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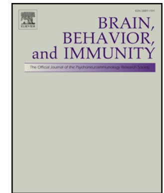

Aberrant inflammatory profile in acute but not recovered anorexia nervosa

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ABSTRACT

Anorexia nervosa (AN) is a severe psychiatric disorder with high mortality and relapse rates. Even though changes in inflammatory markers and cytokines are known to accompany cachexia associated with somatic disorders such as cancer and chronic kidney disorder, studies on inflammatory markers in AN are rare and typically include few individuals. Here, we utilize an Olink Proteomics inflammatory panel to explore the concentrations of 92 preselected inflammation-related proteins in plasma samples from women with active AN (N = 113), recovered from AN (AN-REC, N = 113), and normal weight healthy controls (N = 114). After correction for multiple testing, twenty-five proteins differed significantly between the AN group and controls (lower levels: ADA, CCL19, CD40, CD5, CD8A, CSF1, CXCL1, CXCL5, HGF, IL10RB, IL12B, 1L18R1, LAP TGFß1, MCP3, OSM, TGFα, TNFRSF9, TNFS14 and TRANCE; higher levels: CCL11, CCL25, CST5, DNER, LIFR and OPG). Although more than half of these differences (N = 15) were present in the comparison between AN and AN-REC, no significant differences were seen between AN-REC and controls. Furthermore, twenty-five proteins correlated positively with BMI (ADA, AXIN1, CASP8, CD5, CD40, CSF1, CXCL1, CXCL5, EN-RAGE, HGF, IL6, IL10RB, IL12B, IL18, IL18R1, LAP TGFß1, OSM, SIRT2, STAMBP, TGFα, TNFRSF9, TNFS14, TRANCE, TRAIL and VEGFA) and four proteins correlated negatively with BMI (CCL11, CCL25, CCL28 and DNER).

These results suggest that a dysregulated inflammatory status is associated with AN, but, importantly, seem to be confined to the acute illness state.

1. Introduction

Anorexia nervosa (AN) is a severe psychiatric disorder with high mortality and relapse rates (Berends et al., 2018). In fact, up to 10% of those affected die as consequence of the disorder, making it among the most lethal of the psychiatric disorders (Papadopoulos et al., 2009). AN is characterized by persistent restriction of energy intake leading to severe underweight, combined with body image disturbances, and intense fear of gaining weight. The disorder is often accompanied by behaviours that interfere with weight gain, e.g., purging or extreme physical activity (Association, 2013; Schaumberg, 2017). Twin studies have identified a strong genetic contribution: 58–70% of variance in liability is due to additive genetic factors (Bulik, 2006), and the latest genome-wide association study (GWAS) has identified eight loci associated with AN (Watson, 2019). In addition to genetic factors, environmental and neurobiological factors are known to contribute to the etiology of AN (Schaumberg, 2017). Despite this, the pathophysiology underlying AN is still poorly understood, which makes the development of treatments challenging. To date, no medications exist that effectively target the core biology of the disorder and evidence based treatments are psychotherapeutic in nature.

Although it is widely accepted that inflammation plays an important role in several psychiatric disorders, the role of inflammatory processes in AN is still unclear. Changes in body weight and starvation are

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accompanied by changes in a number of inflammatory markers, rendering it difficult to separate starvation-related from disease-related changes in inflammatory markers. Whilst it is well known that obesity leads to a pro-inflammatory state (Saltiel and Olefsky, 2017), cachexia associated with somatic disorders such as cancer (Tan and Fearon, 2008) and chronic kidney disorder (Pecoits-Filho et al., 2003) is also associated with changes in inflammatory markers and cytokines. In cachexia, in particular, elevated levels of interleukin (IL) 1 and 6, and tumor necrosis factorα (TNFα) have been well documented (Fearon et al., 2012; Patel and Patel, 2017).

