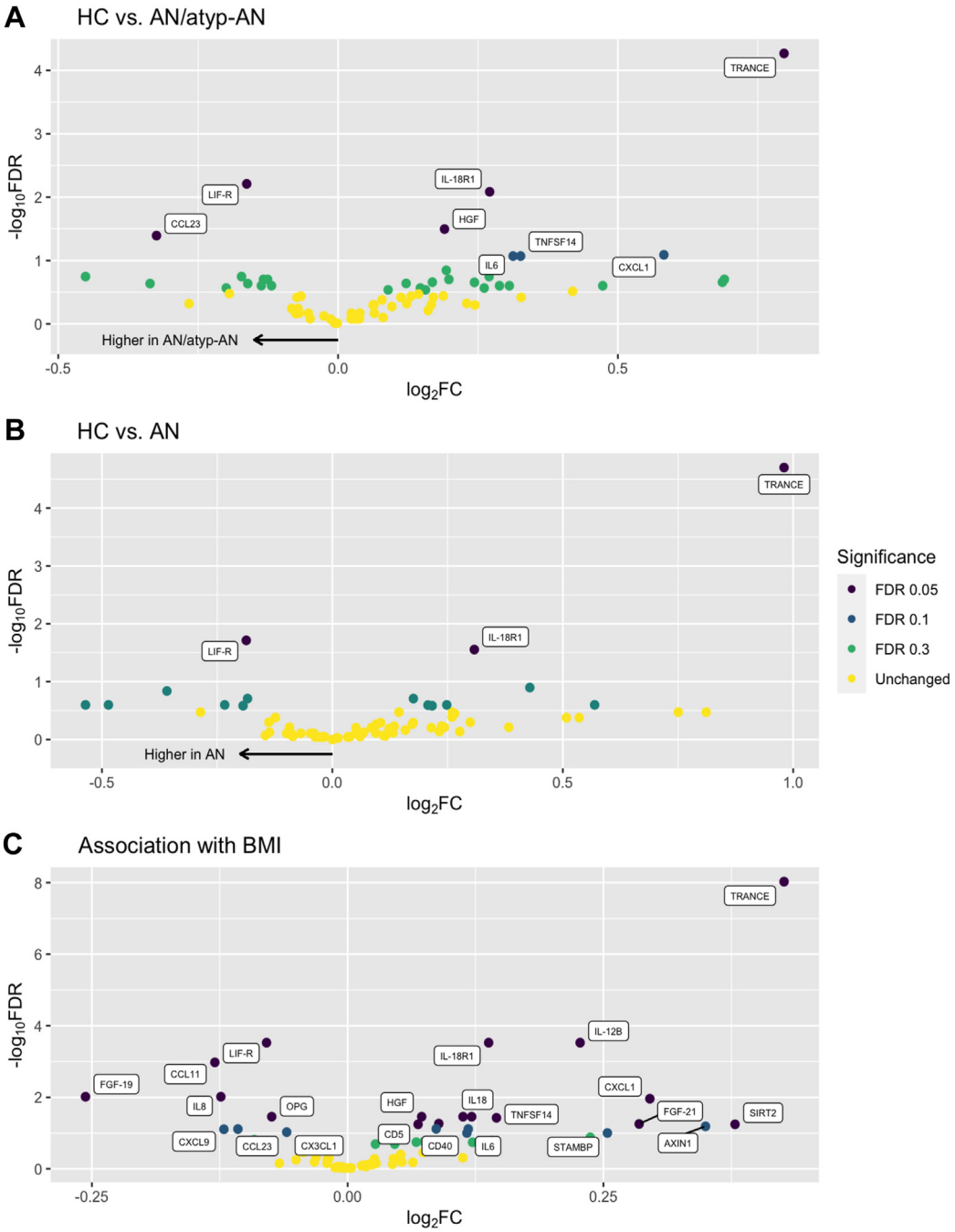

Figure 2. Statistical significance and $\log_2$FC for proteins that were found to differ between HC vs. AN/atyp-AN groups **(A)**, differ between HC vs. AN groups **(B)**, and associated with BMI **(C)** after adjusting for age (model 1). The x-axis represents $\log_2$FC. Because protein concentrations were reported using a $\log_2$ scale (i.e., normalized protein expression), in our analysis, $\log_2$FC is equivalent to the beta coefficient associated with the group **(A, B)** or the slope of BMI in the regression model **(C)**. In **(C)**, it indicates the changes in protein concentration on a $\log_2$ scale for every 1 unit change in BMI. The y-axis depicts $-\log_{10}$FDR, with smaller FDR values corresponding to higher $-\log_{10}$FDR values. A value of 1.3 on the $-\log_{10}$FDR scale corresponds to an FDR of .05. AN, anorexia