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The Expression of Cytokines and Chemokines in the Blood of Patients with Severe Weight Loss from Anorexia Nervosa

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Abstract

Anorexia nervosa (AN) is a serious, potentially life-threatening disorder characterized by severe weight loss, dysregulated eating, and often excessive exercise. While psychiatric illnesses such as depression are associated with increased levels of pro-inflammatory mediators, evidence for such disturbances in patients with AN has been less clear. To elucidate further immune responses in AN, we assayed a panel of cytokines and chemokines in the blood of patients undergoing inpatient treatment, testing the hypothesis that metabolic disturbances in this disease would lead to a pattern of immune disturbances distinct from that of other psychiatric diseases. For this purpose, we evaluated patients by the Beck Depression Inventory-II (BDI-II) and the Eating Disorders Examination-Questionnaire and assessed cytokines and chemokines by enzyme-linked immunosorbent assays. Patients reported a moderate level of depression (mean BDI-II = 22.6) but exhibited few immunologic abnormalities of the kind associated with major depressive disorder [e.g., increased interleukin (IL)-6]; RANTES showed the most frequent elevations and was increased in 4 of the patients studied. Together, these findings suggest that features of AN such as loss of adipose tissue and excessive exercise may attenuate cytokine production and thus modulate the experience of illness that impacts on core features of disease.

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Declaration of Interest

All authors reported no biomedical financial interests or potential conflicts of interest.

1. Introduction

AN is a serious, potentially life-threatening psychiatric illness that affects ~1% of the population. AN disproportionately afflicts females, and is associated with severe weight loss, dysregulated eating, distorted body image, and, often, excessive exercise [1-3]. AN carries substantial morbidity and mortality as well as personal, familial, and societal costs. Nevertheless, patients affected appear to value the ill state and can make extensive efforts to achieve and maintain the starvation that characterizes the illness. Given the clinical features of AN, treatment is uncertain and current approaches to therapy carry significant costs despite limited efficacy [4-6]. Medications targeting the core symptoms of the disorder currently are not available [7-9]. Elucidating the pathogenesis of AN and identifying biomarkers are therefore essential for developing more effective interventions.

AN presents a very complex biological setting that encompasses biochemical, metabolic, and sensory abnormalities [10-17]. Importantly, changes in visceral experience occur prominently in the ill state (e.g., reduced pain sensitivity, reduced detection of visceral changes such as heart beat), with findings that differ from alterations in experience accompanying food restriction that occurs independently of AN [18,19]. Biological alterations that regularly occur during starvation, combined with unique features of the ill state of AN, may help to explain why the ill state appears to be so reinforcing for patients.

A potentially important but understudied aspect of AN is the effect of this disorder on the immune system. Data from existing studies are conflicting and, while some studies suggest a pro-inflammatory state, others indicate few abnormalities [20-30]. In contrast, depression and schizophrenia, among other psychiatric diseases, show abundant cytokine disturbances and define pro-inflammatory states that co-occur with the experience of mental illness [31-35]. The lack of consistent evidence of such a relationship in AN suggests that AN may involve a unique interplay between nervous and immune systems and dissociation of expected cytokine abnormalities from mood and affect.

The lack of more decisive evidence for cytokine disturbances in AN is notable, particularly given that the severe loss of fat in AN would be expected to have important effects on immunity. As now recognized, adipose tissue produces many bioactive substances, including pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα) and interleukin (IL)-6 [36-39]. In addition to their immunological and metabolic effects, these cytokines could play a role in the initiation and maintenance of psychiatric manifestations such as disturbances in mood and affect. Notably, in addition to extreme dietary restriction with subsequent loss of fat mass, AN can be characterized by engagement in a determined excessive exercise routine. Extensive exercise may be relevant to the overall state of the immune system in AN since muscle cells, when undergoing contraction, can also produce cytokines, known as myokines [40-41]. Of the myokines, IL-6 may have anti-inflammatory action at least locally although systemically it can lead to immune activation. Because of extensive exercise, the production of myokines could increase in patients and, depending on the array of these mediators produced, attenuate inflammation [42-45].