In a narrative review, Gibson and Mehler concluded that AN is associated with dysregulation of the immune system, with an elevated CD4/CD8 ratio, increased T cell proliferation, an inflammatory profile that differs from that seen in primary malnutrition, as well as changed concentrations of a number of pro-inflammatory cytokines (Gibson and Mehler, 2019). Indeed, two previous *meta*-analyses have found that TNFα, IL1ß, IL6, and TNF-receptor-II are significantly increased in AN, whereas C-reactive protein and the IL6-receptor are significantly decreased compared to healthy controls (Solmi, 2015; Dalton, 2018). In addition, a study of the genetic correlation between several psychiatric disorders and inflammatory disorders revealed a significant negative genetic correlation between AN and the acute phase reactant CRP, indicating that people with increased genetic risk for AN are more likely to carry genetic variants associated with lower levels of CRP (Tylee, 2018). However, even if several other psychiatric disorders (e.g., ADHD, major depressive disorder, bipolar disorder, and schizophrenia) show positive genetic correlations with inflammatory disorders, this does not seem to be the case with AN (Tylee, 2018).

The aim of this study was to test the hypothesis of a dysregulated immune system in AN, and whether potential alterations in inflammatory markers are state dependent. To these ends, we utilized the Olink Proteomics inflammation panel to assess concentrations of 92 preselected inflammation-related proteins, including IL6 and IL7, as well as TNF-related proteins, in plasma samples from women actively ill with AN, women recovered from AN (AN-REC), and normal weight healthy controls (CTRL).

2. Materials and methods

2.1. Participants

The study participants were identified from the Swedish sample of the Anorexia Nervosa Genetics Initiative (ANGI-SE). Details on the recruitment procedure were described previously (Thornton, 2018). The general inclusion criteria for the AN group for this study were female patients, at least 18 years of age, meeting DSM-IV criteria for AN, with the exception of amenorrhea (Association, 1994), with at minimum one year since AN onset (n = 113). AN *with binge eating* (AN-BE) was defined by documented episode(s) of binge eating during the last two years before blood sampling. AN with *past binge eating (*AN-pBE*)* by more than two years since last documented binge eating episode, and AN without episodes of binge eating (AN-nonBE) by complete absence of documented binge eating episodes. For AN-REC the inclusion criteria were history of DSM-IV AN with subsequent weight restoration (BMI greater than 20 kg/m^2) plus no eating disorder behaviours for at least a year (n = 114). The age-matched normal-weight female controls reported no history of disordered-eating behaviour (CTRL, n = 113). See table 1 for further information on the participants.

The study was approved by the Regional Ethics Review Board in Stockholm. All participants provided written informed consent.

2.2. Blood sampling

Blood was collected using EDTA tubes at the hospital nearest to the participant, sent to Karolinska Institutet Biobank with overnight mail, and processed upon arrival. After centrifugation, plasma samples were stored at −80 °C until shipping to Olink Proteomics (Uppsala, Sweden) for analysis.

Table 1
Sex, age, BMI, years since AN onset, and binge behavior of the study participants by group.

Characteristics	AN	AN-REC	CTRL
n	113	113	114
Females (%)	100%	100%	100%
Age (years) (median [IQR])	27.7 (24.0–31.0)	27.7 (24.0–31.0)	27.8 (24.0–31.0)
BMI (kg/m^2) (median [IQR])	15.8 (15.0–17.0)	23.4 (20.0–24.5)	24.3 (22.0–26.0)
Years since AN onset (median [IQR])	10.8 (6.0–15.0)	10.7 (6.0–14.0)	-
AN with binge episode(s) n (%);			
current	26 (23.0%)	-	-
past	14 (12.4%)	-	-
never	53 (46.9%)	-	-
information not available	20 (17.7%)	-	-

AN = anorexia nervosa, AN-REC = recovered from anorexia nervosa, CTRL = healthy controls, IQR = interquartile range.

2.3. Proximity extension assay

The samples were analyzed with the inflammation reagent kit from Olink Proteomics consisting of 92 preselected proteins, all related to inflammation (Supplementary Table 1). The kit is based on the proximity extension assay (PEA) technology utilizing oligonucleotide labeled antibody probe pairs binding to their respective target protein and a proximity dependent DNA polymerization step, resulting in a reporter sequence that can be quantified by real-time PCR (Assarsson, 2014). Limit of detection (LOD) was reported by OLINK for each assay.

