Increased naïve CD4+ and B lymphocyte subsets associated with body mass loss drive relative lymphocytosis in anorexia nervosa patients

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Abstract

Anorexia nervosa (AN) is an atypical form of malnutrition with peculiar changes in the immune system. We hypothesized that different lymphocyte subsets are differentially affected by malnutrition in AN and thus, our aim was to investigate the influence of body mass loss on the variability of lymphocyte subsets in AN patients. A group of 66 adolescent female patients, aged 12-17 years referred for their first episode of either AN or Feeding or Eating Disorders Not Elsewhere Classified (FED-NEC) were studied upon admission (46 AN-restricting subtype [ANR], 11 AN-binge/purging subtype [ANP] and 9 FED-NEC). Ninety healthy adolescents served as controls. White blood cells and lymphocyte subsets were analyzed via flow cytometry. Relationships with the BMI z-score were assessed in lineal models adjusted by diagnostic subtype and age. Leukocyte numbers were lower in AN patients than in controls, and relative lymphocytosis was observed in ANR. Lower CD8+, NK and memory CD8+ counts were found in eating disorder patients compared to controls. No differences were found for CD4+ counts or naïve and memory CD4+ subsets between the groups. Negative associations between lymphocyte percentage and the BMI z-score, as well as between the B cell counts, naïve CD4+ percentage and counts and the BMI z-score were found. In conclusion, increased naïve CD4+ and B lymphocyte subsets associated with body mass loss drive the relative lymphocytosis observed in anorexia nervosa patients, which reflects an adaptive mechanism to preserve the adaptive immune response.

Keywords: anorexia nervosa, body mass loss, lymphocyte subsets, relative lymphocytosis, white blood cells.

Abbreviations:

AN; Anorexia nervosa

ANP; Anorexia nervosa binge/purging subtype

ANR; Anorexia nervosa restricting subtype

BMI; Body mass index

BN; Bulimia nervosa

CD3+; T Mature cells

CD4+; Helper T cells

CD8+; Cytotoxic T cells

CD19+; B cells

CD3+(CD16+56+); Natural killer cells

CD45RA+; Naïve cells

CD45RO+; Memory cells

DSM; Diagnostic and Statistical Manual of Mental Disorders

ED; Eating disorders

FED-NEC; Feeding or Eating Disorders Not Elsewhere Classified

NK; Natural killer cells

WBC; White blood cells

1. INTRODUCTION

Eating disorders (ED) are mental disorders characterized by abnormal cognitions, attitudes and behaviors towards food and body weight and shape. This results in severe physical and psychological complications. Studies on hematological and immune variables have disclosed some features of an impaired immune system status, including an increased frequency of leucopoenia with relative lymphocytosis [1, 2], delayed hypersensitivity skin test response [1, 3], and an impaired cytokine production in response to a stimulus when compared with a control group [4]. Relative lymphocytosis refers to an increased percentage in peripheral lymphocytes among the white blood cells while absolute lymphocyte number is normal [5]. Regarding lymphocyte subsets in ED patients, a rather scarce number of studies have been published [1, 6-12], and the findings are not consistent. Some of these studies have important drawbacks derived from the heterogeneity of the participating patients, including issues like age [7, 8], duration of the disease [7], subtype of anorexia nervosa (AN) or ED categories [6, 7, 8, 9] and history of dieting [13].

According to the diagnostic criteria established in the DSM V [14], AN is a disorder characterized by a persistent restriction of energy intake leading to significantly low body weight, an intense fear of fatness, and a disturbed perception of body image; in bulimia nervosa (BN) the distinguishing feature is binge eating, accompanied with compensatory behaviors, and both syndromes differ in their effects on body weight and nutritional status. Besides, vomiting episodes can be considered to be one of the most common stress-inducing bulimic features that might have an influence on the immune system [15]. Feeding or Eating Disorders Not Elsewhere Classified (FED-NEC)

includes patients that do not meet full criteria for a specific ED but still present some of their clinical features, although less severe or frequent. In addition, BN has recently been described as a continuum of the disease in former patients with AN [16], and for this reason the mean age is superior compared to patients with AN. Within the AN disorder two subtypes have been defined: the restricting type and the binge-purging type, the last one presenting binge-eating and/or purging behavior (e.g. self-induced vomiting, misuse of laxatives, diuretics or enemas).