Since immune mediators can drive metabolic and nervous system disturbances, a focus on their role in AN is conceptually and practically important as it suggests potential interventions with immunomodulatory agents; such agents could interrupt an injurious cycle of metabolic and psychological disturbance. To characterize immune system changes in AN and assess any abnormalities present in other psychiatric disorders, we analyzed a panel of immune chemokines and cytokines in a cohort of well-characterized AN patients undergoing inpatient treatment. Results of these studies indicate that patients with AN show few abnormalities in the expression of cytokines and chemokines. As such, patients with AN may differ from patients with other psychiatric disorders where immune disturbance is more prominent.

2. Method

2.1. Participants

Participants were 30 females ages 15 to 45 who met *DSM-IV* criteria for AN (any subtype), who were admitted for inpatient treatment at the University of North Carolina Center of Excellence for Eating Disorders. Participants were assessed at the time of admission, typically at <75% ideal body weight (IBW). For participants who were unable to complete the assessment at admission, testing occurred within 1 week of admission with one exception (participant ID = 16). Prior to the assessment, participants completed a semi-structured screening interview designed specifically for this investigation to assess recent illness, health history and current medications that could affect cytokine levels. This investigation was approved by the Biomedical Institutional Review Board at the University of North Carolina at Chapel Hill and all participants provided informed consent.

Blood sampling was unsuccessful in 5 participants, who were therefore omitted from the analyses. From the remaining 25 participants, we were unable to assess the full battery of psychological or immunological measures on all patients related in part to availability of material and patient-related issues.

2.2. Measures

2.2.1. Body Composition—Height and weight were assessed using a stadiometer and a calibrated digital scale. Body mass index (BMI) was calculated as weight (kg)/height (m^2). Body fat percent was determined by dual x-ray absorptiometry (DXA).

2.2.2. Eating Disorder and Psychopathology Assessment

2.2.2.1. Structured Clinical Interview for DSM-IV Axis I Disorders - Patient Edition Module H (SCID-I/P) [46]: The SCID-I/P, a well-studied and frequently employed semi-structured interview for Axis I disorders was used to assesses eating pathology.

2.2.2.2. The Eating Disorders Examination-Questionnaire (EDE-Q) [47]: The EDE-Q is a 38-item self-report measure of eating disorder psychopathology over the past 28 days. The EDE-Q is based on the EDE [48], a valid and reliable investigator-administered interview for assessing current eating disorder symptoms. The EDE-Q yields a global score and four

subscale scores (restraint, eating concerns, weight concerns, and shape concerns), each with a range of 0 to 6.

2.2.2.3. Beck Depression Inventory-II (BDI-II) [49]: The BDI is a 21-item self-report questionnaire that is used to assess the severity of current depressive symptoms. Each answer is scored on a 4-point scale (0 to 3), with total scores indicating minimal (0-13), mild (14-19), moderate (20-28), and severe (29-63) depression.

2.2.2.4. Spielberger State-Trait Anxiety Inventory (STAI) [50]: The STAI is a 40-item self-report questionnaire that assesses both state (current, event-related) and trait (characterological) anxiety using two 20-item scales. In this study, the STAI was used to assess state anxiety. Participants completed a 20-item self-report questionnaire based on a 4-point scale ranging from 1 (“not at all”) to 4 (“very much”). Example items include statements such as, “I am calm”, “I am worried”. Low, median, and high scores indicate mild, moderate, and severe forms of anxiety, respectively. A score > 40 is considered high.

2.3. Laboratory

A venous blood sample was obtained from each participant and analyzed for complete blood count as well as a comprehensive metabolic panel to assess current kidney and liver function and electrolyte and acid/base balance. Additional blood samples were frozen at -80°C, stored, and later assayed in batch to measure levels of cytokines.