2.4. Statistical analyses

Given the highly non-normal distribution of several markers, median and inter-quartile range (IQR) of clinical characteristics and marker levels were calculated. All plasma marker concentrations were analysed in the NPX unit, a log2-transformed normalized protein expression unit. Values below the LOD were treated as missing values. Normality was assessed visually using quantile–quantile plots for all markers. To rule out single sample multi-dimensional outliers, sample scores from a principal component analysis (PCA) were computed to summarize the combined proteomic variance and were assessed visually. Differences between groups (AN, AN-REC, and CTRL, as well as between AN-BE, AN-pBE- or AN-nonBE) and associations with BMI were assessed. To facilitate comparisons with studies that do not include patients with low BMI, as well as to assess whether the association with BMI was linear over the whole range, associations with current BMI were reported over the whole range, as well as separately for the AN group (BMI < 18.5) and the non-AN group (BMI ≥ 18.5).

For *normally distributed plasma marker variables*, group differences were assessed using ANOVA followed by post-hoc Tukey HSD test to evaluate pairwise comparisons. The associations between marker levels and BMI were assessed using linear regression with age as a covariate. For variables with non-normal distributions, group differences were assessed using Kruskal-Wallis test followed by post-hoc Dunn's test to evaluate pairwise comparisons. The associations between the median marker levels and BMI were assessed using quantile regression with age as a covariate. As a further post-hoc analysis, given the highly skewed distribution of some of the markers, we assessed the differences in distribution of values above the 90th percentile between the three groups for all non-normal distributed markers. Marker levels were binarized in two groups according to the 90th percentile cutoff and

differences in frequencies between groups were assessed using Chi-square test. Correlations between protein markers were assessed using Spearman correlations and partial Spearman correlations. To analyze structural variation across the full panel, a partial least squares discriminant analysis (PLS-DA) (Brereton and Lloyd, 2014; Wold et al., 2001) model was computed. Permutation tests, in which the predictive performance metric (Q2Y) of the model is tested against n models trained on a permutated response variable, were used to avoid overfitting and indicate significance (Szymanska et al., 2012). Root mean squared error of estimate (RMSEE) was reported.

Statistical analyses were conducted using R programming language version 3.6.1 (including packages quantreg (Koenker, 2019); emmeans and multcomp (Hothorn et al., 2008), and ropls v.1.8.0 (Thevenot et al., 2015) for PCA And PLS). Graphs were built using ggplot2 (Wickham, 2016). In order to avoid overcorrection given the high correlation between some markers violating the assumption of test independence, we estimated the number of effective tests M_{eff} as described by Cheverud and Nyholt et al (Cheverud, 2001; Nyholt, 2004). We adjusted p-values to account for the 71 independent tests (total number of proteins tested: 74) using Bonferroni correction; an alpha level of 0.00070 was therefore considered statistically significant.

3. Results

Clinical characteristics of the study population are summarized in Table 1. Eighteen proteins (Il17A, IL20RA, IL-1α, IL2, TSLP, FGF-5, IL22RA1, IL24, IL13, ARTN, TNF, IL20, IL33, IFNγ, IL4, LIF, NRTN, and IL5) were excluded from further analyses since more than 20% of the data were missing (Supplementary Table 1). No individual had more than three missing values in the remaining 74 proteins. A PCA model for data overview retained 33.5% of the variance over the first three components, and no major multi-dimensional outliers were detected in this model. High correlations were observed between many of the markers in all combined study participants. While most were positive, one pair (TRANCE/RANKL) showed a high negative correlation (Supplementary Fig. 1).

