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A longitudinal analysis of cytokines in anorexia nervosa

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Abstract

Objective: Inflammation has been proposed to have a pathophysiological role in anorexia nervosa (AN) and to contribute to the maintenance of the disorder. Longitudinal cytokine research in AN has focused on only a few pro-inflammatory cytokines. We assessed a broad range of cytokines over time in people undergoing specialised treatment for AN. **Method:** We measured serum concentrations of 27 cytokines in people with AN (n=23). Body mass index (BMI), eating disorder (ED) symptoms and general psychopathology were assessed and blood samples were collected within four weeks of the commencement of specialised ED treatment (baseline) and at 12- and 24-week follow-ups. **Results:** Both BMI and ED symptoms improved over the assessment period. Linear mixed models showed that log IL-6 decreased between baseline and week 12 assessments. By week 12, log IL-6 values were comparable to levels in healthy individuals. Log IL-7 increased from week 12 to week 24. **Discussion:** Initially elevated IL-6 serum concentrations appear to 'normalize' during the first 3-months of specialised treatment for AN and this co-occurs with improvements in eating disorder symptoms. Therefore, IL-6 has the potential to be a state biomarker for AN.

Keywords: anorexia nervosa, cytokines, chemokines, longitudinal

1.1 Introduction

Anorexia nervosa (AN) is a serious psychiatric disorder characterised by restricted food intake and other inappropriate weight-loss behaviours (e.g. self-induced vomiting, laxative misuse, excessive exercise), accompanied by an intense fear of food and weight gain, and body dissatisfaction (American Psychiatric Association, 2013). AN has the highest mortality rate of all psychiatric disorders, with a standardized mortality ratio between 5 and 6 (Himmerich et al., 2018), and current treatment options for adults with AN are only moderately effective (Brockmeyer, Friederich, & Schmidt, 2018). The precise aetiology of AN is still unclear, and without knowledge of these causal factors, opportunities for the development of alternative interventions, in particular biological-targeted treatments, are limited. Thus, investigation into the biological correlates of AN, that may contribute to the development and/or maintenance of the disorder, are crucial (Striegel-Moore & Bulik, 2007).

Cytokines, signalling molecules with particular importance in the immune system, have been suggested to play a pathophysiological role in AN (Dalton et al., 2018a). Specifically, pro-inflammatory cytokines have been reported to be elevated in people with AN compared to healthy controls (HCs), including interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α (Dalton et al., 2018a; Solmi et al., 2015). A limited number of small studies have investigated longitudinal changes in these pro-inflammatory cytokines in patients receiving specialist treatment for AN. Some studies have reported a reduction in pro-inflammatory cytokines following refeeding and associated weight gain (e.g., Allende et al., 1998; Misra et al., 2006; Pomeroy et al., 1994); whereas other studies did not find any longitudinal changes in pro-inflammatory cytokines, despite weight gain and/or psychological improvement (e.g., Brambilla, Bellodi, Brunetta, & Perna, 1998; Nakai, Hamagaki, Takagi, Taniguchi, & Kurimoto, 1999). As cytokines have been associated with overall body weight (Schmidt et al., 2015) and adipose tissue (Fantuzzi, 2005), stress (Ménard, Pfau, Hodes, & Russo, 2016)

and psychiatric disorders which are highly comorbid with AN such as depression (Köhler et al., 2017), we would expect to see cytokine changes following weight gain and/or improvements in psychological eating disorder (ED) symptoms in patients with AN.

The symptom profile of AN comprises of both physiological (i.e. weight) and cognitive (e.g., fear of weight gain) features. However, an increase in body mass index (BMI), as a standardized measure of body weight change, has often been chosen as the main outcome of randomized controlled trials or meta-analyses of treatment efficacy in AN (e.g., Dold, Aigner, Klabunde, Treasure, & Kasper, 2015), and has been the most widely favoured index of recovery (Murray, Loeb, & Le Grange, 2018a). More recently, there has been a paradigm shift with research studies increasingly including analyses of both changes in weight and psychological symptoms separately (e.g., Murray, Quintana, Loeb, Griffiths, & Le Grange, 2018b). An improvement in either dimension would be indicative of partial remission and an overall significant improvement in both body weight and the cognitive symptoms of AN would be a sign of full remission (Murray et al., 2018a). Generally, most studies in AN have focused on cytokine changes as a function of weight gain, rather than improvements in the psychological state of the patient, with respect to both ED and general psychopathology symptoms (e.g., Misra et al., 2006; Pomeroy et al., 1994). Therefore, it would be of scientific interest to explore whether cytokine changes during treatment would be associated with weight gain, improvements in AN psychopathology or with both.

