**Thesis**

# Oxidative stress and Anorexia nervosa


submitted by

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Graz, 20.01.2023

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 20.01.2023 Signature m.p. Fiona Haidacher

## Acknowledgement

When I initially sent an inquiry to the Department of Psychiatry of the Universitätsklinikum Graz, I did not know what subject I would be assigned with. When I heard that I could join the team of the ILSD pilot study that focuses on various aspects of eating disorders, I was happy to join as this topic had been interesting me for a while. Therefore, being a part of the research team has a deep personal meaning to me and I am still excited to have been able to contribute to the generation of new scientific findings in this field.

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## List of Abbreviations

| | |
|---|---|
| AN | Anorexia nervosa |
| ApoB | Apolipoprotein B |
| ATP | Adenosine Triphosphate |
| BDI | Beck Depression Inventory |
| BED | Binge-Eating Disorder |
| BMI | Body Mass Index |
| CAT | Catalase |
| CBT | Cognitive-Behavioral Therapy |
| CNS | Central Nervous System |
| CR | Caloric Restriction |
| DIT | Dietary-Induced Thermogenesis |
| DNA | Deoxyribonucleic Acid |
| DSM | Diagnostic and Statistical Manual of Mental Disorders |
| EAT | Eating Attitudes Test |
| EAT-26 | Eating Attitudes Test-26 |
| EDEQ | Eating Disorder Examination Questionnaire |
| EDTA | Ethylenediaminetetraacetic Acid |
| ENS | Enteric Nervous System |
| EPA | Endogenous Peroxidase Activity |
| GI | Gastrointestinal |
| GPX | Glutathione Peroxidase |
| HA | Hypothalamic Amenorrhea |
| HAMD | Hamilton Depression Rating Scale |
| HC | Healthy controls |
| HPA | Hypothalamic-Pituitary-Gonadal Axis |
| ICD-10 | International Classification of Diseases, tenth revision |
| IL-6 | Interleukin-6 |
| iNOS | inducible NO-Synthase |
| LDL | Low-Density Lipoprotein |
| MANTRA | Maudsley Anorexia Nervosa Treatment for Adults |
| MAPK | Mitogen-Activated Protein Kinase |
| MDS | Mediterranean Diet Score |

| | |
|---|---|
| MMSE | Mini Mental State Examination |
| NCBI | National Center for Biotechnology Information |
| NFkB | Nuclear Factor kappa B |
| NO | Nitric Oxide |
| OCD | Obsessive-Compulsive Disorder |
| oLAb | Autoantibodies against oxidized Low-Density Lipoproteins |
| oxLDL | Oxidized LDL |
| PPm | Polyphenols microtitre |
| REE | Resting Energy Expenditure |
| RNS | Reactive Nitrogen Species |
| ROS | Reactive Oxygen Species |
| SAT | Subcutaneous Adipose Tissue |
| SOD | Superoxide Dismutase |
| SSCM | Specialist Supportive Clinical Management |
| SSRI | Serotonin Reuptake Inhibitor |
| TAC | Total Antioxidative Capacity |
| TCA | Tricyclic Antidepressant |
| TDEE | Total Daily Energy Expenditure |
| TMB | 3,5,3',5'-Tetramethybenzidine |
| TOC | Total Oxidative Capacity |
| WHO | World Health Organization |

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# Abstract in german

**Einleitung**

Der Begriff „Oxidativer Stress“ beschreibt eine Dysbalance zwischen reaktiven Sauerstoffspezies und den körpereigenen anti-oxidativen Schutzsystemen. Die Auswirkungen von oxidativem Stress waren in den letzten Jahren Gegenstand intensiver Forschung und wurden mit der Entstehung verschiedener somatischer, wie auch psychiatrischer Krankheiten in Verbindung gebracht. Im Rahmen dieser Pilotstudie wurden mögliche Zusammenhänge verschiedener oxidativer Stress-Marker und der psychiatrischen Erkrankung Anorexia nervosa untersucht.

**Methoden**

Die Studie inkludierte 40 Teilnehmerinnen (n=20 Patientinnen mit Anorexia nervosa (AN), n=20 gesunde Probandinnen (HC)). Alle Teilnehmerinnen beantworteten Fragebögen bezüglich ihres Essverhaltens (EAT-26, Eating Disorder Examination Questionnaire (EDEQ) und Mediterranean Diet Score (MDS)). Zur Bestimmung oxidativer Stressparameter wurden Blutproben auf fünf verschiedene Marker für oxidativen Stress (Totale oxidative Kapazität (TOC), Totale anti-oxidative Kapazität (TAC), endogene Peroxidase (EPA), Antikörper gegen oxidiertes LDL (oLAb) und Polyphenole (PPm)) untersucht, die Ergebnisse wurden mittels SPSS ausgewertet.

**Ergebnisse**

AN-Patientinnen zeigten eine signifikante Erniedrigung der TAC ($t(34)= -2.181$, $p=.036$; AN: 1.47 (0.62); HC: 1.90 (0.56). Für die vier restlichen Parameter wurden keine signifikanten Unterschiede zwischen AN-Patientinnen und der Kontrollgruppe gefunden.

**Schlussfolgerung**

Anhand der Studienergebnisse konnte festgestellt werden, dass AN Patientinnen eine signifikant reduzierte TAC im Vergleich zu HC haben. Weitere Studien sowie Interventionsstudien sind notwendig, um die Frage zu beantworten, ob Antioxidantien zukünftig als add-on Therapie bei AN eingesetzt werden können.

# Abstract in english

### Introduction

The term oxidative stress refers to a disbalance of Reactive Oxygen Species (ROS) and the body's anti-oxidative defense systems. Recently, the consequences of oxidative stress have become a central field of research and have been found to be linked to the genesis of multiple somatic as well as psychiatric diseases. In this pilot study, different potential links between markers of oxidative stress and the psychiatric condition Anorexia nervosa (AN) have been investigated.

### Methods

The study included 40 participants (n=20 female patients with Anorexia nervosa (AN), n=20 healthy female participants (HC)). All participants completed multiple questionnaires regarding their eating habits (EAT-26, Eating Disorder Examination Questionnaire (EDEQ) and Mediterranean Diet Score (MDS)).To examine parameters of oxidative stress, blood tests were investigated for five different markers of oxidative stress (Total Oxidative Capacity (TOC), Total Antioxidative Capacity (TAC), Endogenous Peroxidase Activity (EPA), Autoantibodies against oxidized LDL (oLAb) and polyphenols (PPm)). Subsequently, the results were interpreted via SPSS.

### Results

Compared with HCs, AN patients showed a significant reduction in TAC (t(34)=-2.181, p=0.36; AN: 1.47 (0.62); HC: 1.90 (0.56), for the other four parameters no significant differences were found between AN patients and the control group.

### Conclusion

This study's results indicate a significant reduction of the TAC in patients with AN in comparison to HCs. Further investigations including interventional studies, will be necessary to determine whether antioxidants can be used as add-on therapy in the treatment of AN.

## Angaben von bereits erfolgten Veröffentlichungen

Wagner-Skacel et al., Oxidative Status in Adult Anorexia Nervosa Patients and Healthy Controls -- Results from a Cross-Sectional Pilot Study. Antioxidants. 2022;11(5):842.

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# 1 Introduction

This thesis is composed of four main chapters. The **introduction** acts as a lead-in to the topics Anorexia nervosa (AN), oxidative stress in the human body and the association between these two areas.

In the second chapter, the **methods** that were applied in the clinical research part of this thesis as well as all other factors surveyed are explained.

The **results** and findings are presented chapter three.
Significant findings are summarized and linked to previous studies in the **discussion**. In this chapter, this studies' limitations are pointed out and an outlook on future research is given.

## *1.1 Anorexia nervosa*

### 1.1.1 Definition

AN is a psychiatric disorder characterized by the pathological craving to reduce body weight, by, for example, massively restricting food intake. Additionally, patients often display disordered behavior patterns around food such as cutting the content of their meals into minute pieces, drinking large quantities of water, or following specific rules in their diet, like not consuming fat. A further symptom is body dysmorphia. Patients with AN often perceive themselves as overweight or obese when they look into the mirror. This applies even when they are already emaciated and/or categorized as underweight in the Body Mass Index (BMI) chart. Beyond Caloric Restriction (CR) vigorous exercise routines are a common technique for weight loss (1).

AN manifests itself in two different subtypes: the restrictive subtype and the binge-purge subtype. The restrictive subtype is characterized by the sole measure of massively reducing caloric intake to achieve weight loss. Patients of the binge-purge subtype show purging behaviors to achieve weight loss, such as extreme physical activity, laxative abuse, or self-induced vomiting (2).

### 1.1.2 Diagnosis

As per the International Classification of Diseases, tenth revision (ICD-10), a diagnosis of AN is made if the individuals' weight is at least 15% below their expected healthy weight according to their age and height. Further diagnostic criteria need to be met. Weight loss is induced intentionally by the patients, by, for example, avoiding specific foods and not exhibited due to other factors such as malignant diseases (1).

AN patients display body dysmorphic disorder. This means that they perceive themselves as overweight and are afraid of gaining weight, regardless of their current body weight. Usually, a very low weight is set as a personal ideal (1).

Another essential diagnostic criterium is endocrine dysfunction. More precisely, a disturbance in the Hypothalamic-Pituitary-Gonadal Axis (HPA). Females typically show Hypothalamic Amenorrhea (HA), whereas males typically show impotence and/or loss of interest in sexuality overall. It is important to note, that while some female patients still show menstrual bleedings, these are usually attributed to hormonal treatments such as contraceptive pills (1).

The diagnosis of AN was first established in 1888 by Sir William Gull. The Diagnostic and Statistical Manual of Mental Disorders (DSM) diagnostic criteria have then been continuously adapted over time, due to a deeper understanding of the symptomatology. Debates and findings that have led to an adaptation include the diversity of chronicity among adolescents and adults, or the denial of symptoms, as is found in some patients. The initial practice of relying on self-reported behaviors was superseded by an observational approach. DSM-5 now consists of three criteria. Firstly, there is no explicit bodyweight cut-off. However, patients must be visibly below their expected weight in regard to age, sex, their state of development as well as physical health. Secondly, fear of weight gain does not have to be self-reported but can also be diagnosed by observation of consistent behaviors that lead to loss or suppression of weight. The third criterion is body dysmorphia. HA has been removed as a diagnostic criterion (3). The changes of the diagnostic criteria as found in the DSM-5 in comparison with the DSM-IV can lead to significant differences, as shown in a study by Fisher et al. (4). While the analysis with DSM-IV would have led to 26.6% out of 309 people being diagnosed with AN, the analysis of the same test group with the updated guideline led to a number of 32.3% qualifying for the diagnosis of AN (4).

Another important addition to the DSM was the introduction of the term "atypical anorexia". It describes patients that show all symptoms of AN but do not display very low body weight at the point of diagnosis. This term does not imply a lower level of severity of atypical AN but rather aims at ensuring that patients with atypical symptoms are provided with adequate professional help (3).