In AN, immune disturbances are most likely the result of nutritional deprivation, and in this sense, normalization with re-feeding and clinical recovery has been reported [9, 13]. The extent of the immune and metabolic changes depends on the duration and severity of the reduced food intake and the amount of weight lost [11, 12, 17]. As a result, different white blood cell types might be differentially affected by the degree of malnutrition. On the other hand recent research suggests that other factors different from weight and linked with alterations in hormones and neuropeptides involved in appetite and satiety control may play an important role in explaining some of the immune system abnormalities [18]. Considering all of the above, we hypothesize that different immune cells and specifically lymphocyte subsets are differentially affected by the malnutrition severity. Thus, the aim of this study was to assess the influence of body mass loss on the variability of lymphocyte subsets in ED patients .

2. METHODS AND MATERIALS

2.1 Participants

The sample size for this study was calculated taking lymphocyte percentage as the main outcome, assuming a mean ± standard deviation of 34.23±8.96 (%) in healthy female adolescents [1]. and considering a 12% mean change (4%) over the values in the population as relevant. For an unbalanced allocation ratio between control and patients groups (1.36), an estimated mean difference of 12% with equal variances, 80% statistical power and an alpha error of 0.05, the minimum number of measures to perform is 156, distributed 90 to 66 in control and patients groups, respectively.

Sixty six adolescent female patients diagnosed with eating disorders were recruited upon admission at the Eating Disorders Unit at Niño Jesús Children University Hospital (NJCUH) in Madrid, Spain, to participate in a comprehensive study of clinical, family and biological variables involved in prognosis of ED (ANABEL study). The following inclusion criteria were taken into account: 1) female gender, 2) age range between 12-17 years, 3) first episode of ED, 4) first admission for ED treatment [19, 20] 5) acceptance to participate in the study. One hundred and five patients were first screened for inclusion in the study during the years of 2010 to 2012; twenty six were excluded because they did not fulfill all inclusion criteria and thirteen patients rejected to participate in the study. The patients were classified as: AN-restricting subtype (N=46), AN-binge/purging subtype (N=11) or FED-NEC (N=9), according to the DSM V criteria [14]. The patients were initially diagnosed according to DSM IV-TR criteria [21] by clinical interview performed by clinical experts, but were later reclassified according to the DSM-V [14, 22] in order to be in line with current operational criteria. This fact allowed to reduce the number of patients with no otherwise specified category [23] from 17 to 9 patients. The control group consisted of 90 healthy adolescents, free of psychiatric or somatic disease as answered in auto-administered and parent's

questionnaires, with the same gender, age and socioeconomic status, who participated in the AFINOS epidemiological study [24]. No patient included in the study had had any vaccine administered within the six weeks prior to the day of blood sampling, nor had they been hospitalized for infections or other illnesses. Most of the patients at study enrollment had been recently admitted for in-hospital treatment (75.76 %), and a lower number were admitted to follow home care hospitalization (21.20 %), and out-patient treatment (3.03 %). Seventy one percent of in-patients were at or below 10th BMI for age percentile [25]. Participants and parents gave their full and informed consent once the purpose and nature of the study had been explained. The study was conducted according to the guidelines of the Declaration of Helsinki, the rules of Good Clinical Practice and the Spanish law 14/2007 on Biomedical Research. The study protocol was approved by the Ethics Committee of the NJCUH.

2.2 Anthropometric measurements

All patients and controls underwent anthropometrical assessment by standard procedures (without shoes and with underwear clothes) measured by a trained investigator. Patients' measurements were performed on their first day of admission or on the following day using a digital electronic weighing scale (Seca 780; 0.100 g precision) with an incorporated telescopic measuring rod (Seca 220), and the measurements were recorded to the nearest 0.5 cm.

From these data, body mass index (BMI) [weight (kg)/height^2 (m^2)] and Z-score of the BMI were calculated according to the Spanish growth reference charts [25].