There has been a strong focus on a few pro-inflammatory cytokines in the literature and given that pro-inflammatory cytokines function within a complex network of other cytokines, it would be important to consider whether longitudinal changes are observed in related cytokines. These include other cytokines with pro- (e.g., IL-8, IL-17) and anti-inflammatory (e.g., IL-10) functions, T-helper (Th)-1 (e.g., IL-2, IL-12, interferon [IFN]-γ) and Th-2 (e.g., IL-4, IL-5, IL-13) cytokines, and chemokines which are a subcategory of smaller cytokines

(e.g., monocyte chemoattractant protein [MCP]). Therefore, the current study aimed to measure a broad range of cytokines over time in individuals undergoing specialised ED treatment for AN. To address this aim, we measured 27 cytokines in serum samples from AN patients at the start of treatment (baseline) and at 12- and 24-week follow-up assessments.

1.2 Materials and methods

1.2.1 Participants and study design

Fifty-five people with AN were recruited as part of a larger study, comprising of a longitudinal naturalistic design (for full study details see: Keyes et al., 2015; Schmidt et al., 2017). Female adults with a primary diagnosis of AN and a BMI <17.5 kg/m^2 were recruited from Specialist Eating Disorder Services. AN diagnosis was confirmed using the research version of the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV Axis I Disorders (First, Gibbon, Spitzer, & Williams, 1996).

Participants were first assessed (baseline assessment) within the first four weeks of specialised ED treatment for AN. Typically, patients in the local services receive a combination of nutritional (e.g., dietetics, meal support) and psychological (e.g. individual psychotherapy, family therapy) intervention and general clinical management, with inpatients receiving additional input from physiotherapy and occupational therapy. Follow-up assessments were then conducted 12- and 24-weeks after the baseline assessment. For the current study, only participants who provided blood samples at the baseline assessment and at least one follow-up assessment were included and participants reporting autoimmune and/or inflammatory diseases were also excluded. This resulted in a total of 23 participants to be included in the current analyses (n=19 completed 12-week follow-up; n=18 completed 24-week follow-up; n=14 completed all three assessments).

A healthy comparison group were also recruited as part of the larger study (Keyes et al., 2015; Schmidt et al., 2017). Normal-weight (BMI 18.5-24.5kg/m^2) female adults with no physical illnesses and no history of or current mental health disorder were recruited via e-mail circulars to students and staff at King's College London. The absence of psychiatric disorders was confirmed using the research version of the Structured Clinical Interview for DSM-IV Axis I Disorders (First et al., 1996). HC participants who provided a blood sample

and completed the measures described below on one occasion were included in the current study (n=13).

Informed consent was obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki and the study received ethical approval from the South East London Research Ethics Committee (REC ref: 09/H0807/4).

1.2.2 Measures

At all assessments, height and body weight were measured, from which BMI was calculated. Body fat percentage and other body composition parameters (not presented here) were measured using a portable and non-invasive Inbody S10 machine (Inbody Co., Ltd., Seoul, South Korea), which uses the Bioelectrical Impedance Analysis (BIA) measurement method. ED symptoms over the previous 28 days were assessed using the Eating Disorder Examination-Questionnaire (EDE-Q) (Fairburn, 2008) and general psychopathology over the previous seven days was measured using the Depression Anxiety Stress Scale 21-Version (DASS-21) (Lovibond & Lovibond, 1995). As well as a total score, scores for the subscales depression, anxiety and stress can be calculated. Blood samples were also collected. A number of other measures were administered but data on these are presented elsewhere (Keyes et al., 2015; Schmidt et al., 2017).

Serum was stored at -80°C prior to use and thawed at room temperature. Concentrations of 40 inflammatory markers (including cytokines, cellular adhesion molecules, and growth factors) were quantified simultaneously using multiplex ELISA-based technology provided by the Meso Scale Discovery V-PLEX Human Biomarker 40-Plex Kit, following the manufacturer's instructions (Meso Scale Discovery, Maryland, USA). Plates were scanned on the Mesoscale Scale Discovery MESO Quickplex SQ 120 reader at the MRC Social, Genetic, and Developmental Centre, King's College London. We used the Meso Scale Discovery V-