nervosa; atyp-AN, atypical anorexia nervosa; BMI, body mass index; FC, fold change; FDR, false discovery rate; HC, healthy control individual.

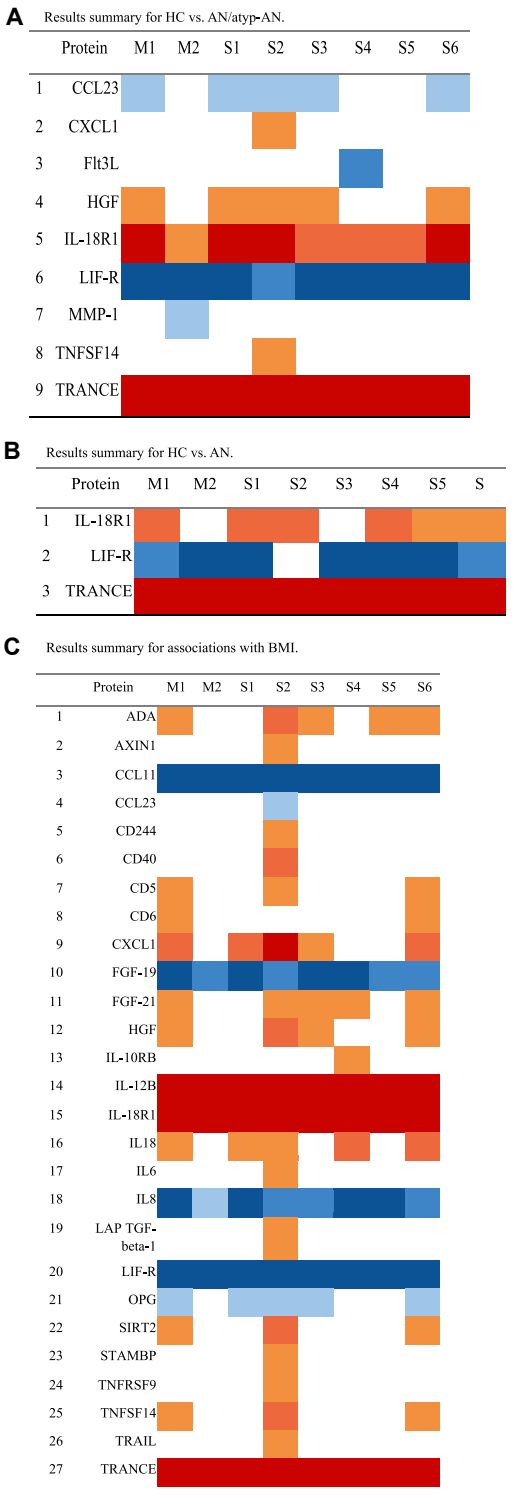

Figure 3. Comparison of results for HC vs. AN/atyp-AN groups **(A)**, HC vs. AN groups **(B)**, and association with BMI **(C)** across models 1 and 2 and secondary models S1 through S6. Red cells represent significantly higher plasma concentrations in the HC group or significant positive associations with BMI (FDR $<$ .05). Blue cells represent significantly lower plasma concentrations in the HC group or negative positive associations with BMI (FDR $<$ .05). Blank cells indicate nonsignificance. Darker colors correspond to smaller *p* values. AN, anorexia nervosa; atyp-AN, atypical anorexia nervosa; BMI, body mass index; FDR, false discovery rate; HC, healthy control individual.