2.3 Blood Analysis

Venous blood samples were obtained from patients and controls between 8-9 AM, after overnight fasting, the next day after enrollment, and they were collected in EDTA-K3E Vacutainer (BD Biosciences) tubes. WBC counts and differentials (percentages and absolute number of white cell types) were determined in patients and control subjects with an automated blood cell counter (ADVIA 120) and an automatic counter (H1 Technicon (Bayer)), respectively. Blood samples from patients were diluted 1:1 with Cytochex™ Reagent (Streck Laboratories, Omaha, NE, USA), a preservative that was used to expand the number of suitable days for blood processing. Subsequently, immune phenotyping analysis of the samples was performed within 7 days from collection. Blood aliquots were incubated for 30 minutes at room temperature in the dark with fluorochrome-conjugated monoclonal antibodies (BD Biosciences, San José, CA, USA) to differentially label those cells positive for the following surface markers: CD45 (pan-leukocyte marker), CD3 (T mature cells), CD4 (helper T cells), CD8 (cytotoxic T cells), CD19 (B cells), CD16+56 (natural killer cells), CD45RO (memory cells) and CD45RA (naïve cells) [26]. A quadruple immunostaining procedure was performed with the following combinations: CD3/CD8/CD45/CD4, CD45RA/CD45RO/CD8/CD3, CD45RA/CD45RO/CD4/CD3 and CD3/CD16^{+}56/CD45/CD19. The antibody in each position was marked with a fluorochrome as follows: FITC/PE/PerCP-Cy5.5/APC. After lysis of the red blood cells, the lymphocytes were analyzed by flow cytometry (FACScan Plus Dual Laser, Becton Dickinson Sunnyvale, CA). The percentage of each lymphocyte subset was obtained, and absolute cell counts were calculated from the total lymphocyte numbers. The $CD4^{+}/CD8^{+}$ ratio was also calculated.

2.4 Statistical analyses

Prior to statistical analyses, all data were examined for normal distributions with the Kolmogov-Smirnov test, as well as for homogeneity of variances with the Levene Test. When necessary, the normal distribution was obtained after logarithmic transformation of the variable. The differences between the whole group of ED patients (ED) and the control group, as well as the differences between each one of the different subtypes and the control group were assessed by Student's t-test. Significant differences between ED diagnostic subtypes were calculated using one-way analysis of variance (ANOVA).When the analysis indicated a significant difference, the Tukey method was used for a pair-wise comparison between the different diagnostic subtypes.

To assess the effect of the BMI Z-score on immuno-hematological variables, a basic lineal regression model was used and corrected by age. In a second model, the diagnostic subtype was also included as a potential influential factor. Differences were considered statistically significant at $P<0.05$. Statistical analysis was performed using the IBM SPSS statistical software version 22.

3. RESULTS

The anthropometric measurements tested are summarized in Table 1. BMI and weight were significantly lower in ED, ANR, ANP and FED-NEC than in the control group.

Among the different diagnostic groups, weight is significantly lower in ANR and ANP compared to FED-NEC, and BMI is lower in ANR than in the other two groups.

Regarding white blood cells, lower leukocyte counts were found in ED girls compared to healthy girls (p=0.019) (Table 2). Both the percentages and total neutrophil, monocyte and basophil cell numbers were significantly decreased in the ED group versus the control group. On the contrary, a significant increase was observed in the lymphocyte percentage, while lymphocyte counts were similar to those in the control group. Comparing the different diagnostic subtypes with the control group separately, a decreasing trend in leukocyte numbers was found in ANR (*P*=0.074) and ANP (P=0.091). The decrease in the monocyte and basophil percentages and counts was observed in all of the diagnostic subtypes, while the increase in the lymphocyte percentage was only significant in the ANR group (P=0.007). In addition, the neutrophil percentage and counts were lower both in ANR and ANP than in the controls, however, in ANP it did not reach statistical significance due to the small group size. The eosinophil population showed a higher percentage in FED-NEC and ANP (p=0.022 and p=0.007, respectively) versus controls, but no differences were found for the eosinophil counts. No relevant differences among ED groups were observed in the different white blood cell types, except for higher eosinophil percentage in ANP compared with ANR.

