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(Article begins on next page)

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Title page

Pro-inflammatory cytokine alterations in recent onset anorexia nervosa adolescent female patients before and after 6 months of integrated therapy: a case–control study.

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Running head: restoration of pro-inflammatory cytokine in anorexia nervosa after 6 months of integrated therapy

Abbreviations:
AN= Anorexia nervosa
ED= Eating disorder
CCK= cholecystokinin
PYY= Peptide YY
TG= test group
CG= control group
BMI= body mass index
DSM-V= Diagnostic and Statistical Manual of Mental Disorders-V
SAFA= Scales for Children and Adolescents
EDI-2= Eating Disorders Inventory II
BDZ= Benzodiazepines
SSRIs= Selective serotonin reuptake inhibitors
CBT= cognitive behavior therapy
EN= enteral nutrition
PTN= parenteral nutrition

ELISA= enzyme-linked immunosorbent assay
HPA= hypothalamic-pituitary-adrenal

Abstract: Anorexia nervosa (AN) is a complex disorder affecting mainly, but not only, teenagers. Researchers agree that AN is deeply associated with a pro-inflammatory state following an impaired immune system, resulting from altered levels of cytokines such as IL-1β and TNF-α, also impacted by the frequent depressive states. Thus, the aim of the present case-control study was to evaluate the relationship between patients suffering from AN undergoing specialized eating disorder (ED) treatment for AN and pro-inflammatory cytokines. To reach our purpose, we assessed eating-related psychopathology and depressive symptoms and measured serum concentration of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α before and after 6 months of integrated therapy (which included psychopharmacotherapy, psychotherapy and nutritional treatment), in order to define whether selected pro-inflammatory cytokines could be considered a pathophysiological marker of the disorder. A sample of 16 young female patients with early diagnosis of AN, and without any previous treatment, and 22 healthy controls matched by age, sex and socioeconomic status were enrolled. After 6 months of integrated therapy a significant decrease of all selected pro-inflammatory cytokines was detected. In addition, an improvement in the anxiety-depressant aspects was also noted. In conclusion, the results obtained suggest that pro-inflammatory cytokines are indeed related to the pathophysiology of AN. However, further investigations, involving larger samples of patients with distinct subtypes of AN, are essential to confirm the current findings.

Key messages of the article:

What is already known on this topic

- Cytokines play an important role in mental health and related mental disorders, which are deeply associated with Eating Disorders, including anorexia nervosa
- Cytokines are cell signaling molecules produced by a variety of cells in the brain and of the immune system and are essential in coordinating re-sponses to infection
- There are few reports describing the effects of refeeding on cytokine levels

What this study adds

- Specialized eating disorder treatments for AN are able to restore cytokines within the reference range
- The anxiety-depressant aspects were improved

How this study might affect research, practice or policy

- The levels of pro-inflammatory cytokines are related to the severity of anorexia nervosa and the response to treatment, therefore they could be considered non-specific markers in the evaluation of these aspects

Keywords: anorexia nervosa; cytokines; eating disorders; immune system; inflammation.

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1. Introduction

Anorexia nervosa (AN) is a complex disorder affecting mainly, but not only, teenage women [1]. It is a mental illness characterized by disordered eating, involving food restriction, and severe weight loss. There is either an intense fear of gaining weight or becoming fat or persistent behavior that interferes with weight gain (even though at a significantly low weight) [2]. AN shows a complex interplay between neurobiological, psychological, and environmental factors and is a chronic disorder with frequent relapse, high treatment costs, and severe disease burden [3].

Although its incidence and prevalence may vary between different population groups, epidemiological studies show that AN afflicts 0.9-4.0% of women in the USA and Europe [1]. In recent decades, the age at onset of AN has been decreasing and more importantly childhood and adolescent AN are on the rise. Age of onset peaks in middle to late adolescence, which affects educational and professional development [4]. The consequences of starvation can have a negative impact on bone density, growth, and brain maturation, especially in children and adolescents [5].

As AN is a psychiatric disorder, psychiatric comorbidity is almost always present (anxiety, depression, obsessive ideation and compulsive behaviors) [4] and this may lead to death; in fact, it has the highest mortality of all psychiatric conditions [6].