PLEX Human Biomarker 40-Plex Kit, as it quantifies TNF-α and IL-6, which were found to be elevated in AN in the latest meta-analysis (Dalton et al., 2018a). It also measures other cytokines, such as IL-1β, IL-10, IL-12, IL-13 and interferon (IFN)-γ, which have been implicated in depression, anxiety disorders and obsessive-compulsive disorders (Himmerich, Patsalos, Lichtblau, Ibrahim, & Dalton, 2019; Renna, O'Toole, Spaeth, Lekander, & Mennin, 2018), the major comorbidities of AN (Marucci et al., 2018). For the purpose of the current study, we limited our analyses to the following cytokines (n=27), rather than focusing broadly on all assayed inflammatory markers: Eotaxin, Eotaxin-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, interferon γ-induced protein (IP)-10, macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemoattractant protein (MCP)-1, MCP-4, thymus and activation-regulated chemokine (TARC), TNF-α, and TNF-β. Cross-sectional comparisons of the baseline inflammatory marker data with HCs are presented in Dalton et al. (2018b).

1.2.3 Statistical analysis

All statistical analyses were performed in Stata 15 (StataCorp, 2017). Standard curves were used to determine absolute quantities (pg/ml) of each cytokine. We transformed cytokine values by natural log to allow for parametric analyses.

Mixed model linear regressions fitted with maximum likelihood were used to identify changes over time in clinical characteristics and log-transformed cytokine values. Linear mixed models allow for all available outcome data to be included in the model simultaneously and provides estimates in the presence of missing data points (within-subjects) (Gueorguieva & Krystal, 2004). With longitudinal data, measures within participants are correlated and linear mixed models take into account this within-subject and also between- subject variability. We constructed a model that specified log-transformed

cytokine values or clinical characteristics (weight, body fat, BMI, EDE-Q global score, and DASS-21 total score) with time point (baseline [week 0], week 12 and week 24) added as fixed effects and participant ID as a random effect. Bonferroni correction for pairwise comparisons was applied to each significant model post-hoc. We further applied a Bonferroni correction to account for multiple testing of cytokine values by dividing the *p* value (0.05) by the number of comparisons made (n=27). This gave a statistical significance threshold of $p = 0.002$.

For cytokines with significant longitudinal changes, we used t-tests to examine differences log-transformed cytokine values between our sample of AN patients and the HC comparison group reported in Dalton et al. (2018b). The level of significance was $p < 0.05$.

1.3 Results

Demographic and clinical characteristics are shown in Table 1. Body weight ($\chi^2(2) = 7.45$, $p = 0.024$) and BMI ($\chi^2(2) = 9.42$, $p = 0.009$) differed between time points; specifically, as expected, there was an increase in both variables between the baseline assessment and the 24-week follow-up ($z = 2.65$, $p = 0.024$). However, there were no changes in these variables between the baseline assessment and the intermediate week 12 timepoint. Body fat percentage ($\chi^2(2) = 3.92$, $p = 0.141$) did not differ between the three time points. ED symptoms showed an improvement ($\chi^2(2) = 14.23$, $p = 0.001$) at both 12-weeks ($z = -3.04$, $p = 0.007$) and 24-weeks ($z = -3.28$, $p = 0.003$), compared to baseline. At both follow-up assessments, the mean EDE-Q global score was below the commonly used clinical cut-off score of four (e.g., Mond, Hay, Rodgers, & Owen, 2006). No differences between time points were observed for general psychopathology ($\chi^2(2) = 0.01$, $p = 0.996$). DASS-21 severity cut-off scores indicated that participants reported severe depression, anxiety and stress at all three timepoints (Lovibond & Lovibond, 1995).

INSERT TABLE 1 HERE

Log-transformed cytokine values for the three time points are presented in Table 2. The identified longitudinal changes in cytokines did not meet formal significance thresholds ($p = 0.002$), however, we present nominally significant findings here (i.e. without Bonferroni correction for multiple testing). The majority of cytokines were found not to differ between time points. Linear mixed models identified a difference between time points for log TNF-β ($\chi^2(2) = 6.66$, $p = 0.036$); however, these differences did not survive post-hoc correction. A difference in log-IL-6 scores between time points ($\chi^2(2) = 9.42$, $p = 0.009$) was also identified. Bonferroni corrected pairwise comparisons showed that compared to baseline, log IL-6 was reduced at week 12 ($z = -3.03$, $p = 0.007$). Analyses were rerun after removal of two outliers, findings remained the same. Log IL-7 ($\chi2(2) = 7.35$, $p = 0.025$) differed between

time points, with an increase from week 12 to week 24 ($z = 2.66$, $p = 0.023$). Figure 1 shows the serum concentration of IL-6 and IL-7 for AN patients at each time point with a comparison group of HCs.