The assessment of the different lymphocyte subsets (Table 3) showed only a few significant differences among diagnostic groups, while important differences were observed in relation to the control group. The whole group of ED patients showed higher CD4+ and CD19+ lymphocyte percentages and lower CD8+ and NK percentages compared to the controls. In relation to the naïve and memory subsets percentage, ED

patients showed significantly lower CD8RO+ and higher CD8RA+ percentages than the controls. On the contrary, no significant differences in CD4RA+ and CD4RO+ percentages were found when compared with controls. When focusing on the different diagnostic subtypes, the formerly described results are also observed in the ANR group. Referring to ANP, the higher CD4+ lymphocyte percentage observed in relation to controls is even higher than the one observed in the other two subtypes.

Regarding lymphocyte subset counts, it is remarkable that no differences in CD4+ and naïve and memory CD4+ subset counts were observed between the ED subjects and controls. However, lower CD8+, NK and memory CD8+ counts were found with an increase in CD19+ cell numbers in relation to the control group. By diagnostic subtypes, CD8+ cell counts were lower only in the FED-NEC, and CD19+ were higher only in ANR. The changes in CD4+ and CD8+ subsets in ED led to a significantly higher CD4:CD8 ratio in the patients (and in all the different subtypes) than in the control group. ANP and ANR showed higher naïve CD8+ percentage and significantly lower memory CD8+ percentage and counts than controls. Oppositely, no differences were found between these two groups and neither against the control group for naïve and memory CD4+. On the contrary the FED-NEC group showed lower naïve CD4 percentage than controls and a trend towards lower naïve CD8 counts compared to the controls and the other ED subtypes.

The results of the general lineal model assessing the effect of age, BMI Z-score and diagnostic subtype on leukocyte profile and lymphocyte subsets (Tables 4 and 5) showed a BMI Z-score influence in lymphocyte counts and percentage, which is diagnostic subtype independent. The BMI Z-score in the basic model showed a

significantly positive relationship with the CD3+CD4+CD45RO+ and CD45+CD3(CD56+16)+ percentages and a negative relationship with CD3+, CD19+ counts, as well as with CD4+CD45RA+ percentage and counts. No influence of the diagnostic subtype was found when this was included in the model.

4. DISCUSSION

The present study explored the alterations in white blood cells and in the lymphocyte subset profile of AN patients of either the restrictive or binge-purging subtype, as well as in a group of patients with FED-NEC, all in their first episode of the disorder and with a short period of evolution. In agreement with our hypothesis, lymphocyte cell variations were differentially influenced by body mass loss in AN patients. Among all lymphocyte subsets analyzed, the associations with the BMI Z-score were significant for the naïve CD4+ and the B lymphocyte numbers, which seems to reflect an adaptive mechanism to preserve the adaptive immune response.

Our results are in agreement with all previous reports describing leucopoenia with relative lymphocytosis as the most frequent immune abnormality in AN [1, 8,], as well as low neutrophil, monocyte and basophil counts in relation to the control group [27-30]. In our study relative lymphocytosis was not found in the FED-NEC or ANP groups.

The decrease in the percentage and absolute number of CD8+ T cells observed in the patients, seem to be explained by the significant decrease in memory CD8+ T cells. Similar findings for CD8+ cells have been previously described in AN patients by several authors [1, 7, 8, 13]; and particularly Mustafa et al. [7] also reported a