AN is characterized by a multifactorial etiopathogenesis where genetic, biological and endocrinological factors are involved [7]. Unlike most other mental health disorders, in which physical health may be completely

normal, compromised physical health is inextricably connected with this illness. Multiple concomitant medical complications occur throughout the body and become more pronounced as the severity of the illness increases. There can be cardiac, gastrointestinal and pulmonary problems, multiple endocrine abnormalities, loss of muscle and bone, brain atrophy and dermatologic complications [8]. Complications of AN can also involve the immune system. It is well documented from the literature that the immune system of AN subjects is impaired due to prolonged starvation and malnutrition as well as any single nutritional deficiency [9]. Thus, an important etiopathological factor contributing to AN is inflammation. Researchers agree that eating disorders (EDs) are deeply associated with a pro-inflammatory state, and a possible treatment, such as weight restoration, is vanquished by an increase in the pro-inflammatory cytokines [10].

Cytokines are cell signaling molecules produced by a variety of cells in the brain (e.g., microglia, astrocytes) and of the immune system (e.g., by macrophages and T-lymphocytes) and are essential in coordinating responses to infection. They can access the brain through humoral, neural and cellular pathways and therefore may have an effect on the mental state, including learning, memory, affectivity, and behavior through several pathophysiological mechanisms [11]. In recent years, it has been shown that cytokines play an important role in mental health and related mental disorders, which are highly associated with EDs, such as depression, anxiety disorders, post-traumatic stress disorder, and sleep disorders [12].

In addition to the known role played in the immune system, interleukin (IL)-1β and IL-6 are able to interact with leptin and ghrelin, with a consequent anorexigenic effect. Furthermore, tumor necrosis factor α (TNF-α) promotes the production of cholecystokinin (CCK) and Peptide YY (PYY), with an anorexigenic effect [13]. A recent meta-analysis has shown that the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 are elevated in people with AN, compared to healthy individuals [14]. While several studies have addressed the question of starvation effects on immune functions, to our knowledge there are very few reports describing the effects of refeeding [15-17]. In a very recent paper, Nilsson et coll., [18] through a proteomic approach, described a peculiar inflammatory plasma profile in patients actively ill with AN, which was not found in those who had recovered from AN. They were also able to correlate plasma concentrations of many of the inflammatory molecules to BMI.

Thus, the present work aimed at testing the hypothesis that specialized ED treatment (which included psychopharmacotherapy, psychotherapy and nutritional treatment) may reverse the higher levels of pro-inflammatory cytokines in patients diagnosed with anorexia nervosa. In particular, serum IL-1β, IL-6 and IL-8 and TNF-α were measured at enrollment (t0) and after 6 months of integrated therapy (t_1) in a cohort of patients with anorexia nervosa (test group = TG). The obtained data were compared with a control group of healthy individuals (control group = CG).

2. Materials and Methods

2.1 Participants and study design

The study was performed on 16 female adolescent patients suffering from AN, aged between 12 and 18 years (mean age 14.6±1.9 years at enrollment) (TG), with a mean weight of 36.8 ± 3.2 kg and a body mass index (BMI) of 14 ± 0.87 kg/m2, who referred to the Department of Neuropsychiatry, Children's Hospital "G. Salesi", AOU Ospedali Riuniti Ancona, Italy, This group was compared with a control group (CG) of 22 age matched healthy female adolescent individuals with a mean age 15.2±0.8 years. The CG was free from a history of or with current mental health disorders and physical illness and went to the hospital for routine checks.

CG had a mean BMI of 22.5±2.5 kg/m2, and normal values of Fasting Plasma Glucose (80.0±12.1 mg/dL), Fasting Plasma Cholesterol (195.2±27.6 mg/dL), HDL cholesterol (58.2±9.2 mg/dL), Fasting Plasma Triacylglycerols (79.4±24.1 mg/dL), Albumin (4.3±0.8 g/dL), and Prealbumin (29.5±5.2 mg/dL).

All recruited subjects were selected in the period between January 2018 and January 2019. All participants were identified as White European.