3.1. Inflammatory markers and AN

After Bonferroni correction, 25 significant differences were observed between AN and CTRL, while 15 significant differences were observed between AN and AN-REC (Fig. 1, Supplementary Table 2). Separate graphs for all tested markers are provided in the supplement (Supplementary Graphs). Nineteen proteins had *lower* plasma concentrations in AN compared with CTRL (ADA, CCL19, CD40, CD5, CD8A, CSF1, CXCL1, CXCL5, HGF, IL10RB, IL12B, 1L18R1, LAP TGFß1, MCP3, OSM, TGFα, TNFRSF9, TNFS14, and TRANCE), most of which also differed significantly between AN and AN-REC (ADA, CD40, CD5, CSF1, HGF, IL10RB, IL12B, 1L18R1, LAP TGFß1, TGFα, and TRANCE). Six proteins were significantly *higher* in AN compared with CTRL (CCL11, CCL25, CST5, DNER, LIFR, and OPG), three of which (CCL11, CCL25, and LIFR) were also higher in AN than AN-REC. GDNF was siginificantly higher in AN than AN-REC, but did not differ between AN and CTRL. No significant differences were found between AN-REC and CTRL (Fig. 1**A**).

Given the highly skewed distribution of some of the markers, we evaluated whether there was a difference in distribution for values above the 90th quantile between the three groups for all non-normally distributed proteins. After correction for multiple testing, only IL6 showed a significant difference, with 22/34 of the 10% highest IL-6 values being measured in patients of the AN-group ($\chi^2(2) = 20.03$, $p_{adj} = 3.17 \times 10^{-3}$).

No significant differences were seen between AN-BE, AN-pBE, or AN-nonBE (data not shown).

3.2. Inflammatory markers and BMI

Fig. 1B shows that 25 proteins correlated positively with BMI (ADA, AXIN1, CASP8, CD5, CD40, CSF1, CXCL1, CXCL5, EN-RAGE, HGF, IL6, IL10RB, IL12B, IL18, IL18R1, LAP TGFß1, OSM, SIRT2, STAMBP, TGFα, TNFRSF9, TNFS14, TRANCE, TRAIL, and VEGFA) while four proteins correlated negatively with BMI (CCL11, CCL25, CCL28, and DNER) (Supplementary Table 3, Supplementary Graphs). For most of the proteins that were positively correlated with BMI, a correlation with BMI among normal weight (BMI ≥ 18.5) participants could also be seen. However, none of the proteins for which higher concentrations in AN and/or a negative association with BMI were reported (i.e., CCL11, CCL25, CCL28, CST5, DNER, GDNF, LIFR, and OPG) showed any association with BMI among only normal weight individuals. Only one protein, CCL11, was significantly negatively associated with BMI in individuals with a BMI < 18.5.

Although CCL28, CDCP1, FGF21, IL6, STAMBP, and VEGFA were strongly correlated with BMI, no group differences were observed for these proteins.

3.3. Sensitivity analysis

As a sensitivity analysis, calculations were repeated replacing the missing values by the LOD for the proteins that had up to 20% missing values (MCP-3, GDNF, IL-17C, IL-2RB, FGF-23, IL-10RA and FGF-21). Similar results regarding groups and BMI were observed.

3.4. Multivariate analysis

A PLS-DA model (2 components, Q2Y = 0.22, RMSEE = 0.409) discriminated AN from AN-REC and CTRL with significant statistics (p = 0.01) over 100 iterations of permutation testing (Supplementary Figure 2)

4. Discussion

We analysed the hitherto largest battery of inflammatory markers in plasma from women with active AN (N = 113), women who had recovered from AN (AN-REC, N = 113), and healthy female normal-weight controls (N = 114). We found a markedly different proteomic plasma profile of inflammatory markers in women with active AN compared not only with controls, but also compared with women who had recovered from AN. Several of these proteins were negatively or positively correlated with BMI, mostly in line with the differences observed between the study groups (see further discussion below). Importantly, we found no differences between women who had recovered from AN and controls, not even in a multivariate PLS-DA model. This suggests that aberrant inflammatory profile is a state marker associated with active AN and/or low BMI.