INSERT TABLE 2 HERE

We used a t-test to compare log-transformed IL-6 and IL-7 values from the AN participants to a HC comparison group (n=13) from the larger study (see Dalton et al., 2018b for HC sample details), to determine whether cytokine values had normalized. At baseline, the AN participants showed elevated log IL-6 values compared to the HCs ($t(34) = -2.67$, $p = 0.012$). At 12- and 24-weeks, log IL-6 for the AN participants no longer differed from HCs (12-weeks: $t(30) = -1.19$, $p = 0.242$; 24-weeks: $t(29) = -1.85$, $p = 0.075$). Log IL-7 did not differ between groups at any time point.

INSERT FIGURE 1 HERE

1.4 Discussion

Our study is the first to measure a wide range of cytokines longitudinally in a sample of 23 AN patients receiving specialist ED treatment. Many of these cytokines have not previously been assessed over time in this patient group. Most existing studies only considered cytokine changes within the context of physical improvements, such as weight gain (e.g., Misra et al., 2006). We assessed cytokine values in the context of both BMI and psychological ED symptoms. In our sample of patients with significantly low weight and body fat, and clinical levels of ED symptoms, depression, and anxiety, 25 cytokines were unchanged over the follow-up period, despite improvements in BMI and ED psychopathology. Two cytokines were found to change longitudinally: IL-6 decreased between baseline and week 12 assessments, and IL-7 increased between week 12 and week 24.

AN patients are metabolically different to healthy individuals, with significantly lower body weight and fat. Despite this, however, we previously reported that many cytokines were not altered in AN compared to HCs (Dalton et al., 2018b). In the current study, we found that most cytokines remain unchanged by improvements in BMI and ED symptoms. This includes those cytokines shown to be previously altered in AN, such as TNF-β and IL-15 (Dalton et al., 2018b), and those that can be hypothesised to have a role in weight, adipose tissue, appetite and feeding behaviour regulation such as IL-1β (a potently anorexigenic cytokine), IFN-γ, TNF-α, and MCP-1 (e.g., Alam, Ullah, Alam, & Ali, 2013; Chen et al., 2005; Labrecque et al., 2019; Peluso & Palmery, 2016; Rask-Andersen, Olszewski, Levine, & Schiöth, 2010; Wong & Pinkney, 2004).

Not all cytokines shown to be involved in weight regulation have been found to be altered in AN in our cross-sectional study (Dalton et al., 2018b) e.g., IL-1β and IL-10. This suggests that AN may be a specific psychological and physical condition that may not involve all weight-regulating mechanisms. Additionally, the net serum concentration of a cytokine is the

result of various physiological processes. For example, TNF-α has been shown to be elevated in AN in our recent meta-analysis (Dalton et al., 2018b). It might be elevated in this patient group, as it has been shown in animal experiments that psychological stress can lead to increased TNF-α production (Himmerich et al., 2013; Krügel, Fischer, Bauer, Sack, & Himmerich, 2014). TNF-α has been shown to lead to weight, skeletal muscle and adipose tissue loss (Patel & Patel, 2017). However, TNF-α levels have also been shown to correlate with body weight (Himmerich et al., 2006). If a patient with AN improves psychologically, there may be reduced production of TNF-α stress-reactive cells. However, simultaneously, increases in body weight in AN recovery might lead to increased production of TNF-α in other cell types. These two opposing processes might leave the net serum level relatively unchanged.

Despite improvement in the physical and mental states of patients with AN, it may also be that underlying biological processes contribute to the persistent alterations in these cytokines. For example, the gut microbiome could be relevant for alterations in cytokine production and the pathophysiology of AN (Bastiaanssen, Cowan, Claesson, Dinan, & Cryan, 2018; Herpertz-Dahlmann, Seitz, & Baines, 2017; Ruusunen, Rocks, Jacka, & Loughman, 2019) because gut dysbiosis and variation within the gut microbes has been associated with the stimulation of cytokine production (Schirmer et al., 2016). Furthermore, inflammatory responses within the gut can signal to the brain via the vagus nerve, which can subsequently affect brain function and behaviour (Bonaz, Bazin, & Pellissier, 2018; Sherwin, Sandhu, Dinan, & Cryan, 2016). Another mechanism by which the microbiome can activate the immune system is through a ‘leaky’ gut (i.e. increased gut permeability): this allows antigens to cross the barrier into systemic circulation, which leads to an inflammatory response. In patients with AN, the gut barrier does not seem to be altered (Mörkl et al., 2018); however, to date, this has only been investigated in a single study with a small AN sample.