Anorexia nervosa patients were of the restricting subtype according to the Diagnostic and Statistical Manual of Mental Disorders-V (DSM-V) criteria [19]. The study was conducted in accordance with the principles of the Declaration of Helsinki, and was approved by the Review Board of Università Politecnica delle Marche (protocol number 201805). A written, informed consent was obtained from all participants (signed by participant or/and parents or legal guardian).

Inclusion criteria, for AN patients, were: (i) female sex; (ii) first episode of ED; (iii) first admission for ED treatment; (iv) age under 18 years; (v) adherence to integrated therapy, in all its components: psychopharmacotherapy, psychotherapy and nutritional treatment; (vi) acceptance to participate in the study. Exclusion criteria were: (i) male sex; (ii) age over 18 years; (iii) illiteracy; (iv) mental retardation; (v) history of diseases known to interfere with eating behaviors: diabetes mellitus, thyroid disease, and loss of appetite related to cachexia syndrome (e.g. cancer, AIDS, renal failure, advanced liver disease, multiple sclerosis).

Any minor intervention (such as the eventual use of antibiotics, analgesics, or supplements) was implemented upon request and subject to parental consent. All patients had psychiatric comorbidities such as anxiety, depression, phobic ideation and obsessive behavior, consequently the use of some psychopharmacological drugs was necessary.

During the studied period, all enrolled adolescents required hospitalization, which lasted between 7 and 57 days. Among them, only 3 patients had a short hospitalization (7-14 days), whereas the others were hospitalized for more than two weeks.

ED symptoms were assessed according to the following tests for psychodiagnostic assessment: the Self Administrated Psychiatric Scales for Children and Adolescents (SAFA) test [20] and the Eating Disorders Inventory II (EDI-2) [21].

From t0 to t1 all the enrolled subjects underwent a multimodal treatment approach, the so called “integrated therapy” which included psychopharmacotherapy, psychotherapy and nutritional treatment.

According to the *psychopharmacotherapy*, three classes of drugs were used:

1. Benzodiazepines (BDZ): Xanax (Alprazolam) and Tavor (Lorazepam).

2. Selective serotonin reuptake inhibitors (SSRIs): Zoloft (Sertraline), Xeredien (Fluoxetine) and Fevarin (Fluvoxamine).

3. Atypical antipsychotics: Abilify (Aripiprazole), Seroquel (Quetiapine) and Risperidal (Risperidone).

Regarding *psychotherapy*, cognitive behavior therapy (CBT) is the supported treatment and it was used once a week, with the routine involvement of the patient's parents. Each patient was treated by a single therapist who was substituted in the case the primary therapist had to be absent.

Nutritional therapy was performed orally or, in more severe cases, by enteral (EN) (3 patients) or parenteral (PTN) (1 patient) nutrition. All formulations were indicated by a specialized nutritionist (LN) and, in the case of EN or PTN, produced by the hospital pharmacy in customized bags.

The patients, who received EN, had an isocaloric formula, providing 1.5 kcal/mL, and 54% energy from carbohydrate, 29% energy from fat, 17% energy from protein. At first, nasogastric tube feeds were commenced at 35 mL/h for 12 h, and after this period of time feeds were increased to a rate of 70 mL/h by continuous infusion in order to reach a total of 1250 mL = 1900 kcal. Only one patient, who refused EN, received PTN. The nutritional formula contained nutrients for peripheral infusion (both essential and non-essential amino acids, monosaccharides such as glucose, minerals, lipids such as medium-chain triglycerides, ω3 and ω6 fatty acids). The daily caloric supply of 1200 to 1400 total kcal/day was augmented by 200 to 400 kcal/day every 3 to 4 days to 60 kcal/kg/day. PTN was used for less than 2 weeks.

The main goal of the nutritional therapy was the restoration of a healthy body weight which implied the resumption of menses and an age-appropriate growth.

The patients had no supplementary therapeutic input, either from physicians, nutritionist or other health professionals unless there was a specific indication for the management of medical complications and/or comorbid conditions.

2.2 Anthropometric measurements

All patients underwent anthropometrical assessment by standard procedures (without shoes and dressed only in underwear) measured by a trained investigator (LN). Patients' measurements were performed on their first day of admission (t0), and after 6 months of integrated therapy (t_1), using a digital electronic weighing scale (Seca 780, Hamburg, Deutschland; 0.100-g precision) with an incorporated telescopic measuring rod and measurements were recorded to the nearest 0.5 cm. From these data, the BMI [weight (kg)/height^2 (m^2)] was calculated.