Immune assays (R&D Systems, Minneapolis, MN, quantitative ELISA) included high-sensitivity interleukin (IL) 6 and 8 (IL-6, IL-8), IL-1 receptor antagonist (IL1-ra), C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α) and soluble TNF receptor (sTNFR75), and monocyte chemotactic protein-1 (MCP-1). sTNFR75 provides another index of the function of TNF-α. CRP was assessed as a general measure of inflammation and acute phase reactant production. Assays were performed at the Cytokine Analysis Facility of the Bioanalytical Core Laboratory at the University of North Carolina at Chapel Hill (IL-6, CRP, TNF-a, sTNFR75) and University Hospital of Geneva, Switzerland (IL-8, IL1-Ra, MCP-1, RANTES).

For some determinants, IL-8, IL-1Ra, MCP-1 and CCL5/RANTES were measured by a commercially available multiplex beads immunoassay, based on the Luminex platform (Fluorokine MAP Multiplex Human Cytokine Panel, R&D Systems, Minneapolis, USA) according to supplier’s instructions.

2.4 Statistical Analysis

The analyses were mainly descriptive in nature. In addition, exploratory analyses included Pearson’s correlations examining relations between and among psychological and immunological measures. Given the exploratory nature of this study, we did not adjust the p value (alpha = 0.05) for multiple comparisons.

3. Results

3.1. Sample Characteristics

Table 1 provides demographic, psychological, and immunological data on each of the 25 study participants. Abnormally high laboratory values are highlighted in bold. Participants ranged in age from 19 to 57 years. Consistent with a primary diagnosis of AN, participants' BMI ranged from 13.4 to 18.8 kg/m^2 and most reported elevated levels of eating disorder psychopathology, depression, and anxiety. Seventeen individuals completed the EDE-Q, with global scores ranging from 0.6 to 5.7. Eleven of the 17 individuals (65%) had global EDE-Q scores greater than one standard deviation above the normative mean for women ages 18-49 [51], suggesting clinically significant pathology.

BDI-II scores ranged from 5 to 49. Two patients reported minimal depression, 6 each reported mild or moderate depression, and 4 reported severe depression. STAI scores ranged from 20 to 79. Five patients scored < 40; 8 scored between 40 and 59; and 8 scored ≥ 60; thus, 76% (16 of 21) patients reported high levels of state anxiety. Body fat percent ranged from 5.9% to 20.2%. The highest BMI and body fat percent were observed in participant 16, who underwent testing more than 1 week after renourishment began. Table 2 presents the descriptive statistics for these measures in the group as a whole.

As results of these experiments indicate, the levels of cytokines measured were almost all within the normal range. Among the patients, 1 showed an elevation of TNF-α while 2 showed an elevation of IL-6. For the chemokines, MCP-1 showed values within the normal range whereas levels of RANTES were elevated in 4 patients. These patients did not have abnormalities in others of the cytokines and chemokines tested. Of the cytokines measured, IL-6 is commonly viewed as the prototype of a myokine.

3.2 Exploratory Analyses

Scores on the psychological measures were strongly correlated with each other (r range 0.61 to 0.71, p range 0.02 to 0.004). Only one significant correlation was noted between the psychological and immunological measures, with the global EDE-Q score being negatively associated with TNF-α ($r = -0.48$, $p < 0.05$). Correlations among immunological markers revealed strong positive associations between RANTES and IL-8 ($r = 0.73$, $p < 0.0004$) and MCP-1 ($r = 0.62$, $p < 0.002$).

Table 3 displays the psychological measures in the participants with abnormally high (>84000) versus normal RANTES (<84000) level. The two groups appear similar in their levels of psychological distress; however, the number of patients is too small to draw conclusions. Together, these studies support prior studies indicating few cytokine abnormalities in patients with AN.