### 1.1.3 Epidemiology

AN is typically a female disorder. Roughly, only ten percent of patients diagnosed with AN are male. In industrialized countries, the prevalence of psychogenic eating disorders including AN, Bulimia nervosa and Binge-Eating-Disorder (BED) in females is about 3,5%. AN is the least common type of these eating disorders, which, overall, are rarely observed in developing countries (1). Usually, the condition displays two age peaks at 14 years and at 18 years, respectively (5).

A 0.5% to two percent lifetime prevalence of AN has been reported. However, the overall prevalence is thought to be higher, by around three percent, because the change of the diagnostic criteria for eating disorders over the past years must be taken into account (3).

### 1.1.4 Symptoms

AN is associated with multiple complications, largely attributed to the hypometabolic state resulting from the typically observable energy imbalance, owed to the disequilibrium of intake and expenditure. The insufficient caloric intake leads to a number of pathophysiological processes (3).

General health deteriorations include reduced growth, hypothermia, reduced general condition, including nutritional condition and underweight. The listed symptoms are accompanied by problems with skin health, the urogenital system, the circulatory system, the digestive tract, the blood system, bone health, the Central Nervous System (CNS) and the neuroendocrine system. Detailed disturbances in the physiological body functions, as listed above, include dryness of the skin accompanied by hair loss, low status of subcutaneous fat and lanugo hair. Other common symptoms are peripheral edema, sinus bradycardia, arrhythmic tendencies during exercise and hypercholesterinemia(1). Furthermore, patients with AN often experience constipation, hypoglycemia and low levels in the hormone leptin. Leukopenia, osteoporosis, and reversible brain atrophy are a small excerpt of various severe consequences of malnutrition. In addition, the reproductive system is impacted negatively leading to infertility due to hormonal disruptions (1).

Gastrointestinal (GI) problems in patients with AN are caused by decelerated transit time in the GI-tract. This results in multiple symptoms such as diarrhea, the mentioned constipation, gastroparesis, and even liver disease (6).

Osteopenia and osteoporosis can be anticipated chronic complications. Due to the peak in bone growth in adolescence, even complete recovery may result in consistently reduced levels of bone density. Once attained, it is impossible to reverse the bone loss entirely and to make up for missing development of healthy bone mass later in life (3).

Mortality rates are around five to six percent in the initial years of the illness and may rise to 20% in cases of chronic condition (5).

### 1.1.5 Development

The development of an eating disorder is generally a multifactorial occurrence. A selection of the many factors that influence the development of AN will be described in the following paragraph, namely sociocultural, psychotraumatic and genetic factors (1).

Sociocultural influences include the socially determined beauty ideal in the contemporary western world: a skinny, athletic, and youthful body. Personality traits, among others also largely influenced by sociocultural factors, such as perfectionism, a lack of self-confidence and compulsive tendencies can lead to dieting, to conform to the beauty ideal and gaining perceived acceptance (1).
Furthermore, several career goals or hobbies prevalent among (potential) AN patients tend to be body-oriented, like professional ballet, acting, or modeling careers (1).

Another contributing factor to disordered eating behaviors can include psychological as well as physical traumatic experiences in early life such as sexual abuse, or negative experiences during first sexual relationships (1).

Genetics also plays a role: Multiple studies, e.g. by Strober et al. 2000 (7) and Strober et al. 2001 (8) have shown that eating disorders and other psychiatric disorders, such as depression, occur more often in first-degree relatives of people affected, than in relatives of healthy individuals.
The significance of genetical factors is further demonstrated by scrutinizing the prevalence of restrictive AN amongst identical twins, which shows that in 66% of cases both siblings are affected. Mothers of AN patients tend to show higher rates of Obsessive-Compulsive Disorders (OCD) (1). It is assumed that around 50 to 74% of the cases are correlated with genetic factors. The child of a pregnant woman with AN is also more likely to develop an eating disorder in the course of a lifetime (3).

Most patients struggle with their identity, show fear of maturing and are lacking healthy coping mechanisms. Often, the onset of AN happens simultaneously with major life transitions, experienced as difficult, like starting university, moving out of home, or experiencing abuse (3).

Additionally, the current diet culture can be seen as a risk factor for developing eating disorders. Dieting during adolescence can lead up to an eighteenfold risk of developing an eating disorder. The risk of disordered behavior changes is further promoted by exposure to western media (3).

### 1.1.6 Treatment

Most international guidelines list outpatient treatment as the first choice in adult patients (9).

Inpatient treatment is advised for more severe cases, where the first option does not prove to be sufficient; for example, in cases of very low absolute body weight, of a BMI under 15, or weight loss of more than 20% within six months. Another indication for inpatient treatment is the instability of a patient's health. The same applies to children and teenagers, with the difference that a slow transition from inpatient to outpatient treatment is advised (9). Outpatient treatment usually results in a weight gain of 0.45kg per week, inpatient settings typically in a gain of 1.36kg per week (3).

Psychotherapy is recommended in a vast number of international eating disorder treatment guidelines. As of yet, no specific type has been proven to be superior to others. Cognitive-Behavioral Therapy (CBT) is often employed, but other options such as Maudsley Anorexia Nervosa Treatment for Adults (MANTRA), or Specialist Supportive Clinical Management (SSCM) have also proven effective (9).

International guidelines do not always correspond to one another. In some countries, additional support, such as meal support and physical therapies, like yoga, are suggested. According to Resmark et al., 2019 (9) some international guidelines strongly emphasize on employment of an interdisciplinary approach.

With severely malnourished patients a cautious approach must be taken, to avoid refeeding syndrome. This complication is caused by the effect of electrolyte shifts, as a response to the sudden availability of energy in form of Adenosine Triphosphate (ATP). The consequences can be detrimental, including delirium, coma and even death. To prevent this state, a slow approach should be taken, with an initial caloric intake of around 1000-1400 calories daily. The caloric goals are then elevated by 100-200 calories a day until the desired intake for achieving weight gain is reached (3).

Irregularities in the Resting Energy Expenditure (REE) also have to be taken into account when calculating caloric needs. Individuals with AN show higher REE than would be expected when applying formulas that are used for healthy individuals to calculate energy expenditure. This increase in REE do not correlate with body mass, but it correlates with the level of caloric intake. Therefore individual caloric requirements should be calculated according to the patients' response to the refeeding protocol (10).

The primary focus in treatment should be a sufficient daily caloric intake to enable weight restoration to a healthy BMI. This does not only improve physical complications, but also reduces psychological side-effects like disordered thoughts that promote eating disorder behaviors. Setting a goal or target weight can prove difficult, as there is no universal consent on the ideal body weight (3).Nevertheless, the World Health Organization (WHO) defines a BMI of 18.5 to 24.9 kg/m$^2$ as the healthy weight range (11).

Opposed to the more general focus of sufficient caloric intake, as with adult patients, in children and growing adolescents, the goal in treatment is returning to one's personal growth curve, to allow for sufficient development to adulthood. Other goals that do not directly regard the bodyweight, can be the recovery of a regular menstrual cycle and the re-establishment of medical and nutritional stability (3).

### 1.1.7 Psychiatric comorbidities

AN is not only correlated with a number of somatic diseases, but also with multiple psychiatric comorbidities. These include OCD, avoidant personality disorder, self-induced harm, suicidal tendencies, anxiety disorder and depressive disorders (3). A 2007 study by Salbach-Andrae et al. (12) identified mood disorder in around 60% of AN patients. With 20% to 55%, anxiety disorders, like social phobia, are highly prevalent and OCD seems to present itself in 17% to 41% of the cases (3).

Both depression and anxiety symptoms can either be a result or a contributing factor to AN. Low concentration, low energy levels, low mood and irritability are listed as possible indicators for all three illnesses. Self-induced harm and suicidal thoughts tend to correlate with AN 27% of deaths have been attributed to suicide (3).

### 1.1.8 Metabolism in AN

Multiple metabolic disturbances have been described in individuals with AN. Amongst others, these include decreased body temperature and energy expenditure, low resting heart rate (bradycardia), lower levels of hormone production (e.g. thyroxine) and HA (1).

Weight gain is difficult to pursue for patients with AN. While this is often attributed to a patients' lack of compliance with treatment suggestions, it might partly stem from changes in metabolism. These changes may hinder weight restoration and slow down the process of weight gain in AN treatment (13).

In addition to the protective properties of the body's fat mass for organs and its role as storage of energy, it also works as endocrine tissue. Adipose tissue is responsible for the production of cytokines and the hormones leptin and adiponectin. Leptin production is proportional to fat mass, while adiponectin production shows an inverse correlation. Patients with AN show lowered levels of leptin and higher levels of adiponectin since the disease is connected to depleted body fat storage (14).

The body's Total Daily Energy Expenditure (TDEE) consists of three categories; Dietary-Induced Thermogenesis (DIT), REE and energy consumption through physical activity (15).

Changes in metabolic response to light stressors such as moderate exercise and glucose ingestion can be observed in AN. Patients exhibit increased DIT after glucose consumption and an increase in blood glucose levels, in response to low-intensity exercise. Raised DIT could be related to the need for tissue repair in underweight individuals. Particularly important for weight recovery is the sufficient conversion from carbohydrates into adipose tissue (14). Increased DIT, correlating in its magnitude with caloric intake, was measured in AN patients especially at the beginning of refeeding (16).

Acute cases of AN tend to show reduced levels of REE as means of protecting the body from further weight loss. This process is a part of metabolic adaptation (13).

Higher levels of non-exercise physical activity are largely present in AN (13). Every action, excluding eating, sleeping and exercise, that requires energy is attributed to this category (17). Additionally, pathologically high levels of physical exercise is self-reported by up to 80% of patients (18).These reports support a 2015 study (19) investigating physical activity in AN inpatients, AN outpatients, HC and a group with anxiety. AN patients reported 57-92% higher levels in total activity in comparison to the control group. However, these significant differences were not found in objective observation. Physical activity was divided into the subcategories walking, moderate and vigorous exercise. AN inpatients reported more walking, while outpatients reported more walking and more moderate exercise in comparison to HC (19). In order to investigate the reliability of such self-reports, a 2019 study (20) compared objective measurements of activity levels with the participants subjective perceptions; moderate and vigorous activity were accurately reported, light physical activity, however, was considerably under-reported.

High-level exercising patients display similar levels in REE and TDEE as healthy individuals, even though they weigh significantly less. In AN patients performing no to small levels of exercise, REE and TDEE are significantly lower (21). These findings may imply, that this subgroup of patients burns a similar amount of calories compared to HC, while restricting their dietary caloric intake.

In summary, all of these observations made on physical activity levels in AN patients call for individual evaluation of physical activity levels in order to incorporate management strategies to overcome compulsive exercise as well as adjusting meal plans for higher caloric needs.

Several studies have shown conflicting results regarding insulin sensitivity. Some found insulin sensitivity to be elevated in AN (22), some to be decreased (23), while others could not find any significant differences in comparison to HC (24). This inconsistency may be due to the application of different methods used measuring insulin sensitivity and additionally promoted by analyzation of different stages in the weight restoration process within the patient groups (25).

The response of malnourished persons' bodies to weight gain seems to differ considerably from weight gain in individuals with a healthy weight. It has been shown that weight restoration in AN initially leads to unproportionally faster gain of central adipose tissue in comparison to other fat storage sites. Maintenance of the restored weight for a period of around one year, however, leads to redistribution of body fat, comparable to HC (26).