2.3 Blood sampling

Peripheral venous blood was collected into vacutainers tubes, after 12 hours of fasting, between 7 and 9 a.m., and then clot retraction serum was centrifuged at 875 g for 5 min, transferred to a sterile tube and stored at -80 °C, until use.

2.4 Serum cytokine measurements

Different types of cytokines were measured in triplicate by using enzyme-linked immunosorbent assay (ELISA) systems following the manufacturers' protocols: IL-1β, IL-6 and IL-8 and TNF-α (Boster Biological Technology, Pleasanton, CA). The absorbance was read by a plate reader (Synergy HT, Bio-tek, Winooski, VT, USA) at a wavelength of 450 nm.

2.5 Psychological evaluation

2.5.1 The Self Administered Psychiatric Scales for Children and Adolescents (SAFA) test

The SAFA battery is a self-administration diagnostic test. Its strength lies in ensuring a coordinated set of scales that explore a wide range of symptoms and psychiatric states in the area of internal disturbances. The sentences of the articles that formulate the SAFA are adapted to the age of the subject and for the younger ones the number of articles is slightly reduced. This enhances the reliability of the tool in relation to age. In addition, the diagnostic criteria are compatible with the DSM-5.

The entire battery, that lasts between 30 and 60 minutes, includes a total of six scales (each with subscales) that can also be used separately. It evaluates anxiety-related areas (SAFA A), depression-related areas (SAFA D), obsessive–compulsive symptoms (SAFA O), somatic concerns (SAFA S), psychogenic ED (SAFA P) and phobias (SAFA F). These scales have been described elsewhere [22-24]. Based on the obtained scores, it is possible to build a general profile and/or individual profiles within the single scales. The test is organized in order to adapt itself to the comprehension level and the assessment capacities of all age groups. Hence, each scale consists of a version for subjects from 8 to 10 years of age (identified by the letter 'e') and a different version for subjects from 11 to 18 years of age (identified by 'm/s').

2.5.2 The Eating Disorders Inventory II (EDI-2) questionnaire

The self-report Eating Disorder Inventory (EDI-2), is a self-assessment questionnaire consisting of 91 items, divided into 8 subscales, for clinical evaluation of the cognitive or behavioral aspects associated with eating disorders. It can be used as a screening tool in non-clinical populations. The answers are provided by the person on a 6-point Likert scale (from 1 = never to 6 = always), with higher scores indicating the presence of dysfunctional eating behaviors. The Italian version of EDI-2 [25] showed a good internal consistency when administered in clinical populations (Cronbach's α from 0.78 to 0.84 for the individual subscales), while in non-clinical ones the reliability was moderate (Cronbach's α from 0.38 to 0.88). Some subscales concern the impulse to thinness, bulimia and dissatisfaction with the body. An overall measure of concern for weight and diet can be obtained from the sum of these three subscales [26]. Others are related to inadequacy, perfectionism, interpersonal distrust, interoceptive awareness and fear of maturity. The last 3 subscales have been validated more recently and concern asceticism, impulsiveness and social insecurity [27].

2.6 Statistical Analysis

Statistical analysis was carried out using the SAS statistical package (Statistical Analysis System Institute, Cary, NC, USA). All results are expressed as means ± standard deviation. The Kolmogorov-Smirnov test was used to determine whether the data were random samples from a normal distribution. Dependent T-Test for Paired Samples was used to analyze the differences in the values at recruitment (t0) and after 6 months of integrated therapy (t1). The Unpaired T-Test was used for the comparison of TG and CG at t0, as well as for the comparison between TG and CG at t1. Differences with $p < 0.05$ were considered significant.

3. Results

A total of 30 AN patients were initially recruited, 3 were excluded because they refused to take psychiatric drugs, 3 patients rejected to participate in the study and 8 dropped out of therapy (refusing to participate in sessions with the psychologist or continuing treatment in another hospital). Sixteen subjects were enrolled for the study along with 22 healthy female controls.