Five Proteins Differentiate Adolescents With AN/Atyp-AN From HCs

We identified 5 proteins suggesting inflammation in restrictive eating disorders broadly (i.e., AN and atyp-AN) that differed from HCs. Two proteins (LIF-R, CCL23) had higher plasma concentrations in participants with AN/atyp-AN than HCs, and 3 proteins (TRANCE, IL-18R1, and HGF) had lower plasma concentrations in participants with AN/atyp-AN than HCs. Of the 5 proteins, 4 (higher LIF-R; lower TRANCE, IL-18R1, and HGF) were previously identified by Nilsson *et al.* (13), and were in the same direction, in an AN-only adult sample. The data also identify CCL23 for the first time as having higher expression in AN and atyp-AN. CCL23 is a chemokine involved in immune cell migration and inflammation (25).

Interestingly, both of the proteins with higher plasma concentration levels in AN/atyp-AN play a pathological role in the development or progression of autoimmune rheumatoid arthritis (RA). In RA, LIF-R and CCL23 expression promotes an inflammatory response and is upregulated in synovial tissue. Several studies have shown bidirectional associations between AN and autoimmune disorders (26), including RA (26), and comorbid RA and AN have been reported anecdotally (27). However, longitudinal studies that explore the risk of RA following AN require extended longitudinal follow-up (e.g., RA average age of onset is around 44 years) (26,28,29). Notably, the first genome-wide significant association in AN was identified in a region previously implicated in autoimmune diseases (30,31). Genetic polymorphisms and composition play critical roles in the expression of inflammatory proteins (32–35). As such, the elevation of CCL23 and LIF-R observed in our study may reflect the genetic contribution of previously identified genetic regions associated with AN and RA. Future studies that combine genome-wide association and plasma proteomics are necessary to determine the contribution of either genes or state-dependent traits on inflammatory biomarkers and further characterize the utility of these plasma biomarkers in AN diagnosis and disease progression.

Unique Expression Levels of IL-18R1, TRANCE, and LIF-R Differentiate Participants With AN From HCs

Including individuals with AN and atyp-AN allowed us to explore proteins that may be unique to the clinical presentation of restrictive eating marked by low-weight status present in AN. We identified 3 proteins (lower IL-18R1 and TRANCE; higher LIF-R) that were uniquely disrupted in females with AN. Our findings of lower TRANCE and higher LIF-R are consistent with those of with Nilsson *et al.*, but they conflict with 2 previous studies that reported higher concentration of TRANCE in participants with AN than in HCs (36,37). TRANCE is a critical regulator of bone remodeling (38), and the observed low levels of TRANCE among participants with AN and in the combined AN/atyp-AN group suggest that decreased osteoclast activity leads to the reduced bone reabsorption and altered bone remodeling (39,40) that characterize the clinical bone loss observed in AN (41,42). Notably, individuals with atyp-AN may