4.1. Associations between plasma markers and AN

The majority of our observations have not been reported previously. Of the proteins that were higher in AN compared to CTRL and/or AN-REC (CCL11, CCL25, CST5, DNER, GDNF, LIFR and OPG), LIFR (Monteleone et al., 1999) and OPG has previously been reported to be higher in AN than controls (Golabek, 2015; Ohwada et al., 2007; Misra, 2003; Ostrowska, 2016). Of the proteins that were lower in AN than controls (ADA, CCL19, CD40L, CD5, CD8A, CSF-1, CXCL1, CXCL5, HGF, IL10RB, IL12B, 1L18R1, LAP-TGFß 1, OSM, TGFα, TNFRSF9, TNFSF14 and TRANCE), lower TGFß has previously been reported (Ostrowska, 2016), but not in all reports (Solmi, 2015). Our finding of lower CSF1 plasma levels in AN accords a previous report of reduced CSF1 in the supernatant of peripheral blood mono-nuclear cells from AN (Vaisman, 1996). However, we found lower plasma levels of TRANCE in AN, which conflicts two previous studies that reported higher concentration

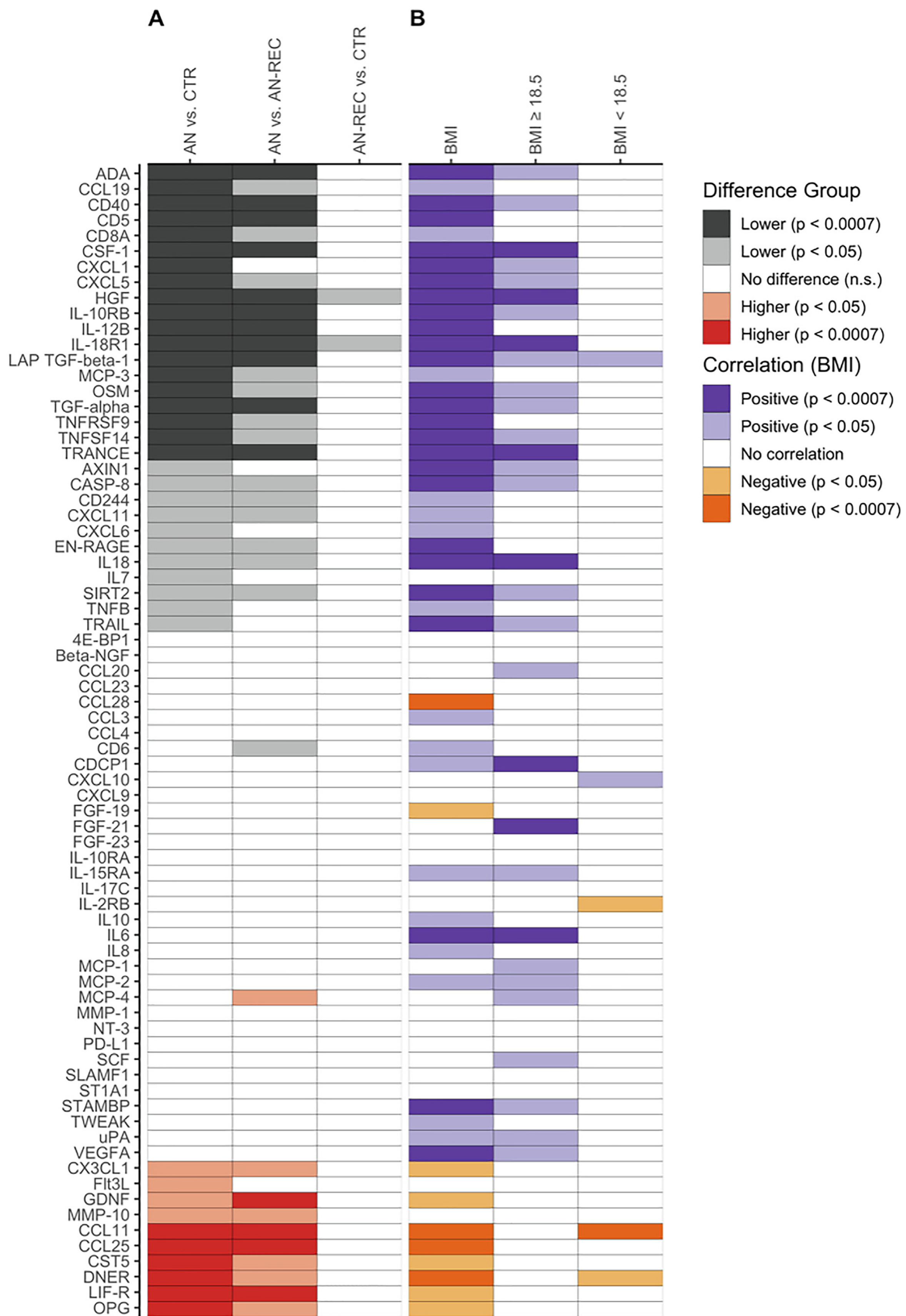

Fig. 1. Plasma protein concentrations changed in AN vs AN-REC and CTRL (A), and associations between plasma protein concentrations and BMI (B). Values are normalized protein expression (NPX) values in $\log^2$ scale. AN = anorexia nervosa, AN-REC = recovered from anorexia nervosa, CTR = normal weight healthy control. For full protein names see Table S1.

in AN than controls (Golabek, 2015; Ostrowska, 2016). A discussion on the proteins that were associated with AN-status and/or BMI at a significance threshold of $p < 0.01$ can be found in the Supplement. Although we did not see any differences within active AN cases with regards to history of binge-eating behavior, our results need replication and should be interpreted with care.

The previous most frequently documented changes in AN with regard to inflammatory markers in blood are high levels of IL6 and TNFα (Solmi, 2015; Dalton, 2018; Dalton, 2018). We did not find higher IL6 concentration in AN. In fact, we observed a positive association between median plasma levels of IL6 and BMI. However, when analyzing the distribution of the patients with values above the 90th percentile for IL6 levels, 22 of 34 cases in fact belonged to the AN group. Speculatively, these could represent a subgroup of AN patients (e.g., recently physically active patients) that if overrepresented in a cohort could lead to higher mean IL6 concentrations in an AN group (Pedersen and Febbraio, 2008). TNFα was unfortunately excluded from our study since 69% of data was missing. Several other proteins that sporadic previous studies have found altered in AN (e.g., IL7 (Germain, 2016), VEGFA (Dalton, 2018), CXC3L1 (Zhang et al., 2017) did not replicate in our sample.