The clinical characteristics of TG and CG are summarized in Table 1. After 6 months of integrated therapy, in each of the 16 patients a statistically significant increase in both BMI and blood glucose was noted, while plasma lipid and protein parameters showed no significant differences (Table 1).

3.1. Pro-inflammatory cytokine levels

In all TG there was an overall significant decrease in the pro-inflammatory cytokine levels between baseline (time at enrollment) and after 6 months (t_1). Data were compared to CG values.

Going into detail, significant differences between the TG at t0 and the CG was found in IL-1b ($p<0.0001$), IL-6 ($p<0.0001$), IL-8 ($p<0.0001$) and TNF-a levels ($p<0.0001$) (Figures 1 A-D). As expected, cytokine levels were higher in TG than in CG.

After 6 months of integrated therapy (t1) all cytokine levels significantly decreased with respect to t0 in TG but they were no longer significantly different from CG. It is interesting to note that in TG, from t0 to t1, IL-1β levels decreased by 60% (being 10.23±1.87 pg/mL at t0 and 4.06±0.44 pg/mL at t1, $p<0.0001$) (Figure 1-A). Likewise, both IL-6 and IL-8 levels diminished by 80% (being respectively 9.48±3.18 pg/mL at t0 and 1.89±0.99 pg/mL at t_1 , $p<0.0001$; and 108.73±86.21 pg/mL at t0 and 22.02±21.05 pg/mL at t_1 , $p<0.001$) (Figure 1 B-C). A similar trend was also found for TNF-α which was reduced by 88% (being 55.17±53.25 pg/mL at t0 and 6.52±2.32 pg/mL at t1; $p<0.0001$) (Figure 1-D).

Remarkably, at the end of the 6 months study period, all observed decreases fell within the CG levels.

3.3. Psychological evaluation

Psychological evaluation was carried out using the SAFA and EDI-2 tests at t0 and after 6 months (t1). At t0 anxiety and depression levels obtained high scores (Table 2) and for this reason psychopharmacological therapy was required. At t1 an improvement in the anxiety-depressant aspects was noted (Table 2), but not the reduction of psychopharmacological therapy. In particular, the scales that reached the statistical significant difference, between t0 and t1, were those related to “School-related Anxiety ($p<0.018$), “Depressed Mood” ($p<0.030$), “Anhedony and Indifference” ($p<0.033$), “Sense of Inadequacy and Low Self Esteem” ($p<0.026$)

and “Insecurity” ($p<0.030$). Considering the other scales, even though they did not show statistically significant differences between t0 and t1, a downward trend was however noticed.

4. Discussion

The aim of the present study was to investigate the role played by inflammation in the pathogenesis of AN in order to define whether cytokines, which are signaling molecules particularly important to the immune system, could be considered a pathophysiological marker of the disorder.

So far, several studies and meta-analysis suggest that pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, are elevated in people with AN compared to healthy controls, and this state is more pronounced in the early stages [14,18,28,29]. Nevertheless, to our knowledge, the studies exploring longitudinal changes in these pro-inflammatory cytokines in patients receiving specialist management for AN are limited, with controversial results.

In the present study after 6 months of specialized ED treatment for AN (i.e. psychopharmacotherapy, psychotherapy and nutritional treatment), a significant decrease in all studied cytokines (IL-1β, IL-6, IL-8 and TNF-α) was found in the whole group of patients, with a concomitant and significant increase in BMI and blood glucose levels. At the end of the therapy, the cytokine levels in AN patients were similar to those measured in healthy controls.

In agreement with our results, some authors have reported a reduction in pro-inflammatory cytokines after refeeding, with associated weight gain [15, 30]. In addition, Corcos et al. showed an increase in pro-inflammatory cytokine concentrations during AN, in particular TNF-α and IL-6 [31]. These data are also confirmed by another meta-analysis, in which TNF-α, IL-1β and IL-6 are higher in AN patients, but, unlike our study, the concentration of these cytokines did not change with weight gain [29].