4. Discussion

Patients with AN who had been admitted to an inpatient unit for a program for weight restoration showed little evidence of immune system abnormality as evidenced by assays of cytokines and chemokines. Of analytes measured, RANTES was elevated in 4 patients while

IL-6 was elevated in two; only one patient had an elevated in TNF-α. Together, these findings suggest that, despite the many potential physical and psychological triggers to immune disturbance in AN, a surprising degree of immune system regulation is maintained even in severely ill patients.

Previous studies on cytokine disturbances in AN have yielded conflicting results. Pomeroy et al. [23] reported elevations of IL-6 and TGF-β in patients with AN during periods of starvation although these levels returned to normal with re-feeding. As in our study, these investigators failed to show elevations in TNF-α. Using quantitative PCR to assess cytokine mRNA in peripheral blood cells, Kahl et al. [29] showed increases in levels of TNF-α and IL-6 during admission to the hospital; TNF-α mRNA levels remained abnormal with re-feeding, however. Other evidence of immune system activation was found in a study indicating increased nitric oxide (NO) production by patients with AN but not in those with bulimia nervosa as indicated by levels of nitrites [52].

A variety of factors could explain the differences in findings across studies. These factors relate to assay methodology (i.e., blood levels vs. cell-based assays); the features of patients studied; and clinical setting (i.e., inpatient vs. outpatient). Differences in criteria for inpatient admission across centers could affect the severity of illness of patients studied and therefore the extent abnormalities observed. The demographic features of our patients, however, suggest that our clinical sample is representative of individuals undergoing inpatient weight restoration. Moreover, our results are internally consistent since we analyzed several different cytokines yielding similar results.

Among cytokines and chemokines measured, RANTES showed elevations in 4 patients. RANTES was originally cloned on the basis of its expression in T cells and subsequently renamed as CCL5 [53]. While RANTES has chemokine activity, it may act more broadly and impact diseases ranging from HIV infection to malignancy. Like other cytokines and chemokines, CLL5 may act as a neuromodulator and contribute to the symptomatology of those patients with elevated levels [54-56]. Prior studies have suggested increased RANTES levels in several psychiatric diseases [57]. Among the 4 patients in our studies, we did not see any major differences in the psychological measures tested.

The absence of elevation of IL-6 is notable in view of its designation as a myokine, a cytokine produced in response to muscle contraction. While IL-6 can be produced during exercise, the extent of elevation may vary depending on the type and intensity of exercise; the condition of the individual undergoing exercise; and the time of sampling [40,42,58]. Furthermore, IL-6 may act locally and mediate communication between muscle and adipose tissue; in this situation, IL-6 may display anti-inflammatory activity although, when acting systemically, it also can drive inflammation and induce the acute phase response [40,41,43]. The lack of evidence of significant elevations of IL-6 in this study population therefore does not exclude a role of IL-6 in modulating the immune system in AN.

Our study has a number of strengths. We used sensitive immunoassays to assess an array of cytokines and chemokines that correspond to various elements of the immune system, with CRP protein providing a general measure of immune system activation. In addition, we

simultaneously determined psychological measures that reflect various dimensions of AN and associated conditions such as depression. Our study has limitations, however. The analysis involves a limited number of patients who had been hospitalized for their condition and, while we assessed nutritional status and psychological features, we did not measure exercise in a quantifiable way. Another limitation relates to a comparison of laboratory values to population norms rather than a control population matched for age and sex; we also did not have a cohort of patients with depression for direct comparison. Nevertheless, our results are very consistent with those in the literature and indicate the absence of evidence of inflammation in patients with AN despite the presence of eating disorder pathology and related depression and anxiety.