Specifically, people with AN have reduced but also altered diversity of bacterial species within the gut, and there is increased heterogeneity between subjects, as compared to HCs (e.g., Seitz, Trinh, & Herpertz-Dahlmann, 2019). However, the connections between AN, the gut microbiota and the immune system are difficult to disentangle as it has shown that they influence each other. For example, a number of cytokines are involved in the regulation of intestinal microbiota, including TNF-β (Kruglov et al., 2013; Upadhyay & Fu, 2013), and bacteria from the gut microbiome can stimulate the production of cytokines, such as IL-1β, IL-6 and TNF-α (Schirmer et al., 2016; Sherwin et al., 2016).

In AN, longitudinal changes in the gut microbiome associated with nutritional rehabilitation and weight gain are unclear, given the limited research (Ruusunen et al., 2019). Results from two small studies show that differences in the gut microbiome composition in AN patients persist after refeeding (Kleiman et al., 2015; Mack et al., 2016). Taken together, biological processes associated with the microbiome may contribute to the cytokine disturbances seen in AN in cross-sectional studies (Dalton et al., 2018a; Dalton et al., 2018b), but it is unclear whether the gut microbiota changes significantly during the course of AN and whether these potential changes or the persistence of microbiota alterations have an influence on cytokine concentrations in the long-term.

Persistent cytokine disturbances may also be related to depression and anxiety, which was rated as severe by our sample at all time points. These disorders are highly comorbid with AN (Marucci et al., 2018; Ulfvebrand, Birgegard, Norring, Hogdahl, & von Hausswolff-Juhlin, 2015) and have been associated with inflammation and cytokine alterations (Baldwin, Hou, Gordon, Huneke, & Garner, 2017; Köhler et al., 2017). Furthermore, severe stress was present throughout assessment periods and may contribute to the persistent cytokine alterations. Indeed, acute and chronic social stress have been shown to promote cytokine production (Himmerich et al., 2013; Krügel et al., 2014). Therefore, these markers may be

potentially more sensitive as a marker of general psychopathology, rather than to improvements in ED symptoms.

We have previously reported that serum IL-6 levels are increased in people with AN compared to healthy individuals (Dalton et al., 2018a; Dalton et al., 2018b). In the current study, we found that IL-6 decreased between the baseline and the 12-week assessment, with a mean reduction in serum IL-6 concentration of approximately 26%. At 12-weeks, IL-6 levels were comparable to those of HCs. There was no difference between baseline and 24-week IL-6 levels. Inspection of the box plot in Figure 1a also suggests a reduction in heterogeneity of IL-6 values over time in the AN patients. One explanation for this may be that there is a sub-group of AN patients with a pro-inflammatory profile that 'normalizes' with improvement in ED symptoms.

We found that ED symptom severity improved during the first 12 weeks of treatment and the mean EDE-Q global score dropped below the typically used clinical cut-off (e.g., Mond et al., 2006). Thus, the normalization of IL-6 levels may not be strictly related to weight changes, but also to psychological improvement. This suggests that IL-6 has the potential to be a state marker of AN. Similar findings have been reported in unmedicated patients with depression, where a reduction in IL-6 concentrations was associated with improvements in symptom severity following psychotherapy (Dahl et al., 2016; Del Grande da Silva et al., 2016); however, it is important to note that in these depressed patients, any weight changes were not reported.

IL-6 has been associated with psychological symptoms (including anxiety and social stress (Niraula, Witcher, Sheridan, & Godbout, 2018), compulsivity, and mood changes (Lichtblau, Schmidt, Schumann, Kirkby, & Himmerich, 2013)), physical exercise (Lavebratt et al., 2017), and appetite regulation (Paulsen et al., 2017), all of which have been implicated in AN

(Munro, Randell, & Lawrie, 2017). From a molecular perspective, peripheral cytokines, such as IL-6, have been shown to enter the brain and influence brain functioning and development, through humoral, neural and cellular pathways, and thus impact on psychological state (Capuron & Miller, 2011). Specifically, cytokines have been shown to affect neuroendocrine pathways and alter neurotransmitter production and signalling. For example, cytokines can activate the hypothalamic-pituitary-adrenal (HPA) axis and stimulate the expression and release of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol (Capuron & Miller, 2011), all of which are reported to be elevated in patients with AN (Misra & Klibanski, 2014). Furthermore, relative hypercortisolemia in AN has been associated with greater ED psychopathology (Lawson et al., 2011).