As described by Allende et al., the immune system of patients with AN is characterized by high instability, resulting from severe chronic malnutrition. The dysregulation of the immune system would seem to begin with the increase in IL-1β and TNF-α, which results in impaired balance and stimulation of T cells and consequently in changes in terms of activation and function of B cells, as found in our study. Following nutritional

treatment, the parameters of the immune system are regularized and the immunodeficiency present in selected cases is reversible [15], completely reversible according to our results. In another older study it was shown that nutritional treatment and dietary rehabilitation in individuals with AN led to a significant normalization of the secretion of pro-inflammatory cytokines such as IL-6 and TNF-α [32]. Conversely, other researchers did not find any longitudinal changes in pro-inflammatory cytokines, after BMI increase and/or psychological improvement [33,34].

It is well documented in the literature that a situation of profound stress, typical of AN, can induce the release and increase in the production of pro-inflammatory cytokines [35]. Numerous studies, on animal models, have demonstrated that acute and chronic stress can cause an overproduction of such mediators [36], whilst in humans, psychological stress plays an important role in determining the increase of cytokines, more evident in pathologies such as anxiety and depression, which are highly comorbid with AN [37].

As reported in the present study, anxiety and depression levels had high scores at t0 making it necessary to initiate a psychopharmacological therapy. Hence, the significantly high levels of pro-inflammatory cytokines might be justified by the high levels of anxiety and depression, as described by other authors [38], but not present in the present paper. Numerous meta-analyses have highlighted that, in the case of depressive and cognitive symptoms associated with AN, the concentrations of IL-6 and TNFα are particularly elevated [14, 39-42]. Therefore, the inflammatory state present during AN cannot be considered a pathognomonic alteration of the pathology [43], since the presence of elevated levels of cytokines in many psychiatric and inflammatory pathologies does not allow them to assume the role of biomarkers of AN. On the contrary, it would seem more appropriate to consider them as a non-specific marker that correlates very well with the severity of the disorder and the response to treatment. At t1 an improvement in the anxiety-depressant aspects was observed, which might justify, at least in part, the decrease in pro-inflammatory cytokines, even though no correlations were found between these two parameters, and also did not warrant the reduction of psychopharmacological therapy. Many reports have shown that some pro-inflammatory cytokines (i.e. IL-1 and TNF-α) are also able to interact with different hormones and neurotransmitters involved in appetite regulation, in particular ghrelin, leptin, serotonin and dopamine, even though these relations are not fully understood [44–47]. According to

these studies, it might be conceivable to find a change in cytokine levels after weight gain and/or improvements in the psychological symptoms in AN patients, correlations that were not found in the present work.

Although chronic stress and the frequent depressive states during AN have also been shown to have a significant impact on the immune system, many authors claim that biologic drugs can be valid in the development of a new strategy for the treatment of patients with AN [48]. A relevant compound able to modulate the immune system and important in mood disorders (i.e. anxiety and depression) is Vitamin D [49,50]. Although vitamin D levels were not evaluated in the present study, people with AN show impaired bone quality, and increased fracture risk, as well as low levels of this vitamin [51].

Since cytokines play a central role in the immune response, it is useful to understand how the AN immune system is modified compared to healthy controls. In this pathology, the occurring metabolic changes are able to cause serious biochemical disorders, with consequent dysfunction in organs and systems, including the immune system, which is in close correlation with the neuroendocrine system [11]. Marked endocrine changes take place in AN patients, especially those affecting the sex hormones, the hypothalamic-pituitary-adrenal (HPA) system and various neurotransmitters (in particular serotonin and dopamine), which in turn determine functional modifications of the immune system [52].

In this regard, Holden et al. [53], in their immunological model of AN, proposed inflammatory cytokines as the fundamental regulators of body metabolism and thus decisive factors in the etiopathogenesis of AN. Specifically, the relationship between weight loss and increase in TNF-α is highlighted, which in turn induces noradrenaline elevation and decreased serotonin levels. Another study demonstrated the correlation between BMI and pro-inflammatory disease cytokines, especially TNF-α, suggesting a significant association of these factors in the development and progression of AN [54]. Although cytokines play an important role in catabolism, in the control of infections and inflammatory reactions, it is conceivable that they could represent a key component in the development of immunodeficiency, especially in AN patients. However, despite the significant state of malnutrition, patients with AN are not subjected to a greater risk of infection, since it is very rare in this pathology [44] even though laboratory studies have shown neutrophil abnormalities, with a consequent decreased adhesion and a reduced bactericidal capacity [55].