There is a bidirectional relationship between inflammation and food intake. On the one hand, reduced food intake is a common symptom of sickness behavior (Dantzer et al., 2008) and several cytokines have been shown to be anorexigenic (Gautron and Layé, 2010). On the other hand, increased (e.g., obesity) as well as reduced body weight (e.g., malnutrition, wasting) have been associated with changes in inflammatory status (Gibson and Mehler, 2019; de Heredia et al., 2012). While obesity is a proinflammatory state, reduced body weight seems to have a more complex impact on the immune system and might differ depending on the cause of weight loss; malnourished patients are at increased risk for infections whereas AN patients are not (Gibson and Mehler, 2019; Bowers and Eckert, 1978). From this perspective, a study comparing inflammatory markers in other populations characterized by low BMI (e.g., constitutional thinness, other medical conditions marked by low weight) would be informative.

4.2. Associations between plasma markers and BMI

Several of the associations with BMI that we found have been previously reported (ADA, CD40, CSF1, CXCL5, HGF, IL10RB, LAP-TGFß, OSM, TGFα, TNFRSF9, IL18, SIRT2), but we also report several novel associations (CD4, IL12B, TNFSF14, TRANCE, AXIN1, CASP8, EN-RAGE, TRAIL). Separate posthoc correlation analyses below and above BMI 18.5 revealed that some associations were mainly driven by individuals with very low BMI. This means that studies that do not include indivduals with very low BMI are less likely to detect such correlations. Interestingly, many of the proteins we find to be associated with BMI have previously been shown to be functionally involved in weight gain or loss in experimental animal models, such as CSF1 (Wei, 2005), CCL19/CCR7 signalling (Sano, 2015), CXCL1 (Pedersen et al., 2012), TNFRSF9 (Kim, 2011), TNFSF14 (Saunders, 2018), TRANCE (Yi, 2017), CX3CL1 (Dorfman, 2017; Morari, 2014), TGFα (Luetteke et al., 1993), DNER (Park, 2010), GDNF (Mwangi, 2014; Tumer, 2006; Manfredsson, 2009), LIF/LIFR (Plata-Salaman, 1996; Arora, 2018), IL18/IL18R1 (Murphy, 2016). For further discussion, see the Supplement.