also be at risk for clinical bone loss, although results have been inconclusive to date (43,44), likely due to the heterogeneity of atyp-AN presentations (3). LIF-R is an inflammatory cytokine implicated in muscle and fat wasting in cancer-related cachexia (45). LIF binds to a receptor that is similar to the IL-6 receptor, both of which act via Jak2/stat3 signaling (46,47). Dysregulated Jak2/stat3 signaling contributes to muscle atrophy in an estrogen-dependent manner (48,49). That is, only ovariectomized mice show muscle atrophy from dysregulated Jak2/stat3 signaling (48,49). Estrogen deficiency is common in AN (~60%) and may contribute to the inflammation patterns observed in the low-weight state of AN. The observed peripheral elevations of LIF-R in AN may suggest that muscle size and health cannot be maintained in this low-weight state. Because we observed elevations of LIF-R in the combined AN and atyp-AN group, weight restoration >90% of expected body weight and restoration of menses are likely needed for muscle restoration. LIF-R, TRANCE, and IL-18R1 were all correlated with BMI in the total sample; normalization of these proteins may serve as a more accurate indicator of healthy weight than BMI values in AN. Inflammation levels could serve as an additional indicator of health beyond BMI, thereby supporting treatment engagement or marking progress. Inflammation, as evidenced by specific immunological markers, provides a window into the systemic effects of AN beyond traditional measures of disease severity such as BMI. This perspective is consistent with emerging research suggesting that inflammation plays a critical role in the pathophysiology of AN and could be pivotal in understanding treatment responses and outcomes.

Seventeen Proteins Are Correlated With BMI in the Total Sample

We identified 17 proteins correlated with BMI in the total sample. By employing the same targeted proteomics approach as Nilsson *et al.* (13), we replicated the association (and directionality of relationship) between inflammation protein expression levels and BMI for 11 of 17 proteins identified previously (ADA, CCL11, CD5, CXCL1, HGF, IL12B, IL-18R1, IL18, SIRT2, TNFS14, and TRANCE) (13).

We also identified 6 novel proteins associated with BMI (positive association with CD6 and FGF-21; negative association with FGF-19, IL8, LIF-R, and OPG). Both FGF-21 and FGF-19 have shown previous associations with BMI (50–53). FGF-21 and FGF-19 are hormones secreted broadly (FGF-21) and by the small intestine (FGF-19) (54); both play a critical role in regulating energy metabolism, glucose homeostasis, and lipid metabolism. Elevated circulating levels of FGF-21 are evident in individuals with obesity, insulin resistance, type 2 diabetes, and fatty liver disease (55). In contrast, peripheral levels of FGF-19 have been shown to be lower among individuals with obesity, and low FGF-19 levels have been linked to impaired glucose metabolism, insulin resistance, and dyslipidemia (56,57).

Overall, our results converge with those of previous studies and provide an important replication of the association between specific immune-related proteins and BMI, quantitative differences in these proteins in participants with AN compared with HCs, and extension through detailing these changes in AN and atyp-AN during adolescence, a critical period during which these conditions modally onset.

Strengths and Limitations

Strengths of the current study include the use of a state-of-the-art multiplex proteomics approach to examine a comprehensive and validated profile of inflammation proteins, inclusion of an adolescent sample, and inclusion of individuals with atyp-AN and AN. In addition, to reduce confounding effects on blood-based biomarkers, we collected fasting samples in the morning, placed samples on ice immediately to prevent degradation of markers, and used immediate centrifugation (58,59) to allow for more accurate and reliable measurements across participants. Our deeply phenotyped samples allowed us to conduct complementary sensitivity analyses to ensure that results were not driven by confounding factors (smoking status, acute sickness, psychiatric severity, depression/anxiety, antidepressant/antihistamine use). These secondary analyses did not substantially change the findings, indicating the robustness of our results.

Nonetheless, this study has several limitations. First, because the data are cross-sectional, we cannot determine whether the observed alterations in protein expression are a consequence or part of the etiology of AN/atyp-AN. Because leukocyte count is commonly used as a clinical marker of malnutrition, leukocyte (and lymphocyte) count may be helpful in combination with inflammation proteins markers in future studies of AN/atyp-AN. Given our results demonstrating protein expression levels that were unique to the low-weight AN group, the strong associations of certain protein levels with BMI, and previous findings by Nilsson *et al.*, we hypothesize that several of these inflammation findings are state specific. Longitudinal follow-up studies are necessary to confirm this hypothesis. Our sample of atyp-AN only included individuals who were ≤90% of expected body weight. To understand the complete picture of atyp-AN, larger samples that include individuals who have restrictive eating across the full weight spectrum are needed (3,60).