Findings regarding altered metabolism in AN are important to consider for treatment. They show that slow or missing response to initial weight gain should not automatically be attributed to a lack of compliance. On the contrary, it might be the patients' change in metabolism that may slow down the process of weight restoration, even when adhering to the treatment protocol (13).

#### 1.1.8.1 Microbiome alterations in AN

In previous academic discourse (27), the question was raised whether a change in the microbiome may be a prolonging factor for AN. An interventional study, using rodents, found that transplanting stool from individuals with AN into a mouse resulted in the animal presenting features common for this eating disorder. Furthermore, even the next generation of mice displayed lower body weight, a reduction in food consumption, as well as a reduction in levels of serotonin in the brain stem (28).

Microbiome alteration in AN leads to a decreased rate of energy intake from the consumed food and suppression of body weight, and therefore acts as an aggravating factor in weight restoration (29).

### 1.1.9 Cognitive consequences of AN

Some of the alterations in cognitive processes described in AN patients have also been found in non-diseased first-degree relatives, which may be an indicator for increased predisposition of eating disorder development within families. Traits similar to OCD behaviors in parents of AN patients may lead to over-parenting and consequently contribute to the development of AN in their children (30). OCD, as discussed in section 1.1.7, has been described as a comorbidity in over 40% of cases (3).

AN patients have been witnessed to struggle with set-shifting, meaning they struggle with switching between different tasks (31). Additionally lowered activation of the striatum and raised activation of frontal and parietal brain regions were found, and the focus on specific details is strongly elevated (30).

Further abnormalities include a decline in central coherence (difficulties in looking at the abstract full picture and exhibiting an increased focus on details), as well as poor global integration. This leads to a balance out of equilibrium between global and detailed integration in acute cases of AN (30).

Altered socio-emotional processing may result from a combination of the malnourished state as well as genetic predisposition (30).

A study comparing 10 restrictive-subtype AN patients with 12 HC (32) found similar striatal responses in both cohorts regarding positive feedback. However, AN patients seemed to have an aggravated response to negative feedback. They showed elevated activity in the areas of the caudate nucleus and cognitive cingulate cortex. This may be one of the reasons for the focus on negative consequences of happenings, as has been reported in patients with AN in previous research (32).

While some research (33) on learning in adolescents with AN showed better performance in patients in comparison to HC in explicit and explicit learning, a 2013 study (34) found patients exhibiting poorer performance regarding implicit learning tasks. Implicit learning refers to unconscious learning processes, that cannot be controlled through conscious thoughts (34).

## 1.2 *Oxidative stress*

### 1.2.1 Definition

The term oxidative stress was first introduced in 1985 by German scientist Helmut Sies. His definition has since been reviewed and adapted several times (35).
The word oxidative describes aerobic metabolism, which is physiologically balanced, with neither oxidation nor reduction outweighing the other. The term stress refers to physical stress responses triggered by imbalances of pro- and antioxidative mechanisms (35).

Ideally, the amounts of pro-oxidants and antioxidants should be balanced to protect the body from harmful chemical reactions. These types of reactions serve multiple purposes within human physiology (35). If there is a balance between the antioxidant defense systems and Reactive Oxygen Species (ROS) the body rests in its physiological state. If this balance starts to shift towards ROS, oxidative stress occurs, including consequences like Deoxyribonucleic Acid (DNA) mutation, protein damage and lipid peroxidation in membranes (36).

### 1.2.2 Development

Figure 1 depicts multiple factors that contribute to the elevation and reduction of oxidative stress within the human body.
ROS include molecules like superoxide radicals ($O_2{\cdot}^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radicals ($\cdot OH$) and singlet oxygen ($^1O_2$). The listed compounds develop in metabolic reactions. While a certain level of these species is physiological and even needed for physiological functions, in excess they can harm cell components such as lipids or even the DNA (37). In low concentrations within the body, these molecules are important for immune response, apoptosis, and cell differentiation. Furthermore, several cell-signaling pathways are depending on the presence of ROS (37).

ROS are generated within mitochondria during enzymatic and nonenzymatic reactions, that naturally occur in metabolism. Apart from these endogenous sources, exogenous sources contribute to the levels of oxidated molecules within the human body. These include radiation, cigarette smoke, heavy metal exposition, and some drugs (38).

While occurrence of ROS in low concentration is important to maintain physiological functions, an excess of ROS leads to the state of oxidative stress. This makes the body more prone to developing acute as well as chronic diseases, experiencing degenerative processes and sped-up aging processes (37).

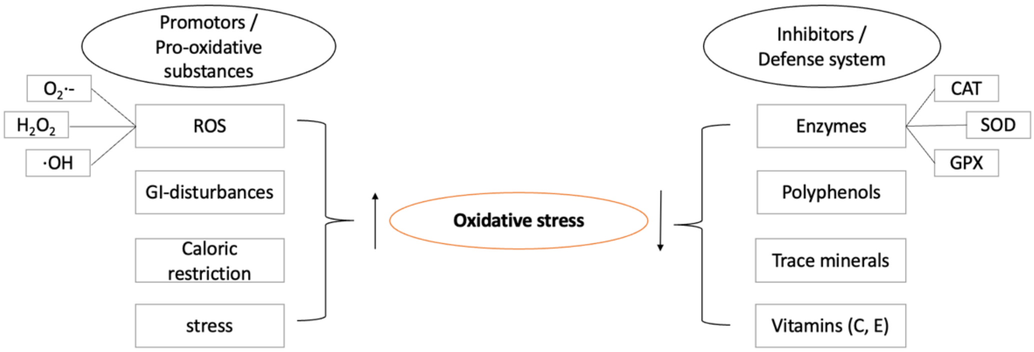


**Figure 1: Factors influencing oxidative stress (own work)**

This figure shows a selection of factors contributing to and on the other hand combating oxidative stress. ROS= Reactive Oxygen Species, CAT= Catalase, SOD= Superoxide Dismutase, GPX= Glutathione Peroxidase, GI= Gastrointestinal

### 1.2.3 Antioxidants

The body displays its own antioxidative defense system, which is made up of various synergistic mechanisms (39).

One part consists of enzymatic activity. The three main enzymes involved are Catalase (CAT), Superoxide Dismutase (SOD), and Glutathione Peroxidase (GPX) (40). These are supported by antioxidants that can usually be found in foods. Multiple vitamins (e.g., Vitamin C and E), trace minerals (e.g. iron, zinc, and selenium), and secondary phytochemicals, such as polyphenols, play important roles in the upkeeping of this system. Some of these dietary factors act as antioxidants on their own, while others are needed as co-factor for enzymatic activity (2).

Data suggests that polyphenol-rich foods improve cardiovascular functions in patients with heart failure, and show protective properties against oxidative stress, vascular diseases, as well as neurodegenerative Alzheimer's disease (40).

### 1.2.4 Oxidative stress and the Central Nervous System (CNS)

Oxidative stress has a neurotoxic effect. This is due to the brain's vulnerability facing oxidative processes. The organ shows remarkably high oxygen demands in relation to its size: It uses around 20% of the body's oxygen while making up only two percent of the total body weight. This leads to high production of ROS, which consequently leads to raised levels of oxidative processes (41).

Moreover, a high concentration of transition metals, which also play an important role in the generating of oxidative stress, is found within the brain (41).

Other influential factors include large quantities of glucose metabolization and mitochondrial activity, as well as neurotransmitters with auto-oxidative mechanisms. Many chronic diseases of the CNS, such as Parkinson's disease or amyotrophic lateral sclerosis, are linked to oxidative stress (41).

Neurotransmitter metabolism (e.g., dopamine and norepinephrine) itself may also lead to ROS: Paired with the non-existent cellular turnover, high amounts of polyunsaturated fatty acids in cell membranes within the brain may lead to oxidative stress (41).

The markers commonly used to determine this pathological status in the CNS comprise lipid-peroxidation products in plasma and cerebrospinal fluid, largely stemming from the reduction of polyunsaturated fatty acid in membranes (42).

### 1.2.5 Oxidative stress and the Gastrointestinal-tract (GI-tract)

Oxidative stress has been linked to disturbances in the function of the GI-tract (43). The brain and gut, i.e., intestines, are connected via two pathways, the Enteric Nervous System (ENS) and the CNS. The ENS, which is situated in the GI-tract, constantly sends afferents to the brain (44).

The CNS affects the ENS via efferences using the vagus nerve. This microbiota-gut-brain-axis is considered a vital point of approach for rising therapy options in operation against neurological diseases (45).

It has been well establishes in previous research (38) that the human gut microbiota influence oxidative stress levels through a range of mechanisms. Microbiota have a crucial role within the intestinal barrier (41).

Dysbiosis, a term that describes disturbance in the microbiome, can lead to a weakening of the intestinal lining, which causes toxins to cross the intestinal barrier, consequently leading to elevated levels of ROS. This process results in systemic inflammation (38).

### 1.2.6 Oxidative stress and fasting

In healthy individuals, CR has been shown to have positive effects on levels of oxidative stress and ROS-production. These mechanisms are based on a transient increase of ROS, which is in turn followed by an upregulation of the antioxidative defense system (46).

A small study (including 10 participants with a BMI > 30) investigating the effect of CR on oxidative stress levels (47) concluded that protein oxidation decreased significantly within two weeks. These findings, however, do not seem to apply to patients with AN, who experience an extreme and prolonged form of CR.

A 2020 study investigated the connection between hypoglycemia, which is also commonly displayed in AN patients (48), and oxidative stress in diabetic patients (49). Episodes of low blood glucose levels seemed to occur together with elevated levels of oxidative stress in individuals with diabetes mellitus type 2. In contrast, healthy individuals did not display increased levels of oxidative stress markers 24 hours after an induced hypoglycemic episode (49). The correlation between oxidative stress and hypoglycemia was also indicated by a 2012 study (50) investigating the effects of hypoglycemia on endothelial cells of human umbilical veins. The results showed an increase in superoxide-production within mitochondria, which caused higher levels of oxidative stress (50).

These findings suggest that low levels of blood sugar might play a role in the development of oxidative stress in AN patients.

### 1.2.7 Oxidative stress and psychiatric diseases

While the connection between oxidative stress and psychiatric illnesses has not been widely researched yet, major depression (51) and schizophrenia (52) have already been investigated in this context. Findings show a correlation with elevated levels of oxidative stress in both, schizophrenia (53), as well as major depression (51).

However, it has not yet been determined if high levels of oxidative stress lead to these diseases or vice-versa. There is a possibility that both, the diagnosis of psychiatric diseases and oxidative stress, interact with each other and lead to a negative cycle. Furthermore, patients with major depression often smoke and consume large quantities of alcohol (54), which are exogenous pro-oxidative sources (42).

### 1.2.8 Investigated oxidative stress markers

#### 1.2.8.1 Total Oxidative Capacity (TOC)

Total Oxidative Capacity (TOC) can be used as a marker to quantify oxidative stress levels within individuals (55).

It is a method to measure the total prevalence of peroxides in the serum of blood (56).

Peroxidation of cell membranes and other cell components is a consequence of exposure to free radicals. A possible effect of membrane peroxidation is the disruption of its structure and consequently inactivation of receptors and enzymes, which then may lead to weakened barriers (39).