diminished population of memory CD8+ cells. A decrease in the capacity of dendritic cells to expand memory T cell clones might explain this finding, according to the results that have also been observed in starvation animal models [31]. In contrast with the CD8+ decrease and as a main finding in the current study, an increase in CD4+ percentage was found in our patients, which could be considered as a successful attempt to preserve CD4+ absolute numbers under restricted intakes and reduced leukocyte numbers. Thus, CD4 + lymphocyte cell levels maintenance could be understood as a fundamental issue within the adaptation process to low intakes, and also a key factor explaining why patients do not suffer an increased infection risk as classical forms of protein-energy malnutrition. The increased CD4+ percentage has been also described in other studies with AN patients [7, 13, 32]. Similarly, the increased B cell counts in our patients are consistent with the generally agreed concept that humoral immunity is well preserved in these patients [2, 33]. This increase was only observed in the ANR group and seems to be linked to the body mass loss as well as the relative lymphocytosis adaptive mechanism, since both lymphocytes and B lymphocytes are negatively associated with the BMI z-score. Similarly, the negative relationship found in our patients between the BMI Z-score and CD4+CD45RA+ cells, both percentage and counts, suggests that as the body weight is depleted, the production of lymphocytes is prioritized over that of other immune cells and, in the case of T lymphocytes, the priority is laid on naïve T helper cells. This might be an attempt to preserve the efficacy of the adaptive immune response under the food deprivation status. It might also be related with the finding of Omodei et al. [27] who observed that the PBMCs from AN patients showed lower activity of its major energy-producing pathways than those from the controls. It might be worth investigating whether the priority in lymphocyte production aims to compensate for a decreased metabolic activity.

We further tested the occurrence of this adaptation mechanism in the most undernourished girls by comparing those girls with <-2SD Z-score BMI (n=13) values with those girls that showed the less undernourished status (Z-score BMI >-1SD; n=12). This analysis showed that the most undernourished girls had significantly higher lymphocyte counts and CD4+CD45RA+ counts than the less undernourished girls (2438±641 vs. 1928±498 and 527±286 vs. 338±168, respectively), and also a trends for a higher percentage of CD4+CD45RA+ (P=0.065).

ANP and ANR showed similar adaptive mechanisms regarding lymphocyte subsets in the context of reduced leukocytes. As in ANR, only NK cells and memory cytotoxic T cells were decreased in ANP; however, the low CD3+CD8+CD45RO+ counts were more significant in ANP than in ANR. Likewise, Nagata et al. [13] found significantly decreased CD8+ counts in 6 patients with ANP compared to controls and a similar non-significant trend in 7 patients with restricting-type AN. The effect of vomiting on lymphocyte subsets has also been analyzed, showing that vomiting as a purging strategy is associated with a more deleterious effect on T cells [15]. The acquisition of purging-compensatory habits might be working as a strain on the already restrained body [33].

FED-NEC patients' NK cells were less significantly diminished than those of both AN subtypes. Malnutrition severity as well as the time of evolution of the disease could be important factors explaining the variability observed since FED-NEC are, in our sample, patients with initial disorders that have relatively shorter periods of evolution than the other groups, however, no conclusions can be derived from this small and

heterogeneous group. Similarly, the low number of patients with ANP compared to ANR might be considered as another limitation in this study; however, this was a convenience sample and reflects the disparity in both subtypes incidence at first episode. Despite this, the results showing a priority in the production of naïve T cells was common to both ED subtypes, and the question arises whether this mechanism aimed to preserve the adaptive immune response is maintained in those patients with longer duration of disease and unsuccessful treatments. Future works with follow-up data from immune assessments performed in this study population will hopefully solve this limitation and will add information on the usefulness of these immunological parameters as biomarkers of disease evolution.

No influence of the BMI Z-score was observed on the NK cell subset in our study, nor was it observed in eosinophil percentage. However, the eosinophil percentage seemed to be influenced by the diagnostic subtype. ANP showed higher values than ANR, but it is not clear if this can be explained exclusively by the prevalence of allergy by groups, which was 33%, 18% and 17% in FED-NEC, ANP and ANR subtypes, respectively. It is important to note the endocrine changes known to occur in patients with AN [34, 35] because pituitary-adrenal-cell mediated immunity interrelationships seem to be disrupted [36]. Furthermore, some issues that frequently co-exist with AN, such as depression or stressful life events [37-40], are themselves known to adversely impact on immune function, and it is difficult to separate their effects. The impact of these processes, or others, derived from the physiological adjustment secondary to ED is likely to confuse the measurement of immune abnormalities.