IL-6 is also reported to be involved in the regulation of adipose tissue (Wedell-Neergaard et al., 2018) and food intake (Wong & Pinkney, 2004), and has been associated with gut microbiome composition (Cooper et al., 2016; Schirmer et al., 2016). Therefore, we may expect changes in IL-6 to coincide with changes in fat mass and/or body weight. Additionally, previous studies reported reductions in IL-6 concentrations, to the level of HCs, with weight gain in AN patients (Misra et al., 2006; Pomeroy et al., 1994). In our sample, however, we did not observe a significant increase in weight, BMI or body fat during this period from baseline to the 12-week assessment, in fact, there was only a 1.5% BMI increase (mean 0.23 kg/m^2) and a 2.0% weight increase (mean 1.0 kg). With respect to body fat, it has been suggested that the BIA measurement method for deriving body fat percentage used in the current study may not be sensitive or reliable in groups with particularly low body weight, dehydration, or electrolyte imbalance, features often seen in patients with AN (Kyle et al., 2004; Mattar, Godart, Melchior, & Pichard, 2011; Mialich, Sicchieri, & Junior, 2014). A more reliable ultrasound method recently reported that subcutaneous adipose tissue differed significantly between AN individuals with the same BMI and that some AN participants had

sufficiently high fat mass despite a low BMI (Lackner et al., 2019). As IL-6 is reported to be involved in the regulation and function of adipose tissue, including both visceral and subcutaneous fat (Wedell-Neergaard et al., 2018), it may be that there is a sub-group of patients with different pro-inflammatory profiles associated with body composition. Taking together the described roles of IL-6 and that refeeding and weight restoration are key factors in specialised AN treatment, it would be important to consider the role of diet and both body and microbiome composition in longitudinal cytokine changes to more fully understand the longitudinal association between IL-6 and AN (Lackner et al., 2019; Wedell-Neergaard et al., 2018).

IL-7 has been less researched in relation to AN and more generally in psychiatry, in comparison to IL-6. IL-7 is predominantly produced by stromal and epithelial cells in various locations in the body periphery (Fry & Mackall, 2002), is required for lymphocyte homeostasis (Khaled & Durum, 2002), and has been shown to induce the production of the pro-inflammatory cytokines IL-1, IL-6, IL-8 and TNF-α (Alderson, Tough, Ziegler, & Grabstein, 1991). IL-7 has also been associated with the regulation of body weight via hypothalamic and adipose tissue control (Lucas et al., 2012; Macia et al., 2010; Makki, Froguel, & Wolowczuk, 2013). For example, in three different models of experimentally induced obesity, administration of IL-7 reduced white adipose tissue mass, protecting mice from obesity (Lucas et al., 2012; Macia et al., 2010). In our study, IL-7 differed between weeks 12 and 24, with a mean increase of approximately 37%. As no significant improvements in ED symptoms, BMI, or general psychopathology were observed during this time (12-week assessment to 24-week assessment), this suggests that these increases in IL-7 were not associated with changes in illness state. It is also important to note that IL-7 values were not altered in comparison to HCs at any time point. This is in contrast to a previous study which found that in 5 patients with AN, already low levels of 24-hour plasma IL-7

levels (compared to HCs) further decreased following weight and menses recovery (Germain et al., 2016). However, these findings need to be considered in view of the small sample size in this study. Given the limited research of IL-7 in relation to weight regulation, food intake, and psychological symptoms in AN, it is unclear why IL-7 changes longitudinally in our sample and in combination with our data and study design, it does not allow for further conclusions.

It is important to consider that a further level of complexity is introduced by the fact that cytokines should be interpreted against the background of genetics, epigenetics and gene expression. It has been shown that cytokine production is also regulated at a genetic level (Li et al., 2016). For example, a single nucleotide polymorphism in the promoter of the IL-6 gene (at position -174 G>C) is reported to regulate peripheral IL-6 concentration, such that the CC genotype was associated with lower plasma IL-6 concentration (Fishman et al., 1998). Molecular genetics research has also suggested that certain IL-6 alleles and genotypes may decrease the risk for schizophrenia (Hudson & Miller, 2018) and depression (Khandaker, Zammit, Burgess, Lewis, & Jones, 2018). While IL-6 may be a promising biomarker of AN, genotyping studies in AN have focused on TNF-α (Ando et al., 2001; Kanbur et al., 2008), a cytokine which has been less consistently associated with AN. Future research should consider genotyping, and epigenetic and gene expression analyses of the IL-6 promotor gene to improve understanding of findings related to peripheral cytokine levels and individual differences in cytokines in patients with AN.