At the time of our literature research, we could not find studies investigating TOC levels in AN patients before.

#### 1.2.8.2 Polyphenols microtitre (PPm)

The total Polyphenols microtitre (Ppm) determines total polyphenol content in serum samples (56).

Polyphenols are compounds that can be found in plant foods such as vegetables, grains, fruits, herbs, and spices. They act as the largest group of natural antioxidants and can be subdivided into three main categories; flavonoids, non-flavonoids, and phenolic acids. Polyphenols are part of the body's antioxidative defense system and mainly act via three pathways. The compounds directly interact with ROS and inactivate their harmful properties, they have an impact on the signaling of oxidative stress and they activate cellular signaling (57).

Polyphenols show lower bioavailability in comparison to other dietary antioxidants like vitamins. Consumption of polyphenols, whether naturally or by using supplements, has been linked with restoration of oxidative balance. This positive effect is partly caused by agonistic interactions with antioxidative enzymes (58). Furthermore, polyphenols reduce inflammation by inhibiting pro-inflammatory pathways including molecules like Nuclear Factor kappa B (NFkB) and Mitogen-Activated Protein Kinase (MAPK). These interactions are thought to be the main reason for the great antioxidative effect of polyphenols since direct scavenging activity has only shown significant levels at concentrations much higher than given in vivo (58).

Higher polyphenol contents within the blood have been directly linked to lower morbidity and/or the progression of various diseases. These include cancer, cardiovascular diseases as well as neurodegenerative diseases (59).

Since polyphenols affect various neurophysiological activities like brain plasticity, behavior, and cognition, it can be assumed that they also play a role in AN (60).
However, there is a lack of research regarding polyphenols in the context of AN.

#### 1.2.8.3 Endogenous peroxidase activity (EPA)

The Endogenous Peroxidase Activity (EPA) assay is used to measure the activity of all peroxidases and pseudo-peroxidases, such as SOD (61).
The main enzymes contributing to the degradation of peroxides are GPX (62), also called endogenous peroxidase, SOD (63) and CAT (64).

There are two forms of GPX and one of them is dependent on selenium. The enzyme is responsible for catalyzing the reduction of pro-oxidative compounds to water or alcohol. The presence of glutathione is vital for these reactions. It has been found to be one of the first-line defense mechanisms against ROS in the body (65).

#### 1.2.8.4 Total Antioxidative Capacity (TAC)

Total Antioxidative Capacity (TAC) is used to measure the total capacity of the body to inactivate prooxidative radicals. It is a non-enzymatic marker that consists of many individual defense mechanisms. Some of the components are dietary antioxidants like vitamin C, E, polyphenols, and beta-carotene. This marker can also be used to determine the antioxidative effect of different diets, e.g. the Mediterranean diet (66).

A recent 2021 study, conducted on female patients with AN (n=111) showed lower levels of plasma TAC compared to a group of healthy individuals (n=29) (67).

#### 1.2.8.5 Autoantibodies against oxidized Low-Density Lipoproteins (oLAb)

Autoantibodies against oxidized Low-Density Lipoproteins (oLAb) can be used to measure the body's immune response to Low-Density Lipoprotein (LDL). They may be a way to determine oxidative stress and have been correlated to cardiovascular diseases (40).

Patients with hypercholesteremia have shown higher levels of total cholesterol and lower oLAb titers. A 2011 randomized clinical control trial with 200 healthy participants investigating the effect of exogenous polyphenol supplementation on oLAb levels found an inverse correlation for oLAb and oxLDL circulating. Furthermore, oLAb inversely correlated with oxidized glutathione and total cholesterol (68). These findings are supported by an Italian study, comparing 44 subjects with hypercholesterolemia with 41 HC. The HC group showed an inverse relation between oxidized LDL and oLAb, the hypercholesterolemic patients showed significantly lower oLAb titers (69).

A 2019 study examining the Subcutaneous Adipose Tissue (SAT) of 18 individuals with a mean BMI of 15.3 found low levels of oLAb (70). As patients with AN often show hypercholesterolemia (71), high levels of cholesterol must be considered as a possible cause of decreased oLAb levels.

## *1.3 Anorexia nervosa and oxidative stress*

As established in section 1.2.7, oxidative stress is a factor co-existing with some psychiatric disorders.

As previously mentioned in section 1.2.4, the brain consumes about one-fifth of the body's oxygen and is largely made up of lipid structures. Although nerve cells within these structures show increased risk of oxidative damage, the brain does not possess stronger defense mechanisms against oxidative stress. Contrastingly, antioxidative enzyme activity seems to be even lower than in other body cells. As oxidative stress and mental illnesses behave reciprocally to each other, it is unclear to what extent increased levels of oxidative stress in brain cells stem from an existing psychiatric disorder and vice versa (72).

A big 2015 meta-analysis, analyzing 29 studies with a total of 1,729 participants (AN=895, HC=834) on the relationship between antioxidant markers and oxidative stress with AN (72), found a correlation between some altered oxidative stress parameters and AN. Several studies included found that AN patients showed higher levels in some oxidative stress indicators, such as exhaled NO and plasma nitrates, and lower levels in specific antioxidative agents, such as SOD, than HC. Not all of the reviewed parameters did show significant differences between the two groups. This may be due to the various contributing factors to oxidative stress (72).

Among the significantly elevated parameters in patients with AN were **Apolipoprotein B (ApoB)**- and **Nitric Oxide (NO)** (72).
Both plasma nitrites and exhaled NO were raised in AN compared to HC. The whole NO-metabolism is impacted by the disorder, possibly due to elevated inducible NO-Synthase (iNOS) activity. This leads to raised nitrite production and subsequently higher levels of **RNS (Reactive Nitrogen Species)**, which are linked to protein damage (72).

High ApoB in serum and increased levels of **oxLDL** might both be causes for higher risk of experiencing negative cardiovascular complications, including the development of heart disease, in AN (72).

Regarding the antioxidant status, some patients with AN showed very high levels of **beta-carotene** (which is found in various foods with few calories, such as carrots, often favored by AN patients). While at physiological levels it acts as an antioxidant, elevated levels can have an opposite effect. This is especially applicable to individuals with already high amounts of oxidated molecules and pro-oxidants. Other antioxidant markers, including **glutathione** and **cysteine,** were decreased in AN patients (72).

Generally, individuals with AN show an increase in a number of oxidative markers and a decrease in certain antioxidative markers. Thus, it can be assumed that their anti-oxidative barriers are weakened. However, it is not yet clear whether AN leads to oxidative stress, or vice versa (72).

### 1.3.1 Anorexia nervosa and stress

The term stress refers to a disruption of homeostasis within the body. Imagined or real threats to the individual, so-called stressors, lead to a physiological stress response with the aim of regaining an equilibrium and ending the exposure to harm (73). Immune, nervous and endocrine system are activated and lead to a series of defensive mechanisms that prepare the body to fight off external threats and are supposed to serve as short-term protection (74). Chronic exposure to stress can impair the body's well-being and lead to negative consequences psychologically, namely depression and anxiety, as well as physically, such as heart disease (75).

In AN side effects of malnutrition might act as a stressor (76).

#### 1.3.1.1 Psychological stress

AN patients are in a state of high psychological stress (77).

As previously described in section 1.1.5, many factors can lead to an increased risk of developing AN, such as adolescence, problems within the family, personal problems, and the extreme fear of gaining weight. Experiencing these stressors can lead to an increased in oxidative stress markers and elevated cytokine production, e.g. Interleukin-6 (IL-6), which stimulates the generation of ROS. A higher rate of catecholamine production occurs under stress, and these hormones are known to be able to auto-oxidate into ROS (36).

This indicates that higher levels of oxidative stress in patients with AN are influenced by external stress factors.

#### 1.3.1.2 Physiological stress

Patients with AN are exposed to a variety of physical stressors, including exercise (78), malnutrition (79) and low amounts of body fat (70) among others.

One of the body's main sources of ROS is the mitochondrial respiratory chain mechanism. Its processes are increased during exercise and result in a higher ROS-concentration within the body. Excessive physical activity leads to increased IL-6, which in turn leads to an imbalance in the oxidant/antioxidant system. Healthy individuals are generally able to adapt to these raised levels of oxidative stress by upregulation of their anti-oxidative defense systems (80). In AN, important substrates are missing to fully counteract the elevated levels of oxidants. Many individual suffering from AN undergo vigorous physical exercise routines in addition to CR to suppress their body weight (80). The combination of elevated ROS production and missing adaptation leads to raised levels of oxidative stress within the body (36).

Higher levels of physical activity in AN lead to hypercortisolemia (13).

Malnutrition in general (79), as well as low amounts of body fat specifically can also be seen as physiological stressor which is supported by findings of raised oxidative stress levels in individuals with reduced SAT (70).

External factors contributing to raised oxidative stress in patients with AN include smoking and alcohol consumption. Individuals with AN generally show a higher rate of smoking and patients of the binge-purge subtype also display higher rates of alcohol abuse (72).

### 1.3.2 Aims and hypotheses

This study aims to investigate markers of oxidative stress that have not yet been researched in AN patients (TOC, PPm), where data are contradictory (EPA) or insufficient (TAC, oLAb) along with parameters of eating pathology, which were collected using well-established questionnaires. All blood tests and questionnaires were conducted on a group of 20 AN patients and a control group of 20 HC.

Most studies that yielded data in this field before did not primarily intend to investigate specific oxidative stress parameters in patients with AN but reported them in the context of a different research focus. As there is a lack of research for these oxidative stress parameters in the context of AN, this study has been conducted to further investigate links between the oxidative stress parameters TOC, PPm, EPA, TAC and oLAb in AN patients.

Discovery of alterations in specific mechanisms could lead to the development of new treatment options.

The following hypotheses were formulated for this study:

1) AN patients show a distinct profile of oxidative stress parameters in comparison to HC.
2) Oxidative stress parameters correlate with scores of eating pathology and depression.

# 2 Material and Methods

## *2.1 Study design*

This was a cross-sectional, monocentric pilot study on oxidative stress parameters in AN patients, in comparison to healthy controls (HC). The patients recruited to take part in this study were inpatients of the Department of Psychiatry and Psychotherapeutic Medicine at the Medical University of Graz. They were informed about the study and asked to participate during their routine inpatient stay. As a control group, healthy individuals, of comparable age range to the AN group, were recruited in the personal and occupational environment of and by the medical students and staff involved in the project. All participants were educated on the goals and procedures of this study and gave written informed consent before participation. The study was approved by the ethics committee of the Medical University of Graz (EK-number: EK 27-481 ex 14/15).

The ethical principles of the current version of the declaration of Helsinki were applied.

Firstly, blood samples were drawn to measure oxidative stress markers in the serum and secondly, a series of questionnaires and tests were completed, with the support and guidance of medical staff.

The surveys and tests included questions regarding personal information, such as medication, age, height, and weight, as well as sociodemographic factors, like sex, education, marital status, the extent of education and current profession. The questionnaires Mini-Mental State Examination (MMSE), Beck Depression Inventory (BDI), Hamilton Depression Rating Scale (HAMD) and Mediterranean Diet Score (MDS) were conducted. The study's participants were additionally asked to complete the Eating Disorder Examination Questionnaire (EDEQ) and the Eating Attitudes Test-26 (EAT-26).