In conclusion, our study of patients suffering their first episode of AN or FED-NEC showed that relative lymphocytosis results from an enhanced production of naïve CD4+ T cells and B cells as body mass loss progresses. This seems to be an adaptation mechanism to maintain lymphocyte numbers, and likely, the adaptive immune response.

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Table 1. Anthropometric parameters in controls and patients with eating disorders.

	CONTROL	ED	FEDNEC	ANP	ANR	
	n=90	n=66	n=9	n=11	n=46	P value[#]
Age (years)	14.86±1.17	14.62±1.38	14.44±1.24	15.27±1.42	14.50±1.38	0.230
Weight (kg)	56.98±8.97	42.08±6.45***	49.92±5.65*[a]	43.92±3.80*** [b]	40.11±5.84***[b]	0.000
Height (m)	1.63±0.06	1.61±0.07°	1.61±0.05	1.63±0.04	1.61±0.08°	0.850
BMI (kg/m^2)	21.29±2.99	16.05±1.84***	19.12±1.23* [a]	16.45±0.75*** [b]	15.36±1.45*** [c]	0.000
Duration of disease (months)		10.97±8.16	7.11±3.41	15.18±8.85	10.72±8.32	0.110

Values are expressed as means±SD

BMI, body mass index; ED: eating disorder patients; FED-NEC: Feeding or eating disorders not elsewhere classified; ANP: AN purging subtype; ANR: AN restricting subtype.

Asterisks indicate differences versus control; Student´s t test. *P<0.05, **P<0.01, ***P<0.001, °P<0.1

[#]ANOVA among ED groups.

Different letters indicate significant differences between ED groups. Tukey test; $P<0.05$.

Table 2. White blood cell counts and differential in adolescents with AN and FEDNEC.

	CONTROL n=90	ED n=66	FED-NEC n=9	ANP n=11	ANR n=46	P value[#]
Leukocytes (cel./µL)	6612±1736	5962±1612*	5841±1590	5673±1602°	6055±1643°	0.78
Lymphocytes (%)	35.6±9.1	38.9±9.5*	35.5±12.6	36.7±11.1	40.0±8.3**	0.153
Lymphocytes (cel./µL)	2300±617	2260±636	1950±494	2049±771	2370±606	0.011
Neutrophils (%)	53.7±9.6	50.5±10.2*	54.4±14.2	49.4±12.8	50.1±8.6*	0.156
Neutrophils (cel./µL)	3624±1406	3094±1305*	3264±1740	2869±1484°	3115±1186*	0.871
Monocytes (%)	7.0±1.5	4.4±1.5***[a, b]	4.6±1.5***[a, b]	5.3±2.7**[a]	4.1±1.0***[b]	0.092
Monocytes (cel./µL)	461±151	259±100***	272±126***	281±88***	251±99***	0.424
Basophils (%)	1.4±0.5	0.9 ±0.4***	0.8±0.4**	1.0±0.5*	0.9±0.4***	0.466
Basophils (cel./µL)	94±41	53±27***	43±17***	55±31**	55±28***	0.297
Eosinophils (%)	2.2±1.1	2.7±2.1	3.4±2.1*[a,b]	3.6±2.2**[a]	2.4±2.1[b]	0.039
Eosinophils (cel./µL)	150±84	159±115	188±100	196±111	144±118	0.358

Values are expressed as means ±SD

ED: eating disorder patients; FED-NEC: feeding or eating disorders not elsewhere classified; ANP: AN purging subtype; ANR: AN restricting subtype.

Asterisks indicate differences versus control; Student´s t test: *P<0.05, **P<0.01, ***P<0.001, °P<0.1

[#]ANOVA among ED groups adjusted by age.

Different letters indicate significant differences between ED groups. Tukey test; P<0.05

Table 3. Lymphocyte subsets in adolescents with anorexia nervosa and FED-NEC.