1.4.1 Strengths and limitations

This is the first study to assess a broad range of cytokines and chemokines longitudinally in patients with AN, using validated electrochemiluminescence methods, but as such, it has some limitations. We did not systematically document the various treatment elements the patients received nor recorded treatment status/engagement at the two follow-up assessments.

Also, anti-depressant and other psychotropic medication were not recorded. As anti-depressants are reported to be immune-modulating and hypothesised to exert an anti-inflammatory effect, we cannot be sure that changes in cytokines are associated with symptom improvement rather than psychotropic medication. We did not conduct an a priori power analysis, as our intention was to perform an exploratory study with a broad range of cytokines that have not previously been measured in patients with AN. While we initially corrected for multiple testing of cytokine values, longitudinal changes in cytokines did not meet these formal significance thresholds and therefore, the findings presented have not been adjusted for multiple comparisons (i.e. they were only nominally significant). Due to the small sample size and limited power, we were unable to include confounding factors in our analyses that might influence cytokines (e.g., age, smoking, physical activity) and in turn, may have affected our results or statistically assess the relationship between cytokine changes and changes in AN presentation.

1.4.2 Conclusion

In patients with AN receiving specialist ED treatment, we measured 27 cytokines at three time points over a 24-week period. We found that IL-7 increased between weeks 12 and 24 in people with AN. Little research on this cytokine exists in AN and other related psychiatric disorders, therefore, research is needed to improve understanding of the role it may play in AN. Our findings also show that the IL-6 serum concentrations, which have been reported to be elevated in AN (Dalton et al., 2018a), normalize during the first three months of specialised treatment for AN. This reduction may be associated with an improvement in ED symptom severity. Therefore, IL-6 could be hypothesised as a state marker of AN psychopathology and may present a promising target for future biomarker research and treatment interventions. In our sample, after six months, only 20% of patients had reached a healthy weight (BMI >18.5kg/m^2) and 33% still presented with clinically significant ED

behaviours and symptoms. As such, more research will be needed in larger samples over a longer time frame to increase understanding of the role of IL-6 in AN and recovery.

The temporal sequence and direction of causality of the changes in IL-6 and IL-7 in relation to weight and ED symptom change are unclear from the current data and overall, the role of cytokines in AN is not well understood. Future research would benefit from including prospective longitudinal measurements of cytokines, along with ED-associated physical and mental presentation as well as genotyping, epigenetic characterisation of cytokines genes and the measurement of gene expression, to better understand the role of these cytokines in the development, maintenance and/or recovery from AN.

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Table 1. Demographic, anthropometric and clinical characteristics at baseline and 12- and 24-weeks follow-up.

	Baseline (n=23)	**12-week follow-up (n =19)**	**24-week follow-up (n=18)**
Age [years] (mean ± SD)	32.57 ± 11.81		
Current smoker (n)	5	3	4
Treatment status [inpatient / outpatient] (n)	8 / 15		
AN subtype [AN-R / AN-BP] (n)	10 / 13		
Duration of diagnosis [years] (mean ± SD)	11.68 ± 12.20[a]		
Weight [kg] (mean ± SD)	42.75 ± 8.27	43.76 ± 9.48	46.95 ± 8.89*
BMI [kg/m^2] (mean ± SD)	15.45 ± 2.30	15.68 ± 2.65	16.76 ± 2.61*
Body fat [%] (mean ± SD)	8.20 ± 6.36	7.84 ± 5.76[a]	11.11 ± 7.80[b]
EDE-Q Global (mean ± SD)	4.39 ± 0.97	3.84 ± 1.36*	3.93 ± 1.00*
DASS-21 Total (mean ± SD)	72.78 ± 28.08	70.11 ± 32.45	72.22 ± 29.83
DASS-21 Depression (mean ± SD)	24.52 ± 12.99	24.74 ± 13.78	25.33 ± 10.98
DASS-21 Anxiety (mean ± SD)	19.30 ± 10.12	17.74 ± 11.28	20.00 ± 10.58
DASS-21 Stress (mean ± SD)	28.96 ± 8.48	27.63 ± 11.58	26.89 ± 10.50

[a]n=2 missing; [b]n=1 missing* denotes significantly different from baseline at $p < 0.05$

Abbreviations: SD = standard deviation; AN = anorexia nervosa; AN-R = anorexia nervosa restricting type; AN-BP = anorexia nervosa binge-eating/purging type; BMI = body mass

index; EDE-Q = Eating Disorder Examination – Questionnaire; DASS-21 = Depression Anxiety and Stress Scales – 21 Version

Table 2. Mean (± standard deviation) log-transformed serum cytokine concentrations at baseline and 12- and 24-weeks follow-ups.