## *2.2 Literature search*

The majority of the literature research for this paper was conducted using the biomedical database "PubMed" by the National Center for Biotechnology Information (NCBI). This database contains a large quantity of available and reliable medical literature. The citation program Endnote (Version EndNote X9, Type: 64 bit, Philadelphia, PA: The EndNote Team, Clarivate. 2013) was used to correctly implement citations of relevant scientific research.

## 2.3 *Statistics and graphics*

The statistical analysis of the data, measured in the laboratory and collected via questionnaires, and their graphical representation was performed using IBM SPSS Statistics for MacIntosh, Version 27.0. Armonk, NY: IBM Corp. 2020.

Microsoft Excel for Mac, Version 16.5, Microsoft Corporation and Microsoft PowerPoint for Mac, Version 16.58, Microsoft Corporation, were used to create a graphical presentation of raised data.

The collected anthropometric data, questionnaire evaluations, as well as determined laboratory parameters, were transferred collectively into an SPSS file.
The program IBM SPSS Statistics 27 was subsequently used to calculate *p*-values, which serve to confirm or disprove hypotheses. Statistical significance is given at a value of $p < .05$. Furthermore, this statistics program was used to calculate correlations between oxidative stress parameters and questionnaire results.

For domains that showed a normal distribution, a t-test was calculated as it has most statistical power. On domains that did not display this feature, the Mann-Whitney-U test was applied to obtain statistical data.

For the calculation of correlations, Pearson correlation was used, the exception being TOC data correlations, as these showed multiple outliers in the boxplot diagram. TOC was calculated with Spearman Rho correlation. Significances were interpreted according to the statistical guidelines of Zöfel P., “Statistik für Psychologen”, Pearson Studium, 2003 (81).

## 2.4 *Participants*

The study group consisted of 20 females diagnosed with AN and was compared to a control group of 20 healthy females. All included participants were interviewed on their medical history, and data on their height and weight were collected. These anthropometric measurements were used to calculate each individual’s BMI using the formula: BMI=weight in kg/(height in m)$^2$. Participants in the AN group were on average 23 years old, the average age of the HC group was 26 years. The BMI of the patient group averaged at 13.71, while for HC the average BMI was 22.23.

### 2.4.1 Inclusion criteria

To qualify for the study, the participants had to explicitly agree to take part. For the AN group, a diagnosis of the disorder using the DSM IV criteria and a BMI < 17,5 had to be met.

### 2.4.2 Exclusion criteria

Exclusion criteria included neurological or psychiatric diseases apart from AN, head injuries, Trauma, anamnestic drug- or alcohol-abuse and acute suicidality.

## *2.5 Material*

Venous blood samples were drawn as part of a routine laboratory checkup. These consisted of two Ethylenediaminetetraacetic Acid (EDTA) tubes, one lithium-heparin tube, one serum tube, and one citrate tube. The blood samples were centrifuged and frozen at -20°C, to allow long-term storage for further investigations.

### 2.5.1 Total Oxidative Capacity (TOC)

A rapid enzymatic in vitro diagnostic assay by Labor Diagnostic Nord (Nordhorn, Germany) was used (82). Accordingly, to determine the TOC, a peroxide/peroxidase-reaction is measured photometrically using the substrate 3,5,3',5'-Tetramethybenzidine (TMB). Adding the stop solution causes the color change from blue to yellow and marks the end of the reaction. Consequently, the sample was measured at 450nm wavelength. The levels are given in µmol $H_2O_2$ equivalents (83).

### 2.5.2 Polyphenols microtitre (PPm)

A kit by Omnignostica Ltd. (Höflein/D., Austria) was used to measure the total polyphenol content in serum samples. The reagent Folin-Ciocalteu was employed to detect the reaction of polyphenols with transition metals at a wavelength of 766nm. Serial dilutions of gallic acid were used to determine the standard curve (56).

### 2.5.3 Endogenous Peroxidase Activity (EPA)

The method of Tatzber et al. (56) was used to determine EPA with a colorimetric method supplied by Labor Diagnostic Nord (Nordhorn, Germany). This method also relies on the reaction between hydrogen peroxide, TMB and horseradish peroxidase, turning the solution green to blue. Then again, the addition of a stop solution resulted in a color change. The final color was subsequently measured (84). This enabled the determination of peroxides, as well as peroxidase activity (56).

### 2.5.4 Total Antioxidative Capacity (TAC)

In this study, a colorimetric method by Labor Diagnostic Nord (Nordhorn, Germany) was used to determine TAC. TMB was used as a substrate to show the inhibition of a peroxide/peroxidase reaction. Antioxidants present in the sample stop the reaction of ROS with TMB, which can be measured by using a colorimetric signal (56).

After the combination of the reagents, they were incubated at 4°C for 20 minutes. Then, a stop solution was added, resulting in a change of color from blue to yellow. A wavelength of 450nm was used to measure the absorbance (56).

### 2.5.5 Autoantibodies against oxLDL (oLAb)

A commercial enzyme immunoassay by Biomedica (Vienna, Austria) was used to determine the oLAb titers for this study. It is based on the method of Tatzber and Esterbauer (83).

Cupric ions were used to oxidize LDL, which binds to microtiter wells and consequently start a binding reaction with the samples. This reaction is the basis for this enzyme-linked immunosorbent assay (ELISA). A peroxidase-coupled anti-immunoglobulin G antibody is then bound and, using TMB as substrate, measured colorimetrically. The units used are mU/ml (83).

### 2.5.6 Tasks and Questionnaires

#### 2.5.6.1 Mini-Mental State Examination (MMSE)

The MMSE is the most researched cognitive screening test for dementia. It consists of a questionnaire with 30 questions that test the patient's attention, language proficiency, memory and orientation, as well as visual and motoric skills (85). Although a limitation of the test is the lack of consideration for the testees' educational level, a 2015 meta-analysis (86), including 14 studies, found the test to have a sensitivity of 88.3% and a specificity of 86.2% for detecting dementia (85).

#### 2.5.6.2 Beck Depression Inventory (BDI)

BDI has been established by Beck et al. in 1961, to assess rates of depression within certain groups. It is based on the theory, of negative cognitive impairment as a strong indicator of depression. The inventory consists of 21 questions which are answered by the tested individual. The completion of the BDI, takes an average of five to ten minutes. Each answer is rated on a score from zero to three points (87). The higher the sum score, the higher the indication for a severe level of depression (88).

#### 2.5.6.3 Hamilton Depression Rating Scale (HAMD)

This scale for evaluating severity of depression has been introduced in the 1960s (89). It consists of 17 variables, each rated on a scale from either one to five or one to three depending on the question. The questions cover topics such as depressed mood, difficulties at work and loss of interest in hobbies, agitation, hypochondriasis, GI-symptoms, general somatic symptoms, weight loss, insight into the illness, retardation, and suicidality. All factors listed above can be viewed as indicators of depression in individuals (89).

#### 2.5.6.4 Mediterranean Diet Score (MDS)

MDS is a questionnaire conducted to see how closely individuals adhere to a typical diet from the Mediterranean area. It is usually referred to because the Mediterranean diet supposedly stands for longevity and health. The scale is made up of nine questions, and adhering more rigidly to the classic Mediterranean diet approach results in higher scores (90).

This nutritional approach is defined by a considerably high intake of vegetables, legumes, fresh fruits, nuts, grains, and fish. Olive oil serves as the main source of fat for cooking and contributes to reaching the recommended amount of monounsaturated fatty acids. Furthermore, it is advised to limit the consumption of alcohol (one glass of red wine a day is still considered acceptable), red meat, eggs, dairy products, and commercial sweets (91).

A 2008 study (91), investigated the association of oxidative stress and MDS on 138 pairs and 21 unmatched male twins. The results showed an inverse correlation between higher MDS and levels of oxidative stress, measured by oxidized GPX (91).

#### 2.5.6.5 Eating Disorder Examination Questionnaire (EDEQ)

This self-administered questionnaire contains 28 questions on different topics associated with eating disorders. The questions inquire about emotions about and attitudes towards food within the 28 days prior to completing the questionnaire. The answers are consequently assessed to estimate the respondent's probability of suffering from an eating disorder. Additionally, the questionnaire elicits information on the patient's weight, height, and constancy of menstrual cycle over a period of the past three to four months. Then, the end score is evaluated according to the scoring table (92).

The results are divided into a total score and four subscores. These subscores focus on weight concern, shape concern, eating concern and eating restraint (93). This distinguishment can be used to differentiate individual points of focus in the disordered behavior.

For patients with AN, higher scores are expected than with HC. A 2018 study, investigating the correlation of AN subtypes with subscale results in adolescent with AN, found patients of the restricting subtype to score lower, both on a global scale and subscales, in comparison to binge-purge subtype patients (94).

#### 2.5.6.6 Eating Disorder Attitudes Test (EAT-26)

Originally, the Eating Attitudes Test (EAT) was comprised of a scale with 40 items, the EAT-26 is a shortened version with 26 items that is viewed as a reliable and objective indicator of current eating disorders in tested individuals (95).

Questionnaire results are compared to a table that indicates norm ranges and eating disorder ranges. These test subscores can also be helpful to establish body image issues, bulimic tendencies and psychological manifestations linked to disordered eating (95).

# 3 Results

## *3.1 Group Characteristics*

The study included 20 female patients with AN and 20 healthy individuals, matched in age, as a control group. A Mann-Whitney test indicated that HC (Mdn = 26.50 years) showed no significant statistical difference in age, in comparison to patients with AN (Mdn = 23.50 years), U ($N_{HC}$=20, $N_{AN}$=20) = 145, z=-1.491, p=0.139. As expected, there was a statistically significant difference between the two groups regarding weight and BMI, with the AN group showing a BMI matching the diagnosis and the HC group showing a healthy BMI range. A significant difference was also found regarding the participants' height.

Table 1 shows the anthropometric data of the study participants.

**Table 1: Anthropometric data of study participants**

| | **n (AN/HC)** | **AN**<br>**M (SD) /**<br>**M (Min; Max; IQR)** | **HC**<br>**M (SD) /**<br>**M (Min; Max; IQR)** | ***p*-value** |
|---|---|---|---|---|
| **Height [m]** | n (20/19) | 1.64 (.06) | 1.68 (.07) | **.007**** |
| **Weight [kg]** | n (20/19) | 37.03 (6.78) | 64.32 (12.79) | **<.001***** |
| **BMI [kg/m$^2$]** | n (20/19) | 13.71 (1.97) | 22.23 (3.37) | **<.001***** |
| **Age [years]** | n (20/20) | 23<br>(18; 54; 20-30) | 26<br>(22.00; 27; 25-30.75) | .136 |

AN= Anorexia nervosa, HC= healthy controls, M= mean, SD= standard deviation, Min= minimum, Max= maximum, IQR= interquartile rank, BMI= Body Mass Index, **= $p$≤.01, ***= $p$≤.001

## 3.2 Questionnaires

Table 2 gives an overview of the results of the psychological questionnaires evaluating depression and eating pathology in both cohorts.