4.3. Inflammatory markers and bone physiology

Some proteins included in the OLINK panel have other physiological roles not directly linked to inflammation. For example, we found that a subgroup of proteins involved in bone physiology differed between AN and controls, including OPG, TRANCE (RANKL), CSF1 (MCSF), LAP-TGFß, and CST5. Low bone mineral density and increased risk of fractures is a common complication of AN (Steinman and Shibli-Rahhal, 2019). Several factors have been suggested as being causative, including decreased estrogen, low BMI, elevated cortisol, low IGF1, as well as poor nutrition and a lack of vitamin D (Steinman and Shibli-Rahhal, 2019). Although there is some evidence that body weight restoration can reverse bone loss (Hubel, 2019), not all studies have shown this effect (Reviewed by Steinman and Shibli-Rahhal, 2019). Our results indicate a disturbed balance between bone formation and resorption in acute AN. Importantly, none of the proteins involved in bone physiology differed between AN-REC and controls, suggesting that these effects are reversed upon weight gain/-restoration. However, longitudinal studies are needed for confirmation.

4.4. Strengths and limitations

Previous studies on inflammatory markers in AN are scarce and typically investigate a limited set of inflammatory markers in small samples. Furthermore, most previous studies have not included a recovered AN group, which precludes any conclusions as to whether altered inflammatory profile is a state or trait marker in AN. We investigated a broad array of inflammatory markers in a large sample of individuals with AN. We not only included a control group matched for age, but also an age-matched group with women who had recovered from AN. Even though our study would support the state hypothesis for most markers—as no differences between AN-REC and CTRL were seen—a longitudinal study following people with AN across the course of recovery is necessary to confirm our findings. Hence, one limitation is that our study was cross-sectional and cannot establish cause and effect. Another limitation is the blood sampling procedure, as blood samples were sent via overnight mail before being processed. One study found that a 24 h pre-centrifugation delay significantly affected certain plasma protein concentrations, including 22 proteins analysed in this paper. 4EBP1, ADA, AXIN1, CASP8, CD6, CXCL1, CXCL5, CXCL6, CXCL11, EN-RAGE, HGF, IL7, IL18, LAP-TGFß, MCP2, MCP3, MCP4, MMP1, OSM, STAMBP, TGFα, and TNFSF14 have been shown to be significantly higher after 24 h at room temperature compared with direct centrifugation (Shen, 2018). Even if this is likely to have introduced noise and effectively reduced power, it is unlikely to have affected group differences since all samples were collected and handled in the same way. Further, we lack information on time of day of sampling, which would have been valuable as some inflammatory markers vary with circadian rhythms (Wipfler, 2013). Finally, we lack some phenotypic information that may influence cytokine concentrations (O'Connor, 2009) such as drug use (including illicit drugs), smoking, exercise, sleep, ethnicity, socioeconomic and hormonal status, psychiatric as well as somatic comorbidities such as autoimmune/inflammatory diseases.

In conclusion, we observed an aberrant inflammatory plasma profile in AN, which was not seen in those who had recovered from AN. We also observed that plasma concentrations of several of the inflammatory proteins correlate with BMI. Although there are currently no data on causal effects of inflammatory markers on AN, our results could be seen as yet another reason to provide prompt and adequate treatment to patients with AN. Further investigations on the long-term effects of an aberrant inflammatory profile in AN are warranted.

Author contributions

IAKN, VM, AG, MS, and ML designed the study. CMB, LT, and ML collected the samples. IAKN, VM, AG and CH analysed the data. IAKN, VM, AG, CMB, and ML wrote the manuscript, which was revised and approved by all authors.

Declaration of Competing Interest

ML declares that, over the past 36 months, he has received lecture

honoraria from Lundbeck pharmaceutical. CMB reports: Shire (grant recipient, Scientific Advisory Board member); Idorsia (consultant); Pearson (author, royalty recipient)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.05.024.

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