As shown in many studies, AN represents a very unusual biological setting that differs from more usual forms of malnutrition with respect to such findings as susceptibility to infection [59-61]. Furthermore, many patients with AN remain physically very active, with excessive exercise an important feature of the condition [44,45,62,63]. Because of the systemic immune effects of exercise, the predicted inflammatory consequences of tissue damage from malnutrition may not occur [64-66]. Thus, despite factors (i.e., psychosocial stress) that could drive inflammation, patients with AN may have strong counter-regulation secondary to their nutritional state, lack of adipose tissue, heightened production of glucocorticoids, and/or excessive exercise. The setting of AN therefore represents an important contrast to other psychiatric disorders where cytokine production may depend on adipose tissue. Furthermore, in other conditions, excessive exercise may not occur, with depression, for example, more commonly associated with decreased physical inactivity.

Our findings may be relevant to the putative effects of self-starvation on hallmark psychological features of AN such as lack of awareness of patients of the seriousness of the illness. As suggested in many studies, self-starvation may represent a strategy to dull an internal experience of dysphoria, anxiety, or stress-proneness in susceptible individuals [67-69]. To the extent that psychological stress may drive the production of inflammatory mediators, our findings are consistent with a hypothesis that features of AN (i.e., starvation, exercise) may attenuate aberrant immune responses expected in psychiatric illness. In the absence of these responses, patients with AN may not experience illness in a typical way and therefore fail to exhibit classic illness behavior (e.g., lack of energy, decreased physical activity, pain behaviors) that occurs in other psychiatric conditions.

A lack of illness experience could also contribute to the perplexing symptom that has been referred to as “denial of illness” and is now considered to be a failure to recognize the seriousness of the illness. Rather than denying or failing to recognize their illness, patients with AN may actually not experience illness as typically conceptualized, thereby contributing to their common unwillingness to undergo treatment. In fact, patients with AN often report feeling markedly less well upon re-nourishment, a time that can be associated with changes in steroid hormones, for example [70,71]. Although these symptoms are typically explained psychologically as a fear of weight gain, our model would suggest that an increase in adipose tissue (an important source of pro-inflammatory mediators during re-feeding) could “reignite” aberrant immune responses, intensifying an experience of illness which had been suppressed by starvation and excessive physical activity. This model clearly

contains speculative elements and requires further study, including a longitudinal design to document changes throughout the course of illness and not just during the acutely low-weight state. These studies are in progress and hopefully will provide new insights into interplay between the immune system on psychological features of AN.