Significant differences were found for the EDEQ questionnaire in the total score as well as all four subscores. Interestingly, out of the four EDEQ subscores the HC group scored highest for weight concern. AN patients also scored significantly higher in the EAT-26. These findings match their diagnosis and helped prove, that participants in the HC cohort did not display eating disorder thoughts or behaviors.

Results of the depression rating scales HAMD and BDI showed significantly higher levels in the AN cohort, indicating higher rates of depression. These results match

No significant differences were determined in the MDS, with a higher median in the HC group.

**Table 2: Results of psychological questionnaires**

| Name of Questionnaire | n (AN/HC) | AN<br>M (SD) /<br>M (Min; Max; IQR) | HC<br>M (SD) /<br>M (Min; Max; IQR) | *p*-value |
|---|---|---|---|---|
| **EDEQ shape concern** | n (16/14) | 4.03 (1.43) | .69 (.66) | **<.001*** ** |
| **EDEQ restraint** | n (16/14) | 3.4<br>(0; 6; .25-4.95) | .3<br>(0; 22; 0-.85) | **<.001*** ** |
| **EDEQ eating concern** | n (16/14) | 4<br>(0; 5.2; 1.3-4.4) | 0<br>(0; 1.2; 0-.2) | **<.001*** ** |
| **EDEQ weight concern** | n (16/14) | 2.7<br>(1.6; 6; 2.2-4) | 2<br>(0; 2.2; 0-1) | **<.001*** ** |
| **EDEQ total** | n (17/16) | 3.1<br>(.76; 5.7; 1.54-4.47) | .37<br>(0; 57; .23-.71) | **<.001*** ** |
| **EAT-26** | n (16/16) | 26<br>(6; 51; 18.75-36.75) | 2<br>(0; 8; 0-5.5) | **<.001*** ** |
| **MDS** | n (18/18) | 7<br>(2; 11; 3.75-9) | 8<br>(5; 12; 7-9) | .266 |
| **HAMD** | n (19/20) | 18<br>(4; 31; 11-18) | 1<br>(0;8; 0-1) | **<.001*** ** |
| **BDI** | n (16/20) | 25.5<br>(1;44; 13.5-33.5) | 1<br>(0; 17; 0-1) | **.001** ** |

AN= Anorexia nervosa, HC= healthy controls, M= mean, SD= standard deviation, Min= minimum, Max= maximum, IQR= interquartile rank, EDEQ= Eating Disorder Examination Questionnaire, EAT-26= Eating Attitudes Test-26, MDS= Mediterranean Diet Score, HAMD= Hamilton Depression Rating Scale, BDI= Beck's Depression Inventory, **= *p*≤..01, ***= *p*≤..001

## 3.3 *Oxidative stress parameters*

Among all tested parameters of oxidative stress, only TAC differed significantly between AN patients (M=1.474, SD=.624) and HC (M=1.907, SD=.563), t(34)=-2.181, *p=.036*. Although the remaining parameters also showed differences between the two groups, these were not statistically significant. Median TOC and oLAb were lower in the AN group, PPm, were almost identical in both groups, EPA was lower in the control group, as illustrated in Table 3.

The significantly lower level of TAC in AN patients, compared to HC, is depicted in Figure 2.

**Table 3: Oxidative stress parameters**

| **Ox Stress Parameters** | **n (AN/HC)** | **AN M (SD)/ M (Min; Max; IQR)** | **HC M (SD) / M (Min; Max; IQR)** | ***p*-value** |
|---|---|---|---|---|
| **TAC [mmol/l]** | n (16/20) | 1.47 (.62) | 1.91 (.56) | **.036*** |
| **PPm [mmol/l]** | n (20/20) | 7.79 (.52) | 7.7 (.34) | .476 |
| **TOC [µmol/l]** | n (17/20) | 138 (23; 355; 96-171) | 144 (85; 573; 131-204.5) | .427 |
| **oLAb [mU/ml]** | n (20/20) | 513 (185; 6000; 185-2729.5) | 513 (185; 6000; 366.5-1811.5) | .408 |
| **EPA [U/l]** | n (17/20) | 2.98 (1.82; 10.42; 2.40-7.67) | 3.07 (1.11; 12.92; 2.55-7.53) | .547 |

AN= Anorexia nervosa, HC= healthy controls, M= mean, SD= standard deviation, Min= minimum, Max= maximum, IWR= interquartile rank, t= t-value, df= degrees of freedom, TAC= Total Antioxidative Capacity, PPm= Polyphenols microtitre, TOC= Total Oxidative Capacity, oLAb= Autoantibodies against oxidized Low-Density Lipoproteins, EPA= Endogenous Peroxidase Activity, *= $p \leq .05$

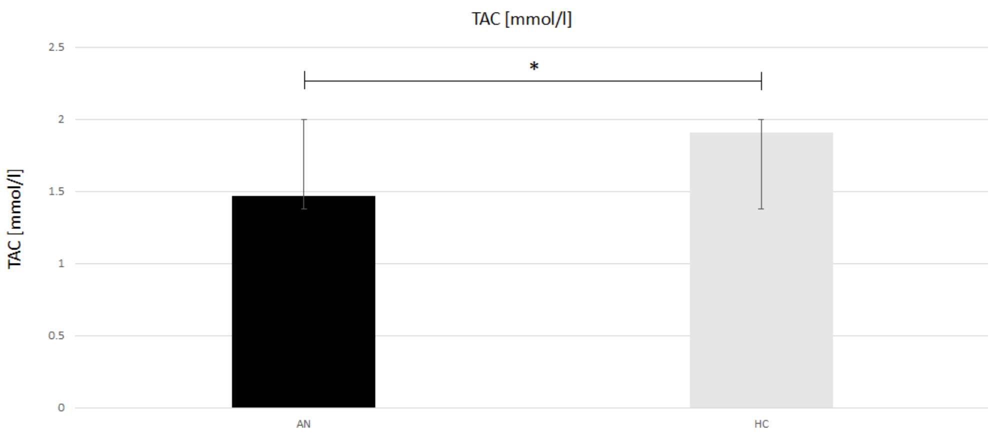


**Figure 2: Comparison of measured TAC-values**

TAC= Total Antioxidative Capacity, AN= Anorexia nervosa, HC= healthy controls

* = significant difference, $p < .05$

## *3.4 Correlations of oxidative stress parameters and questionnaire scores*

A number of statistically significant correlations between the examined oxidative stress parameters and the results of the conducted questionnaires were found, these are explained in detail in the following subchapters.

### 3.4.1 HC group

Various correlations between a number of parameters can be observed in our control group.

TOC was correlated with three parameters. It showed positive correlations with bodyweight ($r_{sp} = .459$, $p = .048$), BMI ($r_{sp} = .739$, $p < .001$), as well as PPm ($r_{sp} = .482$, $p = .031$).

TAC showed statistically significant negative correlations with the EAT-26 questionnaire scores ($r_{sp} = -.574$, $p = .020$) and EPA ($r_{sp} = -.470$, $p = .036$).
EPA also correlated with the participants' bodyweight ($r_{sp} = .505$, $p = .027$).

Regarding the two depression rating scales, the total results of the EDEQ questionnaire did not correlate with any of the investigated oxidative stress parameters. It did, however, correlate significantly with results of BDI ($r_{sp} = .577$, $p = .019$) and EAT-26 ($r_{sp} = .522$, $p = .038$).
HAMD only showed significant correlation with EAT-26 ($r_{sp} = .561$, $p = .024$l).

### 3.4.2 AN group

The AN group showed correlations between a number of questionnaire scores and oxidative stress parameters. TOC correlated to both TAC ($r = -.607$, $p = 0.013$) and the participants height ($r_{sp} = -.572$, $p = .016$). Additionally, TAC showed significant correlation to the oxidative stress parameter EPA ($r_{sp} = -.630$, $p = .009$) and the dietary variable MDS ($r = .644$, $p = .013$).

The questionnaires EDEQ and EAT-26 also displayed significant correlations. EDEQ correlated positively with the depression rating scale HAMD ($r = .599$, $p = .011$) and with the eating disorder assessment test EAT-26 ($r = .846$, $p = 0.001$). Finally, EAT-26 scores in the AN group were correlated with the patients' weight ($r = .755$, $p = .001$) and BMI ($r = .694$, $p = .003$).

# 4 Discussion

In this study, we investigated oxidative stress parameters in AN and conducted an extensive literature review. In summary, studies indicate raised levels of oxidative stress in AN and show that several factors contribute to this.

1) AN patients show a distinct profile of oxidative stress parameters in comparison to HC.
2) Oxidative stress parameters correlate with scores of eating pathology and depression.

The hypotheses of this study will subsequently be discussed in detail in relation to the results.

## *4.1 Changed oxidative stress parameters in AN patients*

We investigated different markers for oxidative stress, aiming to determine whether they were altered in patients with AN and if so, which markers were affected. Uncovering changes in specific markers could be helpful in assessing the changed pathways in AN patients that lead to these alterations and then developing targeted therapies.

Interestingly, the only significant difference among the five oxidative stress parameters studied was found in TAC, which was significantly lower in AN patients. TOC and oLAb were also slightly lower in the AN group, PPm showed almost no difference and EPA was higher in the AN group. These findings suggest that part of the thesis' first hypothesis can be confirmed.

Increased physiological and psychological stress increases the risk of imbalance in the antioxidative/oxidative systems within the body. This causes oxidative stress with a negative impact on health and a raised vulnerability to diseases, associated with this state. AN patients in a state of high psychological stress show increased levels in oxidative stress and elevated cytokine production, e.g., IL-6 (15).

### 4.1.1 TAC-levels in AN and HC

We found TAC to be significantly decreased in our AN cohort. It appears that oxidative stress cannot be reduced to one or several specific markers but is rather a multifactorial event that varies from patient to patient. TAC is determined by a large number of influencing factors. It seems that the other parameters we investigated for the state of oxidative stress are also all shifted towards increased levels of oxidative stress in the AN cohort, but no statistically significant results confirming this observation were generated in this study.

A healthy BMI correlates to a higher TAC (96). Therefore, this parameter might be a valid indicator of nutritional status (71). Our study's results regarding TAC are in line with the results of previously conducted studies. For example, a 1985 study (24) found that patients with AN showed a 24% decrease in their serum TAC in comparison to HC. This study's results should be regarded with caution, as its sample size was rather small, and as as age and gender differences were not taken into account adequately (four female AN patients with a mean age of 23.7 years compared to 12 HC (6 female, 6 male) with a mean age of 53.7 years). Low TAC in 18 AN patients in comparison to reference levels has also been reported (25). Also, similar findings were reported in a 2021 study (67), conducted on 111 female patients with AN, that found a 43% decrease in plasma TAC in the AN cohort. The researchers also described an inverse correlation between duration of disease and this oxidative stress marker (67).

In an interventional study, **TAC increased significantly after achieving a healthy BMI** (from 1033.03 ± 34.38 to 1504.61 ± 99.73 µmol/L; $p < 0.01$) in 22 AN patients. The authors attributed this to an increased intake of iron in patients showing improvement in their TAC status (71). Significant reductions in serum iron concentrations have been found in patients with AN, in comparison to healthy individuals (36), however, iron status was not assessed in our study.