Log-transformed cytokines	Baseline		12-week follow-up		24-week follow-up	
	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD
Eotaxin	23	2.29 ± 0.15	19	2.30 ± 0.17	18	2.35 ± 0.18
Eotaxin-3	23	1.19 ± 0.28	19	1.11 ± 0.26	18	1.18 ± 0.20
GM-CSF	18	-0.84 ± 0.38	18	-0.94 ± 0.54	16	0.76 ± 0.35
IFN-γ	23	0.76 ± 0.40	19	0.71 ± 0.27	18	0.80 ± 0.43
IL-1α	23	0.02 ± 0.27	19	0.02 ± 0.37	18	0.05 ± 0.31
IL-1β	18	-0.88 ± 0.70	14	-1.34 ± 0.99	11	-1.02 ± 0.49
IL-2	11	-0.93 ± 0.58	13	-1.07 ± 0.70	9	-0.78 ± 0.28
IL-4	21	-1.32 ± 0.77	14	-1.22 ± 0.72	15	-1.26 ± 0.67
IL-5	21	-0.02 ± 0.34	14	-0.07 ± 0.40	15	0.02 ± 0.18
IL-6	23	-0.22 ± 0.36	19	-0.42 ± 0.21*	18	-.035 ± 0.22
IL-7	23	1.10 ± 0.17	18	1.02 ± 0.19	18	1.14 ± 0.14†
IL-8	23	1.46 ± 0.57	19	1.40 ± 0.55	18	1.25 ± 0.33
IL-10	23	-0.65 ± 0.46	19	-0.84 ± 0.44	16	-0.86 ± 0.51
IL-12/IL-23p40	23	1.95 ± 0.19	19	1.83 ± 0.45	18	1.97 ± 0.14
IL-12p70	21	-0.80 ± 0.41	17	-0.78 ± 0.36	17	-0.79 ± 0.65
IL-13	13	0.33 ± 0.30	10	0.26 ± 0.35	13	0.30 ± 0.32
IL-15	23	0.47 ± 0.11	18	0.49 ± 0.14	18	0.44 ± 0.15
IL-16	23	2.31 ± 0.17	19	2.14 ± 0.43	18	2.25 ± 0.17
IL-17A	23	0.23 ± 0.26	18	0.27 ± 0.30	18	0.29 ± 0.37

IP-10	23	2.17 ± 0.25	19	2.05 ± 0.23	18	2.15 ± 0.22
MCP-1	23	2.28 ± 0.09	19	2.28 ± 0.12	18	2.33 ± 0.08
MCP-4	23	2.07 ± 0.20	19	2.05 ± 0.20	18	2.15 ± 0.12
MIP-1α	23	1.42 ± 0.17	19	1.39 ± 0.17	18	1.41 ± 0.12
MIP-1β	23	1.93 ± 0.19	19	1.92 ± 0.19	18	1.92 ± 0.17
TARC	23	2.60 ± 0.38	19	2.49 ± 0.27	18	2.57 ± 0.29
TNF-α	23	0.22 ± 0.17	19	0.19 ± 0.13	18	0.21 ± 0.14
TNF-β	23	-0.21 ± 0.10	19	-0.31 ± 0.24	18	-0.20 ± 0.12

* nominally significantly different from baseline at $p < 0.05$; † nominally significantly different from week-12

Abbreviations: SD = standard deviation; GM-CSF = granulocyte-macrophage colony-stimulating factor; IFN-γ = interferon- γ; IL = interleukin; IP-10 = interferon γ-induced protein-10; MCP = monocyte chemoattractant protein; MIP = macrophage inflammatory protein; TARC = thymus and activation-regulated chemokine; TNF = tumor necrosis factor

Figure 1. Box plot with individual data points of (a) IL-6 serum concentrations (pg/ml), and (b) IL-7 serum concentrations (pg/ml) at baseline, week 12 and week 24 in patients with anorexia nervosa (AN). Our previously reported data for healthy control (HC) participants (Dalton et al., 2018a) is provided for comparison (n=13). Log IL-6 significantly decreased between baseline and week 12 ($z = -3.03$, $p = 0.007$), with a mean reduction in serum IL-6 concentrations of approximately 26%. Log IL-7 significantly increased between week 12 to week 24 ($z = 2.66$, $p = 0.023$), with a mean increase in serum IL-7 concentrations of approximately 36%. Abbreviations: AN = anorexia nervosa; HC = healthy control; IL = interleukin.