Clinical Relevance and Future Directions

While there are currently no Food and Drug Administration–approved medications for AN, there are several medications that have limited evidence for effectiveness in AN and that our results are consistent with.

First, several proteins that we identified as being dysregulated in participants with AN/atyp-AN compared with HCs are connected with bone and muscle health, either directly or through their roles in inflammation and immune regulation (CXCL1, HGF, IL-18R1, TNFSF14, TRANCE, and LIF-R). Low levels of TRANCE (also known as RANKL) and TNFSF14 as seen in our sample are consistent with emerging evidence for the use of bisphosphonates and the antireceptor activator of nuclear factor-κB ligand (RANKL) antibody agent (denosumab) for the treatment of low bone mineral density (BMD) in AN and atyp-AN. Both are classified as antiresorptives and thus target osteoclasts and inhibit bone resorption to impact TRANCE signaling (bisphosphonates, denosumab) and TNF superfamily members (denosumab). Furthermore, there is limited evidence that a 12-month regimen of bisphosphonate may improve

BMD in the spine and hip in adults with AN (61). However, bisphosphonates decrease rates of bone remodeling (62), which may explain why trials of bisphosphonate in adolescents with AN have shown no impact on BMD (61,63,64). While the effectiveness of long-term use of bisphosphonates is unknown, there is limited evidence from recent case reports that 2-year and 3-year regimens of denosumab, a monoclonal antibody, may improve BMD in adults with AN (65–68). Currently, there are no standard treatment recommendations for patients with AN/atyp-AN who present with low BMD. Our findings and the initial clinical evidence above encourage research on the use of both bisphosphonates and denosumab in AN/atyp-AN to address low BMD.

Second, our study adds to the discourse on the utilization of olanzapine, an atypical antipsychotic, in the treatment of AN and atyp-AN by shedding light on the proteomic alterations associated with AN. In the 2023 updated guidelines on the pharmacological treatment of eating disorders by the World Federation of Societies of Biological Psychiatry, olanzapine received a limited recommendation (69). At a low dose, olanzapine is mostly used to increase appetite in AN and atyp-AN; however, the mechanisms of action of olanzapine are unknown. It is noteworthy that olanzapine has been shown to induce changes in the cytokine system, including increases in TNF-α and its soluble receptor levels (70,71), as observed in patients with schizophrenia (72,73). This raises intriguing questions about the potential impact of olanzapine on the inflammatory state in AN. The observed low levels of TNF-α and associated TNF family proteins (TNFSF14, TNFRSF9, CD40) in the AN and atyp-AN groups, as well as the positive association with BMI across the total sample, may suggest desirable effects of olanzapine through increased levels of TNF-α and associated TNF family proteins. Additional research is warranted to elucidate the intricate interplay between olanzapine, cytokine dysregulation, and clinical outcomes in AN. Additionally, it is important to consider the potential psychopathological implications of olanzapine use in AN. Given its known effects on mood regulation (74,75), olanzapine may influence the course of psychiatric comorbidities commonly associated with AN and atyp-AN, such as anxiety disorders and depression. However, the complex interrelationships between olanzapine, mood symptoms, and AN pathology warrant careful consideration and further investigation.

Conclusions

This is the first study to examine inflammation protein expression in adolescents with AN and atyp-AN using a methodologically rigorous approach. Our findings replicate those from previously published studies in adults with AN that showed an aberrant inflammatory profile while expanding upon those findings through identification of additional novel proteins that had not been reported previously. Across individuals with atyp-AN and AN, we observed disruptions in several inflammation proteins that are key for bone and muscle regulation, providing additional support for the severity of atyp-AN.

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LB designed the study. KTE, EAL, and MM collected the samples. LB and CJ analyzed the data. LB, KTE, EAL, and MM wrote the manuscript, which was revised and approved by all authors.

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