## *4.2 Insignificant investigated oxidative stress parameters*

### 4.2.1 TOC-levels in AN and HC

Interestingly, while TAC was significantly decreased in comparison to our HC group, AN patients also displayed lower levels of TOC, although these analysis were not statistically significant. We expected TOC to be higher in the patient cohort, as it is usually an indicator of increased oxidative stress. However, our findings indicate that while TAC seems to be affected mainly by nutrition and bodyweight, TOC might be influenced more strongly also by other factors.

Previous research has shown an increase in TOC in obese children when compared to a group of nonobese pre-adolescents. The researchers of this study even observed a positive correlation between the duration of the condition and TOC levels (97). These findings combined with our findings could suggest a link between BMI and TOC.

Interestingly, our findings contradict a study, conducted on patients with major depression, that observed raised levels of TOC in comparison to a group of controls and lowered TOC after antidepressive treatment (98), regarding the fact that many of our patients also suffered from depressive symptoms. 11 displayed moderate to severe symptoms of depression according to HAMD and a further five displayed mild depressive symptoms. The self-administered BDI scores indicated mild to moderate depression in 3 participants of the AN cohort and moderate to severe depression in ten of them.

### 4.2.2 PPm-Levels and MDS in AN and HC

We analyzed polyphenols in the serum of our study's participants as a measurement of the intake of antioxidative rich foods. As we expected to find raised levels of oxidative stress, we hypothesized that the restriction of food intake in AN patients could lead to lower intake of polyphenol-rich foods and therefore lower levels of PPm.
However, our results showed no significant differences in the polyphenol titres of both cohorts, the median was even higher in our AN group.

At the point in time this thesis was written, no further studies investigating PPm in patients with AN could be found in the "PubMed" database.
As patients with AN tend to exclude foods deemed to be "unhealthy", high in calories and fat (1) and focus plant foods that are often lower in calories but also higher in antioxidants, e.g. polyphenols, this may explain our findings.

A 2006 study comparing intake of nutrients in adolescents with AN compared to an HC group matched in age found higher levels of fiber and vitamin intake in the patient cohort, partly stemming from supplementation (99). These findings suggest that intake in health-promoting micronutrients in AN patients is higher and therefore support our finding. However, we did not investigate supplement intake in our study population.

Furthermore, MDS results in both our cohorts did not differ significantly. As previously explained, the Mediterranean diet is high in polyphenol rich foods and similar quantitive dietary intake of food-groups that are commonly part of this diet might be an explanation of these results.

### 4.2.3 Anti-oxidative enzymes (CAT, SOD, GPX) in patients with AN / EPA

Previous research investigating anti-oxidative enzymes individually have led to partly contradictory results regarding increase and decrease of activity.

We measured the collective activity of anti-oxidative enzymes in our study cohorts using the EPA assay.

A 2015 study (71) investigating antioxidant status in AN patients throughout nutritional rehabilitation found aberrations in enzymatic functioning in AN patients; The enzyme activity of 25 patients with AN after recovering to a healthy BMI showed a decrease in SOD activity ($p < 0.05$) in all patients and persistence in the GPX activity. CAT activity showed a significant increase in 21 cases (from a mean of $25.04 \pm 1.97$ to $35.54 \pm 2.60$ µmol/min/mL; $p < 0.01$). They concluded that higher levels of CAT activity seemed to be an indicator of an improvement in nutritional status and a repair of the antioxidative defense system (71).

These findings are backed up by another study including 82 females with AN that found activities of SOD to be decreased, that of CAT increased and no noticeable difference in enzymes of the glutathione system (100).

Interestingly, this was a comparison between females with AN and reference values, whereas the study mentioned before (71) compared individuals before and after weight gain. This might imply that complete rehabilitation of enzymatic activity is possible for AN patients after weight restoration.

The findings of these two studies are contradicted by a 2015 study including 18 students with a high score in the EAT-26. These participants were found to have increased SOD activity and a decrease in CAT activity (101).

Further studies suggesting both an in- (102) and decrease (70) of GPX activities show that data on enzyme activity in AN patients is highly ambiguous.

One possible reason for inconsistent findings might be nutritious factors disregarded in some studies, as an article analyzing a number of factors contributing to oxidative stress in the context of AN concludes that micronutrient deficiencies could play a role in altered enzyme activity (36). Taking individual micronutrient levels into account more prominently may therefore be helpful in providing more feasible results when analyzing enzymatic activity in AN patients.

Contrastingly to the studies cited above, which looked at various enzymes individually, in our study we measured a general marker for combined enzyme activity. The similar levels of EPA in both our groups may result from not measuring anti-oxidative enzymes individually. However, considering the inconsistency in previous research even among studies analyzing the same enzyme specifically, it is possible that enzymatic anomalies in AN patients might not be strongly related to the illness itself but rather to varying individual micronutrient deficiencies.

## *4.3 Oxidative stress parameters correlate with scores of eating pathology and depression*

As shown in the results, we were able to determine statistically significant correlations between the measured oxidative stress parameters and certain questionnaire scores that are used as indicators for eating pathology and depression.

### 4.3.1 Correlations HC group

The control group showed correlations between TOC and PPm as well as between BMI and weight. Higher levels of TOC might be caused by higher body weight. This hypothesis is supported by a study comparing obese individuals to a group within the normal BMI range, which reported that obesity led to significantly higher TOC (103).
We also found a negative correlation between TAC and EAT-26 results in this cohort. This could imply that less tendency to disordered eating results in higher TAC. As our results showed significantly higher levels of TAC in the HC group compared to AN patients, this statistical finding matches our laboratory results.

### 4.3.2 Correlations AN group

We found a negative correlation between TAC and EPA. This inverse correlation has previously been observed in a study conducted on slaughterhouse workers exposed to stressful working conditions. Additionally, the study reported a negative correlation between TOC and TAC (84), which we also found in our AN cohort. Since both groups displaying higher oxidative stress (i.e., slaughterhouse workers and AN patients) are exposed to higher levels of psychological and/or physical stressors, external stress factors may be a causative factor.

We found a correlation between TAC and MDS in AN patients, which reflects findings of a 2022 study examining the effect of the Mediterranean diet on TAC levels in patients with Parkinson's disease. The randomized control trial (RCT) revealed a significant increase of TAC in the group that was put on a Mediterranean diet (104). These results are also supported by a 2010 large-scale cross-sectional study (105), including 1514 male and 1528 female participants. It showed that 20% higher MDS scores lead to an increase of TAC by six percent (105).

Interestingly, we did not find any correlations between polyphenols and other observed parameters. We expected polyphenol-markers, like the MDS, to correlate to other oxidative stress parameters and questionnaire scores, as a previous study showed a negative correlation between polyphenol intake and BDI results (106). However, these contradicting findings could be explained by differences in participants' age and in the analytical methods applied to determine polyphenol levels. Our study's participants were young women (mean age: 23 years) and we analyzed the polyphenol titer in the serum, whereas the mentioned study's cohort consisted of perimenopausal women and the and the only focus lay on the polyphenol intake.

A series of studies discussed in a comprehensive 2020 literature review (107) on the topic of depression and the Mediterranean diet showed a link between depressive symptoms and MDS. Multiple studies reported a negative correlation between higher polyphenol intake (i.e. via increased tea consumption) and BDI-scores (107). These findings do not correlate with our results, as we could not determine statistically significant differences in MDS between our two cohorts, however both of the investigated depression indices (HAMD and BDI) were significantly elevated only in our AN patient cohort. HAMD correlated with both EDEQ and EAT-26. We expected these findings, as AN has often been shown to co-exist with depressive symptoms (3).

BDI, a self-reported depression scale, did not correlate with the questionnaires used to assess eating disorders. This could be due to altered self-perception in eating disorders (108) and may indicate that screening and diagnostic tests conducted by medical professionals are more valid in AN patients than self-conducted ones.

A link between a negative body image and higher EAT-26 scores has previously been described in a study on 800 students, in the subgroup of overweight and obese individuals (109). Unexpectedly, EAT-26 also correlated to BMI and body weight in our patient group. Perhaps higher BMI and body weight in AN leads to higher levels of body dissatisfaction and restrictive behavior, to suppress body weight even further.

### *4.4 AN patients with co-morbidities*

AN patients commonly display various concomitant psychiatric disorders (110). Our results are in line with this established finding, as we identified statistically higher indices of depression in the AN cohort in comparison to the HC cohort.

In AN patients with underlying depression, the effect of the anti-oxidative capacity seems to be even worse compared to those without. Depressive Symptoms due to a depressive disorder or subsyndromal depressive disorder do not only negatively impact mood and cognitive functions but can also have negative effects on cardiovascular health, lead to inflammation and metabolic consequences (3).

One study (111) comparing the dietary and serum TAC levels in 30 healthy and 30 depressed male university students found significantly lower serum TAC in the depressed group. The researchers concluded that lower dietary intake of antioxidative substances, such as vitamin C and carotenoids, are likely to be the main contributor (111). Comparable results have been reported in a study (98) conducted on a cohort of 57 (46 female) patients with major depression compared to a group of 40 HC. The researchers investigated both TAC as well as TOC and additionally made a comparison of the results pre-and post-treatment in the patient group. It was shown that TOC was elevated, and TAC decreased in pre-treatment patients. After treatment, however, TOC dropped significantly and TAC rose significantly within this group (98).

Similar findings had already been reported in a previous study on TAC in male schizophrenia patients in 1998 (52). Compared to a healthy matched control group, the patients were found to have significantly lower plasma TAC levels. An inverse correlation between the severity of symptoms during unmedicated periods and the parameters was also observed (52).

The only co-morbidity we investigated in our study groups was depression, however, as AN patients also show higher probability of exhibiting further psychiatric illnesses which might also be linked to elevated levels of oxidative stress, diagnosis and treatment of concomitant diseases could be a way to reduce oxidative stress in patients with AN.

## *4.5 Reasons for oxidative stress in AN*

### 4.5.1 Pathophysiology of AN and oxidative stress markers

CR has been shown to be a multifactorial stressor on the human body (36). The severe reduction of metabolic rate in AN patients might cause an imbalance in the redox systems. Another factor that is likely leading to elevated levels of stress is the reduced intake of important antioxidative vitamins (e.g., Vitamin C and Vitamin E) and the lack of co-factors for enzymes with scavenging activities and the enzyme glutathione. The decreased barrier against oxidative factors subsequently leads to raised oxidative stress and damage of cell structures (71).

The severity of nutritive restriction in AN seems to cause raised stress levels including oxidative stress (36). This view is supported by findings suggesting that lower SAT in AN is correlated to higher oxidative stress levels even in comparison to other patients with AN and higher SAT (70).

Glucose depletion can also result in higher levels of ROS. When glucose is not available in mitochondria, glutamine is used instead, which raises the production of ROS. This leads to the hypothesis that low glucose levels are another factor contributing to oxidative stress in AN patients (36).

As our AN cohort showed a median BMI of 13.71 it can be assumed that all these factors played a role in the altered oxidative stress parameter profile. In order to determine to what extent these factors individually influenced our results, more data about the diet of our patients would have been needed.

### 4.5.2 Dietary reasons for raised oxidative stress in AN patients

We used the MDS as a marker of nutritional quality in both our study groups, we did not, however, evaluate specific nutritional deficiencies. As MDS is a self-reported scale, there is no way to verify the truthfulness of the participants' statements.