The results of the present study should be considered in light of several limitations. First, the sample was relatively small, and this restricted the generalizability of the findings. Second, due to the modest size of the sample, we did not control for multiple testing, since our study was exploratory, and the cross-sectional approach limits speculation on the direction of causality. In addition, we did not test for covariates such as demographic, severity of illness, and number of comorbid diagnosis. Larger sample sizes could also allow correction for comorbidities. Third, the relatively small sample differences between patients with distinct subtypes of AN were not analyzed in the present study, even though examining these differences in future investigations would be of great importance.

5. Conclusions

In conclusion the results, taken together, are suggestive of an association between pro-inflammatory cytokines and AN symptomatology. By interacting with different hormones and neurotransmitters, they could interfere with the molecular mechanisms underlying eating behavior. Furthermore, as documented in the literature they are implicated in the development of psychiatric comorbidities associated with AN, in particular anxiety, depression, phobic ideation and obsessive behavior [10,31,56,57]. However, the ways in which the cytokines become protagonists of these processes are not yet completely understood and would be worthy of future study and investigation. Since the levels of pro-inflammatory cytokines are related to the severity of the disorder and the response to treatment, they could be considered non-specific markers in the evaluation of these aspects. In addition, given their implication in numerous inflammatory and psychiatric pathologies, they cannot play the role of pathognomonic markers of AN.

Therefore, investigations in a larger sample of patients, with distinct sub-types of AN, and taking into accounts covariates, are highly warranted, in order to confirm the present findings.

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Table 1. Characteristics of studied subjects.

	$TG_{(t0)}$ (n=16)	$TG_{(t1)}$ (n=16)	p-value
Age (years)	14.6±1.9	15.0±1.8	NS
Weight (kg)	36.8 ± 3.2	47.5 ± 4.0	< 0.001
BMI (Kg/m^2)	14.0±0.8	18.2±1.6	< 0.001
Fasting plasma glucose (mg/dL)	73.3±9.4	83.8±8.2	< 0.001
Fasting plasma cholesterol (mg/dL)	201.4±23.7	197.8±21.6	NS
HDL cholesterol (mg/dL)	54.6±15.3	56.1±11.6	NS
Fasting plasma triacyl-glycerols (mg/dL)	85.6±29.4	80.9±27.1	NS
Albumin (g/dL)	4.6±0.6	4.4±0.4	NS
Prealbumin (mg/dL)	26.3±7.4	30.0±6.2	NS

Data are expressed as Mean ± SD; NS= not significant

Table 2. Self-Administered Psychiatric Scales for Children and Adolescents (SAFA).

	TG $_{(t0)}$ (n=16)	TG $_{(t1)}$ (n=16)	95%CI	p-value
General Anxiety	65.31 ± 5.59	61.62 ± 5.20	-0.213; 7.588	NS
Social Anxiety	65.12 ± 6.80	61.94 ± 5.67	-1.341;7.716	NS
Separation-related Anxiety	60.00 ± 8.43	57.12 ± 7.27	-2.815;8.565	NS
School-related Anxiety	57.19 ± 5.70	52.25 ± 5.40	0.927;8.948	0.018
Depressed Mood	69.19 ± 5.75	64.81 ± 5.09	0.451;8.299	0.030
Anhedony and Indifference	63.38 ± 4.70	59.88 ± 4.10	0.313;6.687	0.033
Irritable Mood	61.50 ± 5.65	58.69 ± 5.12	-1.081;6.706	NS
Sense of Inadequacy and Low Self Esteem	67.00 ± 5.50	62.75 ± 4.71	0.548;7.952	0.026
Insecurity	62.88 ± 4.75	58.94 ± 4.99	0.417;7.457	0.030
Guilt	55.81 ± 8.68	52.56 ± 7.33	-2.561;9.061	NS
Hopelessness	58.69 ± 5.75	55.19 ± 5.02	-0.399;7.399	NS

Data are expressed as Mean ±SD; t0: Baseline; t1: 6 months after the integrated therapy.
NS= not significant.

Figure legends

Figure 1. Comparison of the mean serum levels of IL-1β (A), IL-6 (B), IL-8 (C), and TNF-α (D) between TG at t0 and t1, and CG. Data are expressed as Mean ± SD.