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Table 1

ID	AGE (yrs)	EDEQ	BDI	STAI	BMI (kg/m^2)	Body Fat %	IL-1RA (pg/mL)	IL_6 (pg/mL)	IL_8 (pg/mL)	TNFα (pg/mL)	srTNF75 (ng/mL)	MCP-1 (pg/mL)	RANTES (pg/mL)	CRP (pg/mL)
1	22	4.1	19	46	17.2	------	317.2	0.11	2.08	1.87	1.00	62.6	45568.8	4318636.5*
3	40	5.6	------	79	16.4	5.9	198.7	0.41	2.98	0.76	1.24	84.3	28803.3	158277.2
4	20	5.7	------	79	17.0	13.7	245.7	0.34	2.20	0.87	1.39	54.2	32291.5	26861.9
5	57	5.1	32	68	16.1	19.7	385.0	0.74	3.81	1.09	0.86	42.9	2294.6	2852667.5
6	25	------	15	40	14.8	12.0	217.0	0.18	1.12	3.10	1.00	82.3	3252.0	314053.1
7	20	------	15	57	13.4	7.6	233.1	0.03	1.45	0.90	0.96	56.3	16793.7	2969434.5
8	29	------	------	------	16.4	18.0	275.1	0.78	1.39	0.68	0.95	79.8	2959.1	242675.0
9	27	------	26	67	18.8	19.4	129.2	0.10	1.39	0.84	1.06	76.8	4540.4	136003.7
10	32	5.4	33	70	16.8	14.0	151.8	0.17	1.68	0.66	0.74	70.2	4383.0	104706.6
11	25	5.6	25	35	17.0	18.3	243.1	**6.40**	------	1.14	1.31	49.5	5050.0	75098.3
13	35	2.7	11	20	17.7	14.7	313.9	0.82	2.17	2.16	1.02	73.0	35386.9	118983.8
14	19	2.6	5	38	16.8	18.0	184.0	------	------	0.75	0.95	46.3	6246.3	81227.9
15	20	0.6	5	37	17.1	13.7	241.0	0.71	2.23	1.99	1.46	64.4	8944.6	115895.1
16	28	------	18	43	18.5	20.2	231.7	0.33	3.31	1.44	1.63	114.1	**126213.1**	652373.4
18	34	------	------	------	13.8	16.5	417.9	0.17	2.74	1.35	1.65	117.0	**125111.1**	313338.0
19	16	------	------	------	16.5	16.5	395.2	0.66	------	1.33	1.27	67.7	8736.4	369672.4
20	22	2.3	28	46	14.6	8.3	220.0	------	4.18	0.66	1.28	98.0	**235468.1**	158490.7
21	39	2.0	26	------	16.3	16.5	318.8	**2.02**	1.68	1.17	0.92	59.0	17303.1	7030199.5*
22	22	5.3	49	65	16.8	9.4	210.0	0.70	1.74	1.03	1.45	32.2	6972.0	486747.5
23	19	5.1	22	70	14.9	13.3	161.4	------	2.20	1.04	1.27	78.6	14650.6	53258.4
24	31	------	17	40	14.8	7.2	253.2	0.00	1.48	0.71	1.00	73.6	22367.8	32789.5
25	24	1.6	12	52	15.1	10.0	157.0	1.36	------	**4.62**	1.30	46.4	3819.2	93306.1
26	18	5.8	47	53	17.2	18.2	193.3	1.14	3.68	1.05	0.85	69.0	**132864.1**	679760.6
27	26	5.4	35	67	16.3	12.5	------	0.67	------	1.87	------	------	------	59064.5
30	15	4.4	14	39	16.1	18.5	------	------	------	0.94	------	------	------	382666.0

*Value extrapolated beyond standard range

Table 2

Variable	N	Mean (SD)	Range	Normal Range[a]
IL-1RA (pg/mL)	23	247.5 (78.8)	129.2 – 417.9	20 – 880
IL-6 (pg/mL)	21	0.85 (1.36)	0.00 – 6.40	< 1.50
IL-8 (pg/mL)	19	2.29 (0.91)	1.12 – 4.18	< 90
TNFα (pg/mL)	25	1.36 (0.89)	0.65 – 4.62	< 4.00
sR_TNF75 (ng.mL)	23	1.15 (0.25)	0.74 – 1.65	0 – 5
CRP (pg/mL)	25	873047.5 (1680450.7)	26861.9 – 7030199.5	.
MCP_1 (pg/mL)	23	69.5 (21.2)	32.2 – 117.0	50 – 260
RANTES (pg/mL)	23	38696.5 (59193.8)	2294.6 – 235468.1	1200 – 84000

[a]Values are laboratory-specific rather than population “norms”

Table 3

Psych Summary Descriptive Data by RANTES Group

Variable	Abnormal RANTES (> 84000)	Normal RANTES (≤ 84000)
Global EDE-Q score[a]	4.0 (2.5)	4.0 (1.8)
Restraint[b]	3.1 (3.2)	3.5 (2.2)
Eating Concern[b]	4.1 (2.7)	3.5 (1.8)
Shape Concern[a]	4.6 (1.9)	4.7 (1.6)
Weight Concern[a]	4.4 (2.3)	4.0 (2.0)
BDI[c]	31.0 (14.7)	20.8 (11.7)
STAI[d]	47.3 (5.1)	54.0 (17.9)

Due to missing data, N varied in the Abnormal and Normal RANTES groups, respectively, as follows:

[a] 2, 13;

[b] 2, 15;

[c] 3, 15;

[d] 3, 16