A study analyzing antioxidant status and antioxidative enzyme activity in AN patients revealed a significant reduction in tocopherol (Vitamin E) compared to reference levels. 21% of participants had tocopherol levels under the reference interval. This might stem from reduced vitamin intake like folate or increased utilization of antioxidative systems with improper recovery (100). These findings are conclusive to various studies (112) (100) (113) that found levels of Vitamin E to be lower in AN patients, compared to healthy individuals.

Vitamin E is a major fat-soluble antioxidant, that counteracts the development of lipid peroxyl radicals induced by ROS. A deficiency can directly impact antioxidative enzymes like hepatic CAT, GPX and glutathione reductase (36).

A selenium deficiency, which is a co-factor for GPX and acts as a synergist to Vitamin C, E and carotenoids, has been reported in 58 cases in a micronutrient study, including 153 patients with AN (2).

Hypercarotenemia is a common feature in AN patients (114). The causing factors for this are excessive dietary intake, lowered storage capacities due to less tissue storage or supplementation. Many antioxidants can paradoxically act pro-oxidative, if present at too high levels (100). In a study investigating carotene levels, the comparison of patients with AN to a healthy control group showed an elevation in alpha-carotene and no change to elevated concentration of beta-carotene (36). These findings are supported by another study, including 101 patients with AN which found an elevation in serum beta-carotene (>200µg/dl) in 62% (114). Low body fat content seems to interfere with metabolism and carotenoid regulation (70).

A study investigating the connection of TAC and intake of antioxidants via diet, conducted on adults of normal weight as well as obese adults, found a direct link between dietary antioxidant intake and serum TAC levels (115). As we evaluated antioxidants in the serum (PPm) but not the dietary intake specifically, it would be interesting to extend our research in this direction.

These findings were backed by another study focusing on AN patients after oral re-alimentation. The TAC levels increased after dietary intervention (71). This leads to the conclusion that either the improvement of the quality and quantity of nutritional intake or the raise in body weight and return to a healthy metabolic state in former AN patients can decrease oxidative stress in these individuals.

Further studies controlling the concrete micronutrient intake in patients and analyzing the change in oxidative stress parameters throughout nutritional rehabilitation could lead to further insights regarding their connection to specific micronutrient-imbalances.

### 4.5.3 Alterations in gut-brain axis

Changes in oxidative stress parameters may also rely on alterations of the gut microbiota in AN patients (116). Recent research showed that patients suffering from AN display less varied microbiota in their gut. During weight restoration, however, the diversity increased significantly and approached that of healthy control groups (116).

Constipation, occurring often in patients with AN (117), can contribute to dysbiosis (118). Miscolonization of the GI-tract acts as promotor for oxidative stress and inflammatory responses (119). This contributing element to elevated oxidative stress levels in AN patients, may be hypothesized to be unbalanced due to a lack in dietary intake of antioxidants. This hypothesis is backed by the findings indicating increased enzymatic defense after weight restoration (71).

A cross-sectional study, including 106 female individuals divided into four groups (AN, HC, obese and athletes), investigated the changes in microbiome associated to body weight and physical activity. It concorded with the hypothesis that microbiome in patients with AN is less diverse. A decrease in alpha-diversity was especially visible in patients with AN and obese women, whereas athletes showed the widest range in this category. Only one single phylotype was increased in AN patients, whereas all other biota were less varied in this cohort compared to the other groups (120).

All of the findings above lead to the conclusion that rehabilitation of the microbiome in patients with AN is helpful in returning to a balance of antioxidative and oxidative states. Therefore, it enforces the importance of dietary rehabilitation in AN treatment plans.

## *4.6 Limitations*

Multiple factors, i.e., the small sample size of our study, the low diversity of participants with regard to gender and age, the duration of sample storage, as well as not taking into account all confounders, might potentially have impacted the results of this study, as discussed in the following sections. However, despite all limitations, this study has provided some valuable insights into the interaction of AN and oxidative stress and has shown which aspects analyzed need to be scrutinized more thoroughly in future research.

### 4.6.1 Sample size

Within the two groups studied, each consisted of only 20 participants, which was a rather small sample size. All of them were local to the same city and therefore it was to be assumed that they had similar environmental influences. In a larger study, participants might be recruited from more than one city or even on an international level. Larger studies are less prone to statistical errors and are more reliable in creating generally applicable findings. Given the small sample size, caution must be employed if the study is to be applied to the general public. However, most studies on AN patients have a low sample size ranging from 4 to 20 participants (121, 122). To our knowledge, this is the largest study yet conducted in AN patients which included an investigation of a variety of oxidative stress parameters.

### 4.6.2 Gender aspects

This study focused on female patients, therefore it would be interesting to investigate these parameters in male individuals in the future to see whether sex influences the results. Nonetheless, the majority of patients with AN are female, making up 90% of cases (1). Consequently, a study on male patients would be unlikely to have large explanatory power due to the low numbers of participants.

While more data, such as a larger number of participants and inclusion of both sexes, could have made the findings in this study even more significant, it would have exceeded the scope of this thesis.

### 4.6.3 Critical evaluation of applied methods

Clinical measurement of oxidative stress parameters is shown to be a difficult procedure. No two TAC assays, for example, are directly comparable. Therefore, only values determined with the same assay can be directly compared. Thus, special care must be taken when comparing the results of multiple studies to one another (123).

Since some markers rapidly degrade in serum, samples should be used as fresh as possible. Samples in this study were centrifuged, pipetted into eprouvettes and frozen within a few hours of blood collection. For evaluation, they were thawed and consequently rapidly analyzed in the laboratory. We have not been able to find literature stating the exact amount of time it takes for all the markers we measured to lose their meaningfulness. Nevertheless, a study conducted on TAC and two other oxidative stress parameters showed no big alterations in the results after storing them between four and 20°C for up to 48 hours (124). We aimed to freeze our samples immediately after preparing them for laboratory analysis. However, in retrospect, it cannot be said with certainty how long each sample was left unfrozen.

Three of the blood samples from the AN group were hemolytic and therefore TAC, TOC and EPA could not be determined and were filed missing in the SPSS file.

### 4.6.4 Confounder

As this was a cross-sectional study, the duration of illness of the AN patients was not factored in. Furthermore, although medications taken were recorded in both groups, they were not evaluated as confounders in the analyses because of the low sample size. The oxidative stress levels of some members of the AN group may have been influenced by the use of drugs. Multiple studies have been conducted to investigate the connections of antidepressant drugs and oxidative stress, findings however, have been inconsistent. While some antidepressants seem to reduce levels of oxidative stress in the brain, others may not have any impact while some even contribute to higher levels (125). Not all antidepressants work using the same mechanisms, therefore it is necessary to consider the different working mechanisms.

The Selective Serotonin Reuptake Inhibitor (SSRI) fluoxetine has led to a decrease in oxidative stress in rat studies (42). An in silico investigation has found a mechanism in which fluoxetine is acting as a scavenger for ROS (126). Doxepin, a common drug used to treat major depression, has strong anti-oxidative effects in-vitro by reducing ferric and cupric ions, showing chelating activity and oxide scavenging activity (42).
One study evaluating the effects of SSRIs and the Tricyclic Antidepressant (TCA) imipramine in mice, found TCA to be leading to elevated levels of oxidative stress through multiple pathways. This TCA, if administered in doses over 10mg/kg, has been shown to raise levels of reduced glutathione, NO, and lead to higher levels of lipid peroxidation (127).

## *4.7 Implications for clinical practice*

Oxidative stress is the result of a complex interaction of numerous internal and external factors. Therefore, it is difficult to identify a distinct trigger for decreased TAC values in the AN group, when compared to the HC group.

Subsequently, therapy with a focus on reducing oxidative stress (i.e., antioxidants) may not be effective for AN patients. Instead, increased attention should be paid to reducing known underlying factors that contribute to increased oxidative stress. These include, for example, excessive exercise and low intake of polyphenols. Furthermore, comorbidities should be treated adequately.

As patients with AN restrict their energy and food intake tremendously, it can be concluded logically, that smaller amounts of antioxidant-rich foods are eaten, when compared to a healthy control group. Increasing the caloric intake of patients and focusing on a variation of antioxidant-rich food groups should be a part of dietary rehabilitation. Specific meal plans including foods rich in Vitamins C and E, trace elements such as selenium, carotenoids and polyphenols could be created by trained dietitians and applied in a multidisciplinary treatment approach.

As found in a meta-analysis on oxidative stress levels after weight gain and dietary interventions, a raised BMI, even before the point of complete weight-restoration, helps decrease oxidative stress markers (128). Therefore, realimentation should remain the basis in AN treatment, even in treatment plans with the spotlight on oxidative stress management.

In addition to dietary treatment, antioxidative therapy could play a small role, if future studies can also support our findings that individuals with AN seem to experience raised levels of oxidative stress.

Various approaches to treat oxidative stress have been investigated including therapies enhancing endogenous systems of antioxidative defense, limiting the development of oxidative stress and molecules repairing damage caused by oxidative stress (129).

Further studies in this area would be valuable to better understand the interactions of oxidative stress in patients with AN. Presumably, this will help answering the question which factor exists initially and causes the other to develop. A more comprehensive knowledge can contribute to development of more specific therapies, focusing on oxidative stress in AN.

## *4.8 Conclusion*

Oxidative stress in patients suffering from AN can be measured through the analysis of various parameters. In our study, out of the assessed parameters, only TAC was shifted toward elevations in oxidative stress levels, when compared to a matched healthy control group. We expected a raised oxidative stress profile in AN patients as well as a correlation between scores of eating disorder pathology and depression. Our hypotheses could partly be confirmed, as not all tested parameters showed statistically significant changes, but all medians showed a difference between the two groups.

While our findings suggest that AN and oxidative stress (elevation of TAC) are correlated, further research is needed to establish whether one specific mechanism in the pathogenesis of AN is responsible for the change in TAC or if it is caused by the interaction of several factors, as we assume based on the results of this study.

### 4.8.1 Future studies

It would be worthwhile to conduct similar studies with larger sample sizes in the future. In addition, it would be insightful, to recruit both female and male participants in patients and control groups, as this would lead to more precise application of the results to our population. However, as majority of patients with AN are female, it is difficult to recruit male patients for studies in this field. An important next step could be interventional studies to test whether better adherence to a Mediterranean diet leads to a reduction of indicators of oxidative stress. Furthermore, investigating manifestations of oxidative stress such as measurements in urine and/or the brain via fMRI could help solidify our results. Since one of this study's limiting factors is the unavailability of one specific oxidative stress biomarker, finding a marker that can be used as a general parameter for the disbalance of ROS and the antioxidative defense system would be of great use. It would simplify comparing various studies and help to decrease human errors that can naturally occur when multiple different markers are applied to indicate oxidative balance or disbalance.

One further interesting question is the extent of the impact that exercise in purging AN subtype has on oxidative stress. Differentiating between the two common subtypes in AN (restricting vs. binge-purge) in future studies looking at oxidative stress could contribute valuable information on the impact of physical exertion. One possible way of comparing the differences between both subtypes could examine AN patients, matched in BMI and duration of disease, divided into two groups: one consisting of restricting subtype and one of purging subtype to attain further insight.

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