		CONTROL n=90	ED n=66	FED-NEC n=9	ANP n=11	ANR n=46	P value[#]
CD45+CD3+ Mature T lymphocytes	%	70.2±6.5	69.4±5.9	65.0±5.8*[a]	74.3±4.7*[b]	69.1±5.4 [a]	0.003
	cel./µL	1613±455	1567±464	1275±374*	1527±609	1634±427	0.023
CD45+CD4+ T-Helper lymphocytes	%	37.8±6.7	41.6±6.3***	40.4±4.5 [a]	46.6±4.8***[b]	40.6±6.5*[a]	0.018
	cel./µL	872±287	933±305	792±245	960±392	955±291°	0.182
CD45+CD8+ T-Cytotoxic lymphocytes	%	26.9±5.7	23.6±4.9***	23.2±5.7°	25.2±4.7	23.4±4.8***	0.676
	cel./µL	619±218	530±184**	452±149*	508±188	551±189°	0.137
CD45+CD3(CD56+16)+ NK cells	%	16.4±6.6	10.4±4.5***	14.6±6.0 [a]	9±5.6***[b]	9.9±3.4***[b]	0.022
	cel./µL	382±193	230±106***	279±134	172±108**	234±96***	0.141
CD45+CD19+ B-lymphocytes	%	10.9±2.8	12.7±4.4**	11.8±4.0	11.0±3.1	13.3±4.6**	0.168
	cel./µL	249±88	293±149*	232±114	236±165	319±147**	0.007
CD3+CD45RO+ Memory T-lymphocytes	%	49.6±9.9	42.9±11.5***	47.6±15.4	40.5±11.2**	42.6±10.6***	0.526
	cel./µL	788±249	656±220*	586±206*	609±248*	683±216*	0.138

		CONTROL n=90	ED n=66	FED-NEC n=9	ANP n=11	ANR n=46	P value[#]
CD4+CD45RA+ Naïve T-helper lymph.	%	48.3±10.4	47.0±12.0	39.6±12.1*	46.3±13.3	48.6±11.4	0.152
	cel./µL	428±181	453±228	331±192	455±240	478±230	0.127
CD3+CD4+CD45RO+ Memory T-helper lymph.	%	26.4±5.0	26.3±7.6	28.8 ±8.7	29.0 ±8.2	25.1 ±7.1	0.116
	cel./µL	420±141	398±133	354±103	443±204	396±116	0.859
CD8+CD45RA+ Naïve T-cytotoxic lymph.	%	58.1±13.2	64.0±12.0**	57.1±18.6 [a]	72.2±8.8**[b]	63.7±11.5*[a,b]	0.117
	cel./µL	360±152	341±134	262±132°	364±132	351±134	0.121
CD3+CD8+CD45RO+ Memory T-cytotoxic lymph.	%	14.5±5.7	11.0±5.0***	12.9±7.2	8.4±3.4**	11.3±4.7**	0.396
	cel./µL	234±118	169±91***	158±97°	127±60***	182±94**	0.127
Ratio CD4+/CD8+		1.49±0.48	1.84±0.52***	1.85±0.55*	1.92±0.47**	1.82±0.54***	0.934

Values are expressed as means ±SD

ED: eating disorder patients; FED-NEC: feeding or eating disorders not elsewhere classified; ANP: AN purging subtype; ANR: AN restricting subtype.

Asterisks indicate differences versus control; Student´s t test. *P≤0.05, **P≤0.01, ***P≤0.001, °P≤0.1

[#]ANOVA among ED groups adjusted by age.

Different letters indicate significant differences between ED groups. Tukey test; $P<0.05$.

Table 4. Associations between BMI Z-score and white blood cells in ED patients.

	Basic model[a]				Basic model + diagnostic subtype[b]		
	BMI-Z score			R^2	BMI-Z score	Diagnostic subtype	R^2
	β	95% CI	P		P	P	
Leukocytes (cel./µL)	-0.042	(-0.863,0.626)	0.752	0.013	0.996	0.880	0.017
Lymphocytes (%)	-0.311	(-9.244,-1.067)	0.014	0.138	0.039	0.815	0.143
Lymphocytes (cel./µL)	-0.329	(-0.631,-0.102)	0.007	0.201	0.081	0.891	0.204
Neutrophils (%)	0.213	(-0.699,8.274)	0.097	0.098	0.121	0.544	0.116
Neutrophils (cel./µL)	0.090	(-0.399,0.809)	0.500	0.009	0.459	0.707	0.020
Monocytes (%)	0.291	(0.008,0.119)	0.026	0.081	0.048	0.079	0.155
Monocytes (cel./µL)	0.192	(-0.012,0.080)	0.143	0.054	0.161	0.519	0.074
Basophils (%)	-0.100	(-0.255,0.114)	0.448	0.027	0.461	0.505	0.049
Basophils (cel./µL)	-0.143	(-0.019,0.006)	0.273	0.05	0.694	0.737	0.060
Eosinophils (%)	0.190	(-0.034,0.218)	0.150	0.045	0.972	0.067	0.126
Eosinophils (cel./µL)	0.135	(-0.026,0.080)	0.307	0.023	0.809	0.403	0.051

[a] Lineal regression adjusted by age.

[b] General lineal model including diagnostic subtype as independent factor and Z-score and age as covariates.

β, Standardized regression coefficient; CI, confidence interval

Table 5. Associations between BMI Z-score and lymphocyte subsets in ED patients.

		Basic model				Basic model + diagnostic subtype[b]		
		BMI-Z score			R^2	BMI-Z score	Diagnostic subtype	R^2
		β	95% CI	P		P	P	
CD45+CD3+	%	-0.200	(-4.692,0.601)	0.127	0.054	0.532	0.005	0.205
Mature T lymphocytes	cel./µL	-0.353	(-482.222,-91.465)	0.005	0.182	0.077	0.884	0.185
CD45+CD4+	%	0.010	(-2.779,3.001)	0.939	0.048	0.894	0.033	0.151
T-Helper lymphocytes	cel./µL	-0.250	(-270.159,2.236)	0.054	0.092	0.189	0.702	0.102
CD45+CD8+	%	0.042	(-1.901,2.619)	0.752	0.011	0.504	0.607	0.028
T-Cytotoxic lymphocytes	cel./µL	-0.221	(-154.180,10.493)	0.086	0.096	0.369	0.928	0.098
CD45+CD3(CD56+16)+	%	0.316	(0.500,4.450)	0.015	0.097	0.420	0.053	0.142
NK cells	cel./µL	0.058	(-38.323,60.070)	0.660	0.014	0.753	0.103	0.085
CD45+CD19+	%	-0.118	(-2.873,1.068)	0.363	0.050	0.938	0.449	0.078
B-lymphocytes	cel./µL	-0.267	(-32.98,-7.325)	0.029	0.188	0.291	0.749	0.196
CD3+CD45RO+	%	0.228	(-0.758,9.775)	0.092	0.047	0.199	0.606	0.063
Memory T-lymphocytes	cel./µL	-0.147	(-155.503,43.536)	0.265	0.079	0.815	0.756	0.088

CD4+CD45RA+	%	-0.279	(-11.228,-0.515)	0.032	0.085	0.352	0.739	0.094
Naïve T-helper lymph.	cel./µL	-0.296	(-219.600,-18.221)	0.021	0.117	0.134	0.839	0.122
CD3+CD4+CD45RO+	%	0.283	(0.374,7.108)	0.030	0.115	0.130	0.697	0.126
Memory T-helper lymph.	cel./µL	-0.060	(-76.307,48,407)	0.656	0.004	0.564	0.572	0.021
CD8+CD45RA+	%	-0.114	(-8.461,3.390)	0.395	0.012	0.802	0.026	0.126
Naïve T-cytotoxic lymph.	cel./µL	-0.235	(-116.232,5.403)	0.073	0.076	0.427	0.443	0.101
CD3+CD8+CD45RO+	%	0.063	(-1.818,2.914)	0.645	0.006	0.621	0.153	0.066
Memory T-cytotoxic lymph.	cel./µL	-0.167	(-67.293,14.946)	0.208	0.075	0.419	0.442	0.100

[a] Lineal regression adjusted by age.

[b] General lineal model including diagnostic subtype as an independent factor and Z-score and age as covariates

β, Standardized regression coefficient; CI